



# Antibacterial Activity of *Azadirachta indica* Leaf Extracts Against Some Pathogenic Standards and Clinical Bacterial Isolates

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## Abstract

**Background:** *Azadirachta indica* A. Juss (Neem) has been used against a few diseases in traditional medicine. This study was conducted to evaluate the antibacterial activity of Neem leaves extract against some antibiotic sensitive and resistant strains of important human pathogenic bacteria.

**Methods:** The antimicrobial activities of ethanol, methanol, and ethyl acetate extracts of *Azadirachta indica* A. juss leaves were evaluated against some pathogenic bacterial strains including *S. aureus* ATCC 6538, *E. faecalis* ATCC 1394, *P. aeruginosa* ATCC 9027, *E. coli*, ATCC 25922, and their clinical isolates using agar well diffusion and MTT microdilution methods. MBC was determined by subculturing from microdilution assays.

**Results:** The results revealed that methanol extract has the strongest growth inhibitory effect on both standard and clinical isolated strains of *P. aeruginosa*. Ethyl acetate and ethanol extracts, showing a growth inhibitory effect on both standard and hospital isolated strains of *S. aureus*. In the case of *E. faecalis*, ethanol and methanol extracts showed the highest growth inhibitory effect against standard and clinical strains, respectively. According to the MIC index results, the methanol extract has a bactericidal activity against both standard and nosocomial strains of *S. aureus* and *P. aeruginosa* and bacteriostatic activity against nosocomial strain of *E. faecalis*. Ethanol extract showed bactericidal activity against both standard and nosocomial strains of *E. faecalis* and *P. aeruginosa* and bacteriostatic activity against nosocomial strain of *S. aureus*. Ethyl acetate extract had shown bactericidal activity against standard strains of *S. aureus* and *P. aeruginosa* and bacteriostatic against nosocomial strain of *S. aureus* and standard strain of *E. faecalis*.

**Conclusions:** Results revealed that Neem may be a prospective therapeutic agent to combat antibiotic resistant bacteria.

**Keywords:** MIC, MBC, MIC Index, MTT Microdilution Method, *Azadirachta indica*

## 1. Background

Antibiotics have revolutionized mankind's health status, allowing treatment of life-threatening infections. However, indiscriminate use of antibiotics is very common in developing countries, being a major cause for development of antimicrobial resistance. Thus, it has now become essential to look for newer antibiotics. Most of the antibiotics come from natural origin, especially from various microbial or marine sources. Plants also produce compounds to protect themselves from microbial attacks (1-3).

*Melia azedarach* L., commonly known as Neem, Persian lilac or chinaberry, belongs to Meliaceae family and is well

known in Iran and its neighboring countries for more than 200 years as one of the most versatile medicinal plant, with a wide spectrum of biological activity (4, 5). Neem is originally native to South India and Myanmar. However, it abundantly grows in southern coast of Iran, locally known as "Charish" (6). Recent studies have shown that Neem possesses anti-inflammatory, anti-arthritic, antipyretic, hypoglycemic, antigastric ulcer, antibacterial, antifungal, and antitumor activities (7). It possesses a wide spectrum of antibacterial action against Gram-positive and Gram negative microorganisms (8).

## 2. Objectives

This study was conducted to determine the antibacterial effect of 3 Neem leaves extracts on some important human pathogenic bacteria to find if it has any prospect as a future antibiotic. These bacteria included both standard and local clinical bacterial isolates, such as *S. aureus*, *P. aeruginosa*, *E. coli*, and *E. faecalis*.

## 3. Methods

### 3.1. Plant Material

Leaves of *A. indica* were collected from Bandar Abbas (Hormozgan province). Voucher specimens were deposited at the herbarium of faculty of pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran. Collected leaves were dried in shade at room temperature and blended using dry blender to obtain the powder for more efficient and effective solvent extraction.

### 3.2. Preparation of the Extracts for Antibacterial Assay

A total of 100 grams of leaves powder was soaked using ethanol, methanol, and ethyl acetate as solvents and kept at room temperature for 24 hours. The extraction process was repeated 2 times (extraction for 2 days). Then, the extracts were filtered (using Whatman filter No. 1) and concentrated at reduced pressure using a rotary evaporator at 40°C. This stock solution of extract was sterilized by filtration through Millipore membrane filter of 0.45 mm pore size, and the dried extracts were kept at 4°C for further use.

