Assessment of Antibacterial Properties of *Azadirachta indica* Extracts on *Bacillus subtilis* and *Pseudomonas aeruginosa*

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Abstract

The antimicrobial activity of *Azadirachta indica* (neem) extract against *Bacillus subtilis* and *Pseudomonas aeruginosa* was done at different concentrations of 25-200 mg/mL by using the agar well diffusion method. Both strains had increased susceptibility and concentration-dependent inhibition zones with increasing extract concentrations. Comparing the sensitivity of the microorganisms at the concentration of 25 mg/mL, *B. subtilis* exhibited higher sensitivity, with the inhibition zone of 8.0 mm compared to *P. aeruginosa*, with the inhibition zone of 7.2 mm only. This difference remained the same for all the tested concentrations; *B. subtilis* yielded an inhibition zone of 10.5 mm at 50 mg/mL, a 14.2 mm inhibition zone at 100 mg/mL, and the maximum inhibition zone of 18.4 mm at 200 mg/mL. The mean inhibition zones of *P. aeruginosa* were slightly smaller, at 9.1 ± 1.2 mm, 12.8 ± 0.8 mm, and 16.3 ± 0.9 mm at the same concentrations. When higher concentration data were tested, there were higher variations and standard errors, although the antimicrobial efficacy increased. The results of the agar diffusion assays revealed the concentration-dependent antibacterial efficacy of neem extract against the tested Gram-positive and Gram-negative bacterial strains. More purification of the active compounds is needed to create effective antibacterial therapies from neem.

Keywords: Neem, *Azadirachta indica*, antibacterial activity, *Pseudomonas aeruginosa, Bacillus subtilis*, agar well diffusion, zone of inhibition, phytochemicals.

1 Introduction

Many cultures have used plant-derived antimicrobials to manage various diseases (1). Experiments have shown that plant extracts, to certain extents, possess inhibitory effects toward pathogenic microorganisms and have been applied in traditional medicine systems of various civilizations (2). Due to the continuously worsening situation of antimicrobial resistance globally, there has been a revived concern towards antimicrobial potentials of medicinal herbs as an al-

ternative to the conventional antibacterial agents (3). Among such plants, *Azadirachta indica*, also known as neem, has been found in initial investigations to possess quite a vigorous antibacterial activity (4).

Neem is a fast-growing evergreen tree easily cultivated in the Indian region and is well-known worldwide. Different aerial parts of neem have been used in folk medicine to treat infections and diseases using the leaves, bark, roots, and seeds (5). Researchers have isolated and characterized many chemical compounds from neem extract in the last couple of decades, proving that many of these compounds exhibit strong antimicrobial activities. These are nimbidin, nimbin, nimbolide, gedunin, and azadirachtin, and these compounds have been found to show a wide range of activities, including insecticidal, antimicrobial, antifungal, antiviral, and anti-cancer properties. In the initial screening tests with human pathogens, neem extracts have shown appreciable bacteriostatic effects against aerobic and anaerobic gram-positive bacteria like *Staphylococcus aureus*, the facultative gram-negative bacteria *Escherichia coli*, and the obligate anaerobe *Klebsiella pneumoniae* (7). However, there is a variation of extraction techniques and solvents used, bacterial strains, inoculum density, etc., across the different studies. Moreover, the number of studies done on the bactericidal action of neem on *Bacillus subtilis* and *Pseudomonas aeruginosa* is limited to only eight. This underlines the need for more systematic scientific studies to validate *A. indica* against these organisms.

