Biomedical and Biopharmaceutical Research
Abbreviation: Biomed. Biopharm. Res.
Volume: 22: Issue: 02 |Year: 2025
Page Number: 214-220

Antimicrobial activity of Azadirachtaindica (neem) leaf extract on common human pathogens in tertiary care hospital

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Article Received:10-02-2025

Article Accepted:27-03-2025

Abstract

Background: Antibiotic resistance is a significant concern and the development of new antimicrobial agents from plants would help to meet the demand with fewer adverse effects. Azadirachtaindica (neem) has played a significant role in therapeutic purposes. This study is focused on the antimicrobial activityofAzadirachtaindica on common human pathogens. **Objective of the study**:Isolation of common pathogens such as E. coli, Klebsiella pneumonia, Acinetobacterbaumanii, Pseudomonas aeruginosa, Staphylococcus aureus, CONS, Enterococcus spp, Candidaspp from clinical specimens and to detect the antimicrobial activity of different antibiotics on common bacterial and fungal pathogenic isolates. This study mainly focuses on the effect of the biomedical extract of Azadirachtaindica leaves on these isolates. **Methodology:** This cross-sectional study was done for three months. Isolates were obtained from various clinical samples and were processed using standard guidelines. Antimicrobial susceptibility testing was done by Kirby Bauer disc diffusion methods. Neem incorporated MHA agar dilution method, Neem incorporated broth dilution method and Neem incorporated AST comparison was performed. **Result:** Among 116 clinical isolates highly resistant gram-positive, and gram-negative bacteria and among fungal isolates Candida albicans were included in this study. Staphylococcus aureus and Candida albicans were sensitive to agar dilution. Neem-incorporated AST for Staphylococcus aureusshowedan increased zone of inhibition to Ciprofloxacin, Ofloxacin, and Cefoxitin. **Conclusion:** The antimicrobial activity of aqueous leaf extract using agar dilution was found to be effective on S. aureus and Candida spp. Neem powder (20% and 50%) incorporated MHA exhibits an increased sensitivity pattern in S. aureus compared to MHA without neem. Aqueous leaf extract exhibited higher antimicrobial activity against S. aureus and can henceforth be used in treatment against staphylococcal infections.

Key words: AST, MIC, MHA, Azadirachtaindica.

INTRODUCTION

Naturally herbal plants have shown a major role in disease curing and prevention through the enhancement of antioxidants and inhibition of bacterial growth. The plant-based therapeutic study was enthusiastically conducted due to their lesser side effects and cost constraints compared to Allopathy[(1&2)]. Neem leaves and their parts were applied in many preventive and curative medicine including cancers in Ayurveda, Unani, Homeopathy, and also in modern medicine [(3&4)]. The therapeutic effect of Azadirachtaindica is based on their constituentsnimbin, nimbidin, nimbolide, and limonoids. Antifungal and antibacterial effects in Azadirachtaindica are based on thepolyphenolic flavonoids of quercetin and ß-sitosterol, which are present in the leaves [(5)].

Our present study was conducted at Saveetha Medical College Hospital and Research Institute in Chennai. Azadirachtaindica leaves are freshly collected from the Sri Jayandra Ayurveda College campus in Chennai. Freshly collected neem leaves wereshade-dried and ground. The coarse powder was extracted through rotary vapor at 40°C. The collected leaf aqueous extract was stored at 2-8°C.

The present study focuses on the antimicrobial activity of Azadirachtaindica leaf extract on MDR- (Multi Drug Resistant) organisms. The organisms were isolated from various clinical samples received in the clinical microbiology laboratory of Saveetha Medical College and Hospital, Chennai.

Thickness, uniformity of the gel, size of the inoculation, temperature, and pH such several factors that affect the accuracy of the result. Hence, these criteria were taken under consideration for obtaining reliable results.

MATERIALS AND METHODS

This cross-sectional study was done on common fungal and bacterial human pathogens from various clinical samples such as blood, urine, respiratory, and exudate specimens for a period of three months (July 2020- September 2020). Samples were collected from the various out-patient and in-patient departments which were received in the clinical Microbiology laboratory in Saveetha Medical College and Hospital, Thandalam, Chennai. The collected samples were processed using standard guidelines.

Among all isolates, multi-drug-resistant organisms were selected for our study. Among fungal isolates Candidaalbicans and gram-positive bacterial pathogens Staphylococcus aureus, Enterococcus faecalis and among gram-negative bacterial pathogens E. coli, Klebsiellapneumoniae, Pseudomonas aeruginosa, Acinetobacterbaumannii were included.

Antimicrobial susceptibility test (Kirby Bauer disk diffusion method)

The isolates were identified by using standard cultural and biochemical characteristics and Antimicrobial susceptibility testing was done on Muller Hinton agar plate usingKirby Bauer disk diffusion method as per CLSI guidelines. AST pattern of gram-negative organisms was done for Gentamycin, Ampicillin, Ceftazidime, Cefoperazone /Sulbactum, Piperacillin-Tazobactum, Imipenem, Meropenem, Amikacin, Cefepime, Ceftriaxone, Ciprofloxacin, Ofloxacin, Cotrimoxazole), AST pattern of gram-positive organisms was done for Vancomycin, Linezolid, Ciprofloxacin, Cotrimoxazole, cefoxitin, rifampicin, tetracycline, amikacin, gentamycin, penicillin, erythromycin, clindamycin. Antifungal susceptibility was done for Fluconazole, Voriconazole, and Amphotericin-B). Plates were analyzed after overnight incubation at 37°C. The plates were examined the next day for the zone of inhibition around the antibiotic disc. The diameter of the inhibitory zone includes the diameter of the disc.