### 3.3. Preparation of Different Concentrations of the Extract

Three different solvents including DMSO, DMSO: Methanol (1:1 v/v) and DMSO: Ethanol (1:1 v/v) were used to prepare stock solution with concentration of 300 mg/mL. Serial dilution was performed to obtain concentrations of 200, 100, and 50 mg/mL of each extract.

### 3.4. Microbial Strains

Antimicrobial activities of extracts of *A. indica* leaves were evaluated against both standard and clinical bacterial isolates. Standard strains included *S. aureus* ATCC 6538, *E. faecalis* ATCC 1394, *P. aeruginosa* ATCC 9027, and *E. coli* ATCC 25922, and clinical bacterial isolates of these same bacteria isolated from clinical specimens of patients hospitalized in Alzahra hospital, Isfahan, Iran, were applied in this study. All the strains were maintained on nutrient agar at 4°C and were subcultured every month.

### 3.5. Antibiogram Analysis

Disk diffusion susceptibility tests were performed on all standard and clinical bacterial isolates according to NCCLS guidelines (9). Antimicrobial agents tested for clinical isolates included cephalixin (30 µg), cefalotin (30 µg), kanamycin (30 µg), cefixime (5 µg), erythromycin (15 µg), ceftazidim (30 µg), ciprofloxacin (5 µg), oxacillin (1 µg), vancomycin (30 µg), and gentamicin (10 µg). Ciprofloxacin was tested against standard bacterial isolates. The disks were prepared from Padtan-Teb Company, Iran.

### 3.6. Quantitative Antibacterial Evaluation

#### 3.6.1. Antibacterial Assay

Antibacterial activity was determined by agar well diffusion method (10). Sterile Mueller-Hinton agar (MHA) plates were prepared. Plates were swabbed with cotton wool impregnated with the bacterial suspension containing  $10^6$  cfu/mL and allowed to dry; 6 wells (6 mm diameter) were bored on the surface of the agar media on each plate. The first 4 wells were filled with 100 µL solution of the extract at concentrations of 300, 200, 100, and 50 mg/mL, respectively. The other 2 wells were filled by the solvent used for extract preparation as negative control. A disk of appropriate antibiotic based on antibiogram of each bacterial strain was used as the positive control. The inoculated plates were kept in the refrigerator at 4°C for 1 hour to allow the diffusion of the extracts into the agar before growth of the organism commenced. Plates were incubated at 37°C for 24 hours, and then the antibacterial activity was assessed based on measurement of the zone diameter of the inhibition formed around the well.

#### 3.6.2. Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentrations (MICs) of the active plant extracts were determined by MTT assay as outlined by Packer et al., with minor modifications (11). Briefly, the plant extracts with highest inhibition zone diameter were diluted to give 60 mg/mL concentration in Mueller-Hinton Broth (MHB) as stock concentration for MIC determination. To all wells, 50 µL of MHB was added. A volume of 50 µL of the stock concentration was pipetted into the first row of the 96-well microtiter plates. Serial dilutions were performed using a multichannel pipette. Tips were discarded after use such that each well had 50 µL of the test material in serially descending concentrations to give final plant sample concentrations of 30 - 0.03 mg/mL. Finally, 10 µL of bacterial suspension ( $10^6$  cfu/mL) was added to each well to achieve a concentration of  $5 \times 10^5$  cfu/mL. Each plate was wrapped loosely with cling film to avoid dehydration. Each plate had a set of controls: a column with

the appropriate antibiotic as positive control according to the antibiogram test (final concentration 10 - 0.01  $\mu\text{g}/\text{mL}$  of the antibiotic), a column with all solutions with the exception of the test compound, and a column with all solutions with the exception of the bacterial solution adding 10  $\mu\text{L}$  of nutrient broth instead. Plates were prepared in duplicate and incubated at 37°C for 18 hours; 5  $\mu\text{L}$  of a sterile methanolic solution (5 mg/mL) of MTT was added to each well as indicator and incubated at 37°C for 60 minutes. Any color changes from yellow to blue were recorded as positive. The MIC was the lowest concentration of test material in which no growth (no blue color) was observed.