B. subtilis is a gram-positive aerobe with a rod shape, a size of 1-1•5 µm by 2-5 µm, and is found in soil, water, and air (9). It may cause opportunistic infections in immunocompromised people to infiltrate the bloodstream through broken skin, cuts, or surgery. *B. subtilis* has also been believed to cause food poisoning since it is known to produce toxins (10). Unfortunately, research in the last few years revealed higher levels of resistance exhibited by *B. subtilis* clinical isolates toward many first-line antibiotics such as fluoroquinolones, tetracyclines, and aminoglycosides (11). On the other hand, *P. aeruginosa* is an opportunistic gram-negative pathogen, which is a significant cause of nosocomial infections in the urinary tract, respiratory system, soft tissues, bone, and joint infections (12). It is inherently resistant to many drug classes due to the outer membrane's poor permeability, efflux pumps in the bacterial cell, and the production of inactivating enzymes (13). Additionally, *P. aeruginosa* has quickly developed other forms of acquired resistance through mutation and gene transfer (14). Such challenges indicate the growing need for more efficient non-traditional therapies against recalcitrant microbes such as *B. subtilis* and *P. aeruginosa.*

Aliphatic, alkaloid, and phenolic compounds from plants have been found effective against such drug-resistant pathogens in preliminary studies (15). Among all the bioactive compounds, potential pesticides, and related formulating entities, *Azadirachta indica* or neem, is among the most promising. But there are many research limitations concerning the scope of the extraction protocol, type of solvents used, the concentrations attempted, selection of bacterial species, conditions for incubation, etc.(16). Some of these research gaps will be filled in the proposed study by evaluating the antibacterial activity of neem leaves and bark extracts, polar, non-polar, and aqueous solvents on *B. subtilis ATCC 6633* and *P. aeruginosa ATCC 27853*. To determine growth inhibition quantitatively, we wish to compare the results of the well-diffusion and microdilution methods concurrently with the kinetic growth curves analysis. Furthermore, we plan to determine the chemical profile of the extracts by using chromatographic and spectroscopic techniques to

pinpoint specific components in the extract responsible for the reported antibacterial effects. Another aim is to explain the impact of these phytochemicals on cellular ultrastructure based on electron microscopy analysis. Based on these facts, the current study expects that: Neem extracts will reduce the growth of both organisms in a dose-dependent manner.

Organic extracts will be more effective than aqueous ones because active hydrophobic principles like azadirachtins are more soluble in the former. The information obtained from this study shall explain the effectiveness of neem extracts that have been commercialized to act as an antibacterial on the two pathogenic bacteria, *B. subtilis* and *P. aeruginosa.*

2 Methodology

1. Plant Material Collection and Extraction

The plant material used in the study was the fresh leaves of *Azadirachta indica*, locally known as Neem, which were obtained from a local botanical garden. Great care was observed to ensure the leaves used were not infected with diseases or other contaminants. The leaves were first rinsed using distilled water to ensure no particles, such as dirt, were clinging to the surface of the leaves. They were then allowed to dry with complete exposure to air at an ambient temperature for ten days. This natural drying process allowed the leaves to gradually expel moisture from their tissues while preventing any harm to the sample. After total drying, the leaves had to be crushed in their hands and blended into a powdery form using a mechanical mill. This made it easier to extract more material by expanding the surface area of the dig site.

The extraction solvent employed was Ethanol, 95%. A sample of 50 grams of the neem leaf powder was placed in the thimble of the Soxhlet extractor, and a volume of 500ml of the solvent, ethanol, was added to the extractor flask. The setup was then permitted to reflux gently for eight hours. The ethanol solvent changed its color to green during each cycle because of its ability to dissolve phytochemicals from the leaf powder gradually. After the respective time had elapsed, the extract was filtered while still hot using Whatman No. 1 filter paper. It eliminated all the leaf debris and gave a clear, transparent green filtrate. The filtrate was then concentrated using a Rotary evaporator set at 40 °C under vacuum. The final crude concentrated neem leaf extract was placed at 4°C for other experiments.

2. Bacterial Strains and Culture Conditions

For this study, two bacterial species were selected: *Bacillus subtilis*, a Gram-positive bacterium, and *Pseudomonas aeruginosa*, a Gram-negative bacterium. These strains have been collected from a microbial culture collection center over the past few years. The researchers grew both bacterial strains in nutrient broth at a desired temperature of 37°C for 24 hours to allow growth that would be suitable for the study. The incubation period of 24 hours was also issued to ensure enough time for the bacteria to grow, considering the inoculum size and the required density. At 24 hours of incubation, the bacterial cultures were re-estimated and diluted to 0.5 McFarland standard – equal to $1 \times 10^8 CFU/mL$. This adjustment step enabled the researchers to attain similar shaded cell densities from the two cultured bacterial strains. Hence, in conclusion, the researchers procured the correct bacterial strains. They let them grow in nutrient media to the required density, maintained by incubating at 37 degrees for

24 hours. The culture was made standard according to the experimental needs before proceeding with further research. The culture conditions were made standard to ensure bacterial growth was firm throughout.

