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

Leila Maleki,1 Tahereh Sadeghian-Rizi,2,7 Mostafa Ghannadian,3 Mohammad Hossein Sanati,4 Shahin Shafizadegan,5 and Hojjat Sadeghi-Aliabadi6,*

1M.Sc. of Biotechnology, Department of Microbial Biotechnology, School of Biology Sciences, Nour Danesh Institute of Higher Education, Meymeh, Isfahan, Iran
2Ph.D. Candidate in Pharmaceutical Biotechnology, Department of Pharmaceutical Biotechnology, School of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran
3Ph.D., Department of Pharmacognosy, School of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran
4Ph.D., Department of Pharmaceutical Biotechnology, School of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran
5Ph.D., Department of Medical Genetics, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran
6Ph.D., Department of Pharmaceutical Biotechnology, School of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran
7Corresponding author: Tahereh Sadeghian-Rizi, Department of Pharmaceutical Biotechnology, School of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran. Tel: +98-9132366325, E-mail: t_sadeghian@pharm.mui.ac.ir
*Corresponding author: Hojjat Sadeghi-Aliabadi, Department of Pharmaceutical Biotechnology, School of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran. Tel: +98-9132366325, E-mail: sadeghi@pharm.mui.ac.ir

Received 2017 May 10; Revised 2017 October 06; Accepted 2017 November 22.

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 ATCC6538, E. faecalis ATCC1994, P. aeroginosa ATCC 9027, E.coli, ATCC25922, 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* ATCC6538, *E. faecalis* ATCC394, *P. aeruginosa* ATCC9027, and *E. coli* ATCC25922, 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/disk), cefotin (30 µg/disk), kanamycin (30 µg/disk), cefixime (5 µg/disk), erythromycin (15 µg/disk), ceftazidim (30 µg/disk), ciprofloxacin (5 µg/disk), oxacillin (1 µg/disk), vancomycin (30 µg/disk), and gentamicin (10 µg/disk). 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. A disk of the plant extracts with highest inhibition zone diameter was repeated 2 times (extraction for 2 days). Then, the extracts were filtered through Whatman filter No. 1 and were subcultured every month.

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 × 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 µg/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 µL of nutrient broth instead. Plates were prepared in duplicate and incubated at 37°C for 18 hours; 5 µL 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 (MBC/MIC < 4) or bacteriostatic (4 < MBC/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 highest 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...
Table 1. Susceptibility of Four Standard and Nosocomial Bacterial Strains to Antibiotics in Nutrient Agar

| Antibiotics, µg/disk | S. aureus | E. faecalis | E. coli | P. aeruginosa |
|----------------------|-----------|-------------|---------|--------------|
|                      | 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        | R           | NA      | 23          | NA       | R          | NA        | R          |
| Ceftazidim (30)      | NA        | 23          | NA      | R           | NA       | R          | 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.

indicated that methanol extract of Neem exhibited antimicrobial activity against S. aureus, P. aeruginosa, and E. coli (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 effects 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 con-
versely, ethyl acetate extract was effective on the standard strain of *P. aeroginosa* but not on nosocomial strain.

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.

**Acknowledgments**

We gratefully acknowledge the support of microbial biotechnology laboratory of Isfahan University of Medical
Financial Disclosure: This paper was extracted from the MSc thesis of Leila Maleki and was financially supported by the department of microbial biotechnology of Nour Danesh institute of higher education, Meymeh, Esfahan, Iran.

References

1. Packer J, Naz T, Yaqgel Community E, Harrington D, Jamie JF, Vemulpad SR. Antimicrobial activity of customary medicinal plants of the Yaqgel Aboriginal community of northern New South Wales, Australia: a preliminary study. BMC Res Notes. 2015;8:276. doi: 10.1186/s13104-015-1258-x. [PubMed: 26262212].

2. Sarker SD, Nahar I, Kumasamy Y. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. Methods. 2007;42(4):321-4. doi: 10.1016/j.ymeth.2007.01.006. [PubMed: 17560319].

3. Chanda S, Nair R. Activity of some medicinal plants against certain pathogenic bacterial strains. Indian J Pharmacol. 2006;38(2):142-4. doi: 10.4103/0253-7613.24625.

4. Ahmed MF, Rao AS. Simultaneous determination of phenolic compounds in mela azedarach. Linn leaves by high-performance liquid chromatography. Indian J Appl Res. 2011;1(1):429-31. doi: 10.5373/ijsrr.2011.02.15.03. [PubMed: 22495558].

5. Jafari S, Saeidnia S, Hajimehdipoor H, Ardekani MR, Faramarzi MA, Malekzadeh F, Ehsanifar H, Shahamat M, Levin M, Colwell RR. Antibacterial activity of black myrobalan (Terminalia chebula Retz) against Helicobacter pylori. Int J Antimicrob Agents. 2015;45(4):369-74. doi: 10.1016/j.ijantimicag.2015.04.005.