### 3.6.3. Minimum Bactericidal Concentration (MBC)

Plant extracts with antimicrobial activity, as determined by the MTT microdilution assay, were assessed for their bactericidal activity by subculturing onto the fresh MHA plates after the assay. The MBC was defined as the concentration of the plant extract in the last well showing no further bacterial growth on subculture (12).

### 3.6.4. MIC Index

The MIC index (MBC/MIC) was calculated for each extract to determine whether an extract is bactericidal ( $\text{MBC}/\text{MIC} < 4$ ) or bacteriostatic ( $4 < \text{MBC}/\text{MIC} < 32$ ) on growth of bacterial test organisms (13, 14).

## 4. Results

### 4.1. Antibiogram Test

The result of antibiogram analysis (Table 1) indicates that all nosocomial bacterial strains were resistant to some widely used broad-spectrum antibiotics. All the standard strains were susceptible to ciprofloxacin; therefore, this antibiotic was used as positive control to test bacteria in antibacterial assays. According to the antibiogram test, gentamicin and ceftazidime were used as positive control for nosocomial strain of *P. aeruginosa* and *E. coli*, respectively. Vancomycin was used for both nosocomial strains, *S. aureus*, and *E. faecalis*.

### 4.2. Antibacterial Activity

Methanol extracts showed strongest inhibition against both standard and clinical isolated strains of *P. aeruginosa*, with biggest inhibition zones for all the concentrations used from 300 mg/mL to 50 mg/mL when compared to the inhibition zones formed by ethanol and ethyl acetate extract for the same bacteria (Figure 1). Ethyl acetate and ethanol extracts had the biggest inhibition zones for standard and hospital isolated strains of *S. aureus*, respectively (Figure 1). Ethanol and methanol extracts showed

strongest growth inhibitory effect on standard and clinical isolated strains of *E. faecalis*, respectively (Figure 1). None of the extracts showed antibacterial activity against *E. coli*.

### 4.3. MIC, MBC, and MIC Index

As the results revealed (Figures 2 - 4), the methanol extract had bactericidal activity against both standard and nosocomial strains of *S. aureus* and *P. aeruginosa*, and bacteriostatic activity against nosocomial strain of *E. faecalis*. Ethanol extract was bactericidal against both standard and nosocomial strains of *E. faecalis* and *P. aeruginosa*, and bacteriostatic activity against nosocomial strain of *S. aureus*. Also, as demonstrated in Figure 4, ethyl acetate extract has bactericidal activity against standard strains of *S. aureus* and *P. aeruginosa* and bacteriostatic against nosocomial strain of *S. aureus* and standard strain of *E. faecalis*.

## 5. Discussion

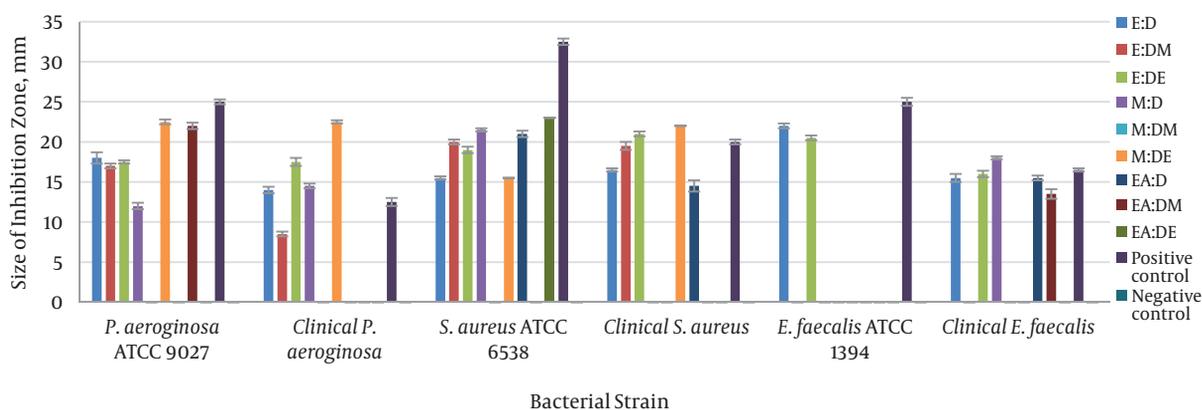
WHO has recently reported that the world is coming into a post-antibiotic era and most of the current antibiotics will become inefficient (15). Appearance of antimicrobial resistance strains is a threat to the public health (16). It imposes huge economic burden due to increasing morbidity and mortality (17, 18). On the other hand, most of the new antibiotics are expensive and have threatening side effects. Research is under way to identify the effective and safe alternatives of current antibiotics from plant sources (19). Studies establish that plants could be a potential origin of drugs against pathogenic organisms (20, 21). In the present study, the Neem leaf extract was subjected to a preliminary screening for antimicrobial activity against *S. aureus*, *E. faecalis*, *E. coli*, and *P. aeruginosa* standard and antibiotic resistance strains. Methanol extract had the strongest growth inhibitory effect on both standard and clinical isolated strains of *P. aeruginosa* with MIC 1.9 and 3.75 mg/mL, respectively. Ethyl acetate and ethanol extracts had the strongest growth inhibitory effect on both standard and hospital isolated strains of *S. aureus*, with MIC 0.06 and 0.234 mg/mL, respectively. Ethanol and methanol extract has the strongest growth inhibitory effect on standard and clinical isolated strains of *E. faecalis*, with average diameter of zone of inhibition 23 mm and 19 mm, respectively.