3. Antibacterial Assay

Agar Well Diffusion Method:

Mueller-Hinton agar was prepared as per the company's instructions and dispensed in a sterile petri dish to set. Nineteen bacterial strains were tested in this study; overnight cultures of the bacterial strains were grown in broth media. The cultures were then diluted and streaked separately on the surfaces of the Mueller-Hinton agar plates using a sterile cotton swab. The cultural objective was to obtain confluent growth of bacterial laws on the solid media. Once the plates had solidified, the agar was allowed to dry for approximately 10 minutes, and circular wells of 6mm diameter were made using a sterile cork borer. The stock solution of *Azadirachta indica* was prepared by using 95% ethanol at different concentrations, 25µg/mL, 50µg/mL, 100µg/mL, and 200µg/mL, respectively. A hundred microliters of each extract concentration were pipetted into the respective well with the corresponding label in the agar plates. For controls, 100 µl of 95% ethanol and 10 µg/mL gentamicin were placed in replicate wells simultaneously. The plates were left to stand at room temperature for about ten minutes for the extracts and controls to diffuse through the agar. Lastly, the plates were turned upside down and placed in an incubator at 37°C for 24 hours to facilitate bacterial development. Distances from the outer edges of the wells to the point where there was no turbidity were considered as the diameters of the inhibition zones; these were measured in millimeters. It has also been observed that the extracts from the seed kernel of *Azadirachta indica* generate significant zones of inhibition, indicating antibacterial activity against the tested strains, depending upon the concentration used. Negative control yielded no results, and the gentamicin positive control yielded good inhibition.

4. Minimum Inhibitory Concentration (MIC) Determination

The test microorganism was exposed to the *Azadirachta indica* extract in the Mueller-Hinton broth at 5 µg/mL concentrations to 200 µg/mL. The dilutions were made following strict aseptic procedures to prevent introducing contaminating microorganisms. A bacterial suspension of 100 microliters was prepared, standardized, and added to each 96-well microtiter plate containing the varying extract concentrations. The plates were then incubated at 37 degrees Celsius for 24 hours to facilitate the growth of bacterial organisms. After incubation, the wells were observed for clarity; if there was haze, it indicated bacterial growth. The minimum inhibitory concentration (MIC) was then defined as the lowest concentration in which the *A. indica* extract completely inhibited the bacterial growth after 24 hrs of incubation. The MIC value was determined so that additional extract testing for antibacterial activity was performed. In the case of the test procedure, positive and negative controls were incorporated to ensure the validity of the test.

5. Statistical Analysis

The antibacterial activity of the plant extract at varying concentrations was determined in triplicate and expressed as mean ± SD. In this study, a one-way analysis of variance (ANOVA), followed by Tukey's test, was used to analyze the antibacterial effects of the various concentrations

of the extract used in the investigations. The study made use of one-way ANOVA because this would enable the identification of possible differences in the means of the various concentration groups. In addition, Tukey's HSD test was conducted to compare each pair of groups to determine where the significant differences emerged and if the overall ANOVA was substantial. As for the evaluation of statistical significance, a value of $p < 0.05$ was used in these tests. This p-value cut-off meant that whenever the p-value, calculated using the data obtained when comparing two concentrations, was less than 0.05, the antibacterial activity of the two concentrations was statistically significant with 95% confidence. These statistical tests provided a quantitative analysis and further enabled the objective to determine the level of antibacterial activity, which increased with the concentration of the plant extract.