6. Zargari A. Treatment with plants, Pharmacogenosis [In Persian]. Tehran: Tehran University Publications; 1990. p. 67.

7. Sharma C, Vas AJ, Gheewala TM, Rizvi TA, Hussain A. Ethanolic neem leaves extract on Klebsiella, Salmonella and Staphylococcus aureus. Indian J Pharmacol. 2014;46(4):389-93. doi: 10.4103/0253-7613.141372. [PubMed: 24803564].

8. Banna QR, Parveen F, Iqbal J. Growth inhibitory effect of ethanolic neem leaves extract on <i>Cleobis biplagiatus</i> and <i>Staphylococcus aureus</i>. Bangladesh J Pharmacol. 2014;9(3):347-50. doi: 10.3329/bjp.v9i3.19454.

9. Ferraro MJ. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard. ninth ed. Wayne, PA: NCCLS; 2012.

10. Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolkner HR. Manual of clinical microbiology. 6th ed. Washington DC: ASM Press; 1995. pp. 15-8.

11. Steenkamp V, Mathivha E, Gouws MC, van Rensburg CE. Studies on antimicrobial, antioxidant and fibroblast growth stimulation of wound healing remedies from South Africa. J Ethnopharmacol. 2004;95(2-3):353-7. doi: 10.1016/j.jep.2004.08.020. [PubMed: 15507359].

12. da Costa LS, Andreazza NL, Correa WR, Cunha IS, Ruiz ALTG, de Carvalho JE, et al. Antiproliferative activity, antioxidant capacity and chemical composition of extracts from the leaves and stem of Chresta sphaerocephala. Rev Brasil Farmacogn. 2015;25(4):369-74. doi: 10.1517/14656566.2015.983077. [PubMed: 25483564].

13. Kone WM, Atindehou KK, Terreaux C, Hostettmann K, Traore D, Dosso M. Traditional medicine in north Cote-d’ivoire: screening of 50 medicinal plants for antibacterial activity. J Ethnopharmacol. 2004;93(1):43-9. doi: 10.1016/j.jep.2004.03.006. [PubMed: 15182903].

14. Malekzadeh F, Ehsanifar H, Shahamat M, Levin M, Colwell RR. Antibacterial activity of black myrobalan (Terminalia chebula Retz) against Helicobacter pylori. Int J Antimicrob Agents. 2000;18(1):35-8. doi: 10.1016/s0924-8579(01)00352-1.

15. Reardon S. Antibiotic resistance sweeping developing world. Nature. 2014;509(7499):141-2. doi: 10.1038/509141a. [PubMed: 24803564].

16. Theuretzbacher U. Global antibacterial resistance: The never-ending story. J Glob Antimicrob Resist. 2013;1(2):63-9. doi: 10.1016/j.jgar.2013.03.010. [PubMed: 23873580].

17. Barriere SL. Clinical, economic and societal impact of antibiotic resistance. Expert Opin Pharmacother. 2015;16(2):351-3. doi: 10.1517/14656566.2015.983077. [PubMed: 25483564].

18. Cassell GH. Emergent antibiotic resistance: health risks and economic impact. FEMS Immunol Med Microbiol. 2007;51(1):271-4. doi: 10.1111/j.1574-695X.2006.00305.x. [PubMed: 17560319].

19. Barriere S. Clinical, economic and societal impact of antibiotic resistance. Expert Opin Pharmacother. 2015;16(2):351-3. doi: 10.1517/14656566.2015.983077. [PubMed: 25483564].

20. Saleem M. Natural Products as Antimicrobial Agents - an Update. 2014;1:95-105. doi: 10.4103/0909-4479.141033. [PubMed: 23799992].

21. Sampedro J, Valdivia ER. Antimicrobial compounds. Berlin Heidelberg: Springer; 2013. pp. 83-114.New antimicrobial agents of plant origin.

22. Tirumalasetty J, Anuradha B, Praveena A. Antimicrobial activity of melianthocin extracts of Azadirachta indica indica, Rosmarinus officinalis and Lagenaria siceraria leaves on some important pathogenic organisms. J Chem Pharm Res. 2014;6(11):429-31. doi: 10.7897/2230-8407.04437.

23. Autade RH, Saini S, Reddy PG, Deorukhkar SC, Padmajakshi G. Effect of Psidium guajava against clinically important bacteria at varying pH and temperature. J Ethnopharmacol. 2015;174(1):141-2. doi: 10.1038/509141a. [PubMed: 24803564].

24. Mamman PH, Mshelia WP, Susbatarus SC, Sambo KW. Antibacterial effects of crude extract of Azadirachta indica against Escherichia coli, salmonella spp and Staphylococcus aureus. Int J Med Sci. 2013;10:988-99. doi: 10.7897/2230-8407.04437.

25. Rasoel M, Malik A, Arooj M, Alam MZ, Alam Q, Awan M.. Evaluation of antimicrobial activity of ethanolic extracts of Azadirachta indica and Psidium guajava against the clinically important bacteria at varying pH and temperature. Biomed Res. 2017;28:134-9.