According to our results, ethanol and ethyl acetate extracts showed highest activity, and this may be related to the polarity of these solvents that could extract more polar and general compounds including antimicrobial agents from this plant. These results are consistent with findings of previous study done by Tirumalasetty et al., (2014) who indicated that methanol extract of Neem exhibited antimicrobial activity against *S. aureus*, *P. aeruginosa*, and *E. coli*

**Table 1.** Susceptibility of Four Standard and Nosocomial Bacterial Strains to Antibiotics in Nutrient Agar

| Antibiotics, $\mu\text{g}$ | Diameter of the Inhibitory Zones, mm |            |                    |            |                |            |                      |            |
|----------------------------|--------------------------------------|------------|--------------------|------------|----------------|------------|----------------------|------------|
|                            | <i>S. aureus</i>                     |            | <i>E. faecalis</i> |            | <i>E. coli</i> |            | <i>P. aeruginosa</i> |            |
|                            | Standard                             | Nosocomial | Standard           | Nosocomial | Standard       | Nosocomial | Standard             | Nosocomial |
| Cephalexin (5)             | NA                                   | R          | NA                 | R          | NA             | R          | NA                   | R          |
| Cefalotin (30)             | NA                                   | R          | NA                 | 12         | NA             | R          | NA                   | R          |
| Kanamycin (30)             | NA                                   | R          | NA                 | 22         | NA             | R          | NA                   | R          |
| Cefixime (5)               | NA                                   | R          | NA                 | 15         | NA             | R          | NA                   | R          |
| Erythromycin (15)          | NA                                   | 23         | NA                 | R          | NA             | R          | NA                   | R          |
| Ceftazidim (30)            | NA                                   | R          | NA                 | R          | NA             | 24         | NA                   | R          |
| Ciprofloxacin (30)         | 32                                   | R          | 25                 | R          | 38             | R          | 27                   | R          |
| Oxacillin (1)              | NA                                   | R          | NA                 | R          | NA             | R          | NA                   | R          |
| Vancomycin (30)            | NA                                   | 20         | NA                 | 23         | NA             | R          | NA                   | R          |
| Gentamicin (30)            | NA                                   | R          | NA                 | R          | NA             | R          | NA                   | 17         |

Abbreviations: NA, Not assigned; R, Resistant.

**Figure 1.** Size of Inhibition Zone Formed by 300 mg/mL Concentration of Neem Leaf Extracts Against Four Reference and Nosocomial Bacterial Strains

E, M and EA refer to ethanol, methanol and ethyl acetate extract, respectively. D, DM and DE refer to DMSO, DMSO-Methanol and DMSO-Ethanol that was used for preparation of different concentrations of each extracts, respectively. Results of other concentrations of extracts (50, 100, 200 mg/mL) were given in the supplementary file appendix 1 - 4.