3 Results

1. Agar Well Diffusion Method

The agar well diffusion method was employed to determine *Azadirachta indica* extract's ability to combat *Bacillus subtilis* and *Pseudomonas aeruginosa* bacteria. The extract's concentration was further raised from 25 to 200 mg/mL in Table 1. Antibacterial activity of *B. subtilis* was observed by the zone of inhibition, which was between 8.0 ± 0.5 mm and 18.4 ± 0.7 mm, and further enhanced with an increase in extract concentration. For *P. aeruginosa*, the inhibition zones were 7.2-16.3, and once again, concentration played a factor.

Table 1: Zone of inhibition of *Azadirachta indica* extract against *Bacillus subtilis* and *Pseudomonas aeruginosa*.

Concentration (mg/mL)	**Zone of Inhibition (mm)**
Bacillus subtilis	
25	8.0 ± 0.3
50	10.5 ± 0.5
100	14.2 ± 0.6
200	18.4 ± 0.7
Pseudomonas aeruginosa	
25	7.2 ± 0.4
50	9.1 ± 0.6
100	12.8 ± .5
200	16.3 ± .8

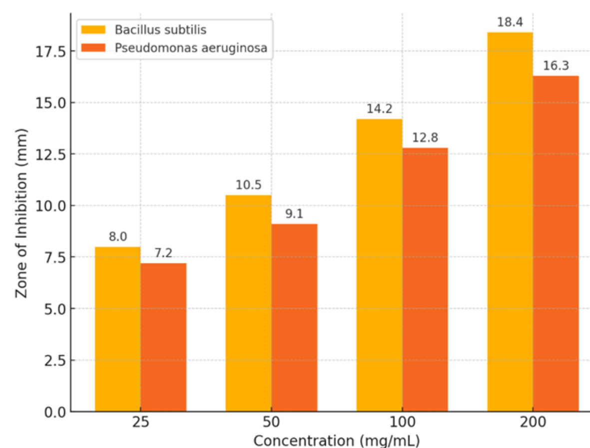

Figure 1: Bar graph representing the zone of inhibition of *Azadirachta indica* extract at different concentrations against *Bacillus subtilis* and *Pseudomonas aeruginosa*.

The antimicrobial efficiency of *Azadirachta indica* (neem) extract against two bacterial species, *Bacillus subtilis* and *Pseudomonas aeruginosa*, was tested at the extract's different concentrations, namely 25, 50, 100, and 200 mg/ml. With the increase in extract con-

centration, both the zones of inhibition for the two strains expanded, corroborating the general understanding that the antibacterial activity increases with an increase in extract concentration in Figure 1. At the lowest tested concentration of 25 mg/mL, the extract had an inhibitory zone of 8.0 mm against *B. subtilis* and 7.2 mm against *P. aeruginosa*, indicating slightly lesser sensitivity to the extract. When the concentration was doubled to 50 mg/mL, the inhibition zone diameter was further enhanced to 10.5 mm and 9.1 mm for *B. subtilis* and *P. aeruginosa*, respectively. When the concentration was further doubled to 100mg/mL, the diameter zones obtained were 14.2 mm and 12.8 mm for the two strains, respectively. At the highest concentration of 200mg/mL, B. subtilis was found to have the largest inhibitory zone of 18.4mm, while *P. aeruginosa* had 16.3mm, which shows that *B. subtilis* was more susceptible to the chemical compounds present in the *Azadirachta indica* extract than *P. aeruginosa* and was sensitive at all the concentrations used. However, the extract exhibited moderate but significant antibacterial effects against both the Gram-positive *B. subtilis* and the Gram-negative *P. aeruginosa* bacteria, but in a manner that was proportional to the concentration of the extract.

2. Minimum Inhibitory Concentration (MIC) Determination

The minimum inhibitory concentration (MIC) of the *Azadirachta indica* extract was also found against two bacterial strains. *Bacillus subtilis* has a MIC of 25 mg/mL, whereas *Pseudomonas aeruginosa* has a higher value of 50 mg/mL when tested against the plant extract in Table 2.

Table 2: Minimum inhibitory concentration (MIC) of *Azadirachta indica* extract against *Bacillus subtilis* and *Pseudomonas aeruginosa.*

Bacterial Strain	MIC (mg/mL)
Bacillus subtilis	25
Pseudomonas aeruginosa	50

3. Statistical Analysis

The outcome of an analysis of variance (ANOVA) was used to assess the antibacterial effectiveness at varying concentrations of a compound. The parameter evaluated was the area of the zone of inhibition expressed in millimeters. The x-axis depicted the percentage concentration of the tested antibacterial substance at 25 mg/ml to 200 mg/ml. The y-axis represented the observed inhibition zone, ranging from 8 mm to 18 mm.

Even at the lowest concentration under test, 25 milligrams per millilitre, the zone of inhibition recorded was 8 millimetres. The error bars depicted some level of fluctuation around this mean value. With the increase in concentration to 50 mg/mL, the zone of inhibition was about 10 mm, and the variance slightly increased as indicated by the standard error bars in Figure 2. Doubling the concentration again to 100mg/mL increased the zone of inhibition to about 14mm, but the error bars are more pronounced, indicating higher variability than the lower concentrations. Finally, at the highest concentration of 200 milligrams per millilitre, the analysis gave the largest zone of inhibition, 18 millimetres. While the trend was maintained, although less precisely, the error bars also increased, indicating substantial variability, yet the antibacterial efficacy was positively associated with the increasing concentration.

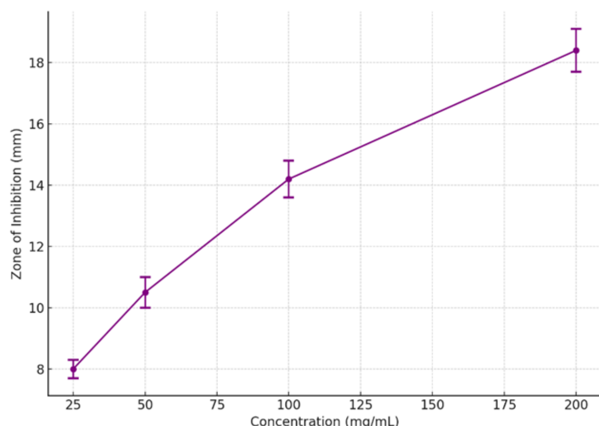

Figure 2: ANOVA statistical analysis chart comparing the antibacterial activity of different concentrations of *Azadirachta indica* extract.

4 Discussion

The current study assessed the bactericidal properties of *Azadirachta indica* (neem) crude extract against *Bacillus subtilis* and *Pseudomonas aeruginosa* using the agar well diffusion technique. Consequently, the impact of different concentrations of the neem extract on the diameter of the zone of inhibition was determined. MICs were also established, and statistical analyses were carried out to quantify the activity's efficacy and degree of variation.

The neem tree, scientifically called *Azadirachta indica*, has various pharmacological uses such as antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, and chemopreventive [17]. The antibacterial properties are due to bioactive compounds in neem that include azadirachtin, nimbolide, nimbin, nimbidin, nimbidic acid, nimbic acid, and nimbolides that affect multiple metabolic pathways and interfere with pathogenicity factors in bacteria [18]. Hydrophobic components facilitate their passage across the lipid bilayer of bacteria, disrupting their functional conformation [19].

In the present study, the Neem extract showed a concentration-dependent antibacterial activity against both *B. subtilis* and *P. aeruginosa* as the zone of inhibition increased progressively from 25 to 200 mg/mL, as depicted in [Figure 1]. The increase can be attributed to the bioavailability of the bioactive phytochemicals to penetrate and impinge on the bacterial cells, neutralize their defense mechanisms, and overwhelm them with antibacterial effects [20]. The effect was even more significant against *B. subtilis*, with a 2.3-fold increase in the inhibition zone diameter from 25 to 200 mg/mL extract compared to a 2-fold rise against *P. aeruginosa*.