(22). Similar results were also observed by Autade et al., (2015) who presented growth inhibitory effect of acetone Neem leave extract on these strains (23). Mamman et al. (2013) found that aqueous and methanol extracts of Neem leaves showed inhibitory effects against *E. coli*, *Salmonella spp.*, and *S. aureus* (24). None of the extracts showed considerable antibacterial activity against *E. coli* even at a concentration of 300 mg/mL. This finding is not supported by most of the previous studies except Autade et al. (2015); they found that chloroform extract of Neem leaves did not inhibit the growth of *E. coli* (23). Rasool et al. (2017) found that ethanol extract of Neem leaves showed inhibitory ef-

fects against *E. coli*, *S. typhi*, *P. aeruginosa*, and *S. aureus* (25).

The importance of the present research was its investigation of the antibacterial effect of Neem leaves extracts against antibiotic resistance bacterial strains isolated from hospital environment, while in the previous studies standard strains were investigated and was more applicable. In this regard, as shown in the results, 1 extract can be effective on nosocomial strain but not on the standard strain or vice versa. For example, methanol extract inhibited nosocomial strain of *E. faecalis* but not standard strain, or conversely, ethyl acetate extract was effective on the standard strain of *P. aeruginosa* but not on nosocomial strain.

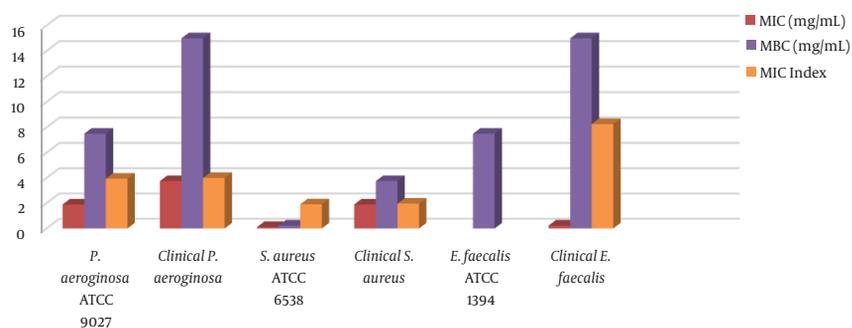


Figure 2. MIC, MBC and MIC Index of Methanol Extract of Neem Leaf

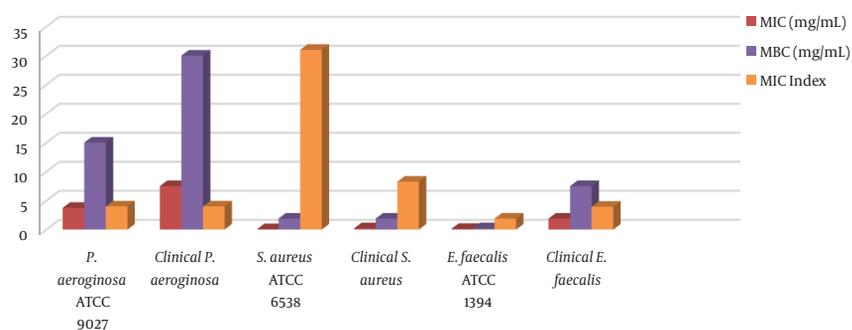


Figure 3. MIC, MBC and MIC Index of Ethanol Extract of Neem Leaf

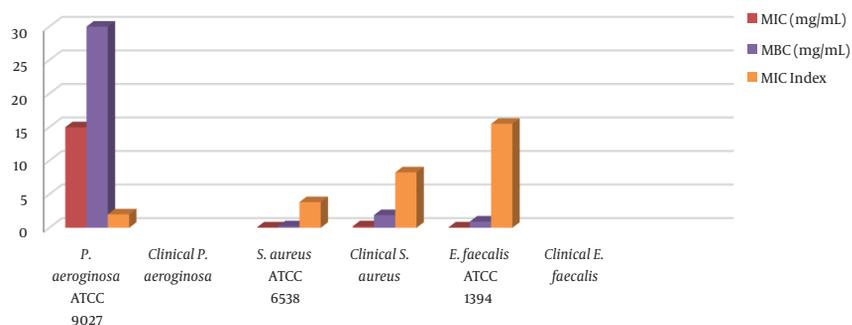


Figure 4. MIC, MBC and MIC Index of Ethyl Acetate Extract of Neem Leaf

Conclusively, these results emphasize that Neem may be a prospective therapeutic agent to combat antibiotic resistant bacteria.

### Supplementary Material

Supplementary material(s) is available [here](#) [To read supplementary materials, please refer to the journal website and open PDF/HTML].

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### Footnote

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