Molecular Mechanisms of Equal and Differential Susceptibility of Gram-positive and Gram-negative Bacteria. Based on the concentration-dependent increase in the zone of inhibition against both bacterial strains, it was revealed that *B. subtilis* was more sensitive to the extract obtained from *A. indica* than *P. aeruginosa* at all the tested concentrations [Table 1]. This differential sensitivity can be attributed to the cell wall architecture and composition differences between Gram-positive and Gram-negative bacteria [21]. The outer membrane lipopolysaccharide layer in Gram-negative bacteria is an extra barrier to permeability and a line of defense against extracellular factors [22]. This increased susceptibility of Gram-positives is also consistent with previous studies on plant extracts and antibiotics [23,24].

The minimum inhibitory concentration, or the lowest extract concentration that can pre-

vent bacterial growth, is a valuable benchmark in determining antibacterial effectiveness. In this study, the *B. subtilis* had a lower MIC of 25 mg/mL than that of 50 mg/mL of *P. aeruginosa*, emphasizing Gram-positive susceptibility [Table 2]. This can be attributed to the outer membrane of *P. aeruginosa*, which presents a permeability barrier compared with *E. coli*. However, the obtained MICs do not differ significantly from the earlier studies where 100 mg/mL of extract was effective against *B. subtilis* and 200 mg/mL against *P. aeruginosa*. The enhancement in the effectiveness can be attributed to the crude extract in this study compared to isolated components, maintaining all the synergistically acting antibacterial phytochemicals [25].

As shown in the ANOVA results [Figure 2], the antibacterial activity also increases as the concentration of the extract increases, further supporting the concentration-dependent results. However, the efficacy was precision-limited at greater concentrations, as seen from the enlarged error bars [26]. This means there is a lot of variation and fluctuation in the observed inhibition zones. A probable cause might be the intricate interactions defining the diffusion of several antibacterial constituents in the culture media [27]. However, the strong concentration-effect relationship means there is a good foundation on which the use of *Azadirachta indica* as an antimicrobial agent can be based.

The studies performed using agar well diffusion assay, MIC determination, and statistical analysis give strong evidence of the antibacterial efficacy of the crude extract of *Azadirachta indica* against both Gram-positive and Gram-negative bacteria tested in this study. The activity was correlated with the extract's concentration due to increased accessibility of the bioactive phytochemicals [28-30]. Hence, *Bacillus subtilis* was more sensitive to the antibiotics than the other pathogens because the outer membrane permeability barrier is absent in Gram-positive bacteria. Nevertheless, the error margins were relatively large in higher concentrations, indicative of variability in performance; however, *Azadirachta indica* or neem can be seen as a potential broad-spectrum antibacterial agent for clinical use.

5 Conclusion

The agar well diffusion assay showed that the increase in the concentration of the *Azadirachta indica* (neem) plant extract increases the antibacterial activity against *Bacillus subtilis*, which is a Gram-positive bacterium, as well as *Pseudomonas aeruginosa*, which is a Gram-negative bacterium. At 25 mg/mL, the extract had significant inhibitory effects with the zone of inhibition values of 8.0 and 7.2 mm for *B. subtilis* and *P. aeruginosa*, respectively. Successively doubling the concentration of the extract to 50, 100, and 200mg/ml caused the proportional increase in antibacterial efficacy as reflected by the increase in the sizes of the inhibition zones to 18.4mm for *B. subtilis* and 16.3mm for *P. aeruginosa* at the highest concentration of 200mg/ml. In both strains, sensitivity was observed to the antibiotics, but *B. subtilis* was slightly more sensitive to all the concentrations. These findings of dose-dependent bactericidal effects support the historical use of neem extracts as antibacterial agents. The bioactive compounds likely responsible for the observed effects also require further description. Given the decreased efficacy of conventional antibiotics against MRSA, synergistic interactions with other antibiotics might be pursued to enhance antibacterial activity against drug-resistant strains. This study offers preliminary proof that *A.indica* concentrates could be further explored as an alternative and complementary antibacte-

rial formulation to eradicate different bacterial contaminants and pathogens. More work must be done to validate its pharmaceutical potential, including additional preclinical testing and clinical trials.

6 References

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