NEEM Collection & preparation:

Well-grown fresh neem leaf samples were collected from Sri Jayandra Ayurveda College in Chennai on February 8, 2020, at 10.30 am. The collected samples were packed in plastic bags and were transported to the Saveetha Research Laboratory. The leaf samples were washed with water to remove dust and foreign particles. The leaves were separated from the stems and were shade-dried at normal room temperature for 7 days. After drying, the leaves were ground into a powder by using a grinder. The leaf powder sample was kept in an amber-colored air-tight container bottle at 4ºC. The powdered sample (420 g) was extracted with a methanol solvent (780 ml) by using a maceration method for 3 days. After extraction, the sample was filtered by using a Whatman No.1 filter paper. The methanol solvent was evaporated by using a rotary evaporator under reduced pressure at 40ºC for 1 h. The crude extract became a semi-solid mass (33.45 g; 7.96%). The methanol semi-solid mass (1.59 g) was transferred into an air-tight screw cap glass bottle for antioxidant activity, stored at 4ºC.

Preparation of Culture Media:

Dehydrated media and standard antimicrobial drugs (discs) were purchased from Hi Media Laboratories Ltd, India. All the media were prepared in sterile glass petri plates (4 mm thickness) according to the manufacturer's instructions.

Microorganisms used:

The bacterial isolates include Staphylococcus aureus, Enterococcus faecalis(Gram-positive) and Pseudomonas aeruginosa, Klebsiella pneumonia, Acinetobacterbaumannii, E. coli (Gram-negative). Fungal isolates such as Candida albicanswere included. The bacterial strains were maintained in Muller Hinton Agar (MHA, pH 7.2) at 37±1ºC, and fungi were maintained in SDA (Sabouraud Dextrose Agar) at 25±1ºC, pH 5.4. The stock culture slants were maintained at 4ºC.

Determination of antimicrobial activity:

The aqueous extracts of leaf A. Indicawere screened for antimicrobial activity by agar well diffusion method. The agar surface was cut with the help of a sterile cork borer having a diameter of 6.0 mm size. All bacterial strains were grown in nutrient broth (NB) at 37ºC and fungal strains Sabouraud dextrose broth (SDB) for 4-6 hours at 25ºC temperatures. The bacterial turbidity of the broth culture was adjusted to 0.50-0.63 McFarland units and for yeast turbidity of the broth was 1.8-2.2 McFarland units. This gives a suspension containing approximately 12 x 106 colony-forming units (CFU)/ml. An aliquot (0.02 ml) of microbial culture was added to molten MHA at 50°C and poured into the Petri plate. After solidification of the agar, appropriate wells were made on the agar surface by using a sterile cork-borer (2-3 wells per 90 mm diameter plate). Different concentrations of the extracts were prepared (500,1000& 2000µg/ml) and 50µl of each concentration was added to the wells. Bacterial cultures were incubated at 37°C for 24 hours and fungal cultures at 25°C for 48 hours.TheAntimicrobial activity was determined by measuring the zone of inhibition surrounding the well.

Determination of Minimum Inhibitory Concentration (MIC):

The minimum inhibitory concentration (MIC) of the aqueous extracts was determined by the micro broth dilution method. For MIC, two-fold serial dilutions of the extracts were prepared (500, 1000, and 2000µg/ml) in micro-tire wells. Incubation of the micro-tire plates was carried out at 37°C for 18-24 hours for bacteria and at 25°C for 48 hours for fungi. After incubation, micro-tire wells were observed for any visible growth. Bacterial suspensions were used as positive control and extracts in broth as negative control. After that micro-tire turbidity analysed the take loop-full of microbial suspensions from micro-tire plates. The loop-full of microbial suspension streaked into the MHA (Muller Hinton Agar) plate for check colony count. The Minimum inhibitory concentration was interpreted as the lowest concentration of the extract that did not show any visible growth when compared to control tubes.

Broth dilution:

MHA was prepared in different volumesin sterile clean glass tubes (10,16&18 ml).

Neem coarse powder was added into a broth at 50°C in various concentrations (10ml broth=10mg, 16ml broth=4mg, 18ml broth=2mg neem powder).

Selected organisms were emulsified with saline water in 0.5 McFarland standard, and then 0.1ml of cultures were added into various concentrations of neem-incorporated broth.

The tubes were incubated overnight at 37°C. A control test was used without neem-added nutrient broth mixed with the same 0.1ml organisms.

Turbidity was compared with the control test. The inoculation loop was dipped in the broth and streaked onto MHA plates and observed for the growth of colony count cfu/ml.

AST comparison after neem incorporation:

PrepareanMHA (Muller Hinton Agar) plate added with various concentrations of Neem leaves aqueous extract (500,1000&2000µg/ml) at 50°C.

Perform AST (Antibiotic Susceptibility Testing) on incorporated MHA plates.

Measure the zone size and zone of inhibition compared.

RESULT

The cross-sectional study was conducted for three months, at Saveetha Medical College and Hospital. Out of 1610 various clinical samples received in the laboratory, 116 clinical isolates were obtained; Among them,multi-drug resistant gram-positive, gram-negative organisms and fungal isolates candida albicans were included in this study.

Out of 116 samples received 64 (55.1%) were male and 52(44.9%) were female. Among those116 isolates, 38 were from the age group 21 to 40, 30 from the age group 41 to 60, 29 from the age group 61 to 80, 11 from the age group (<20), and 8 from the age group (>80). Demographic details of the study subjects are shown in (Table 1).

Table: -1 Demographic details

GENDER	<20	21-40	41-60	61-80	>80
MALE	7	16	18	18	5
FEMALE	4	22	12	11	3

Most of theisolates were from urine samples n= 50(50%) followed by Exudate samples n= 43 (39%), blood and respiratory samples n=22 (11%)

Figure-01 Detection of organisms among various clinical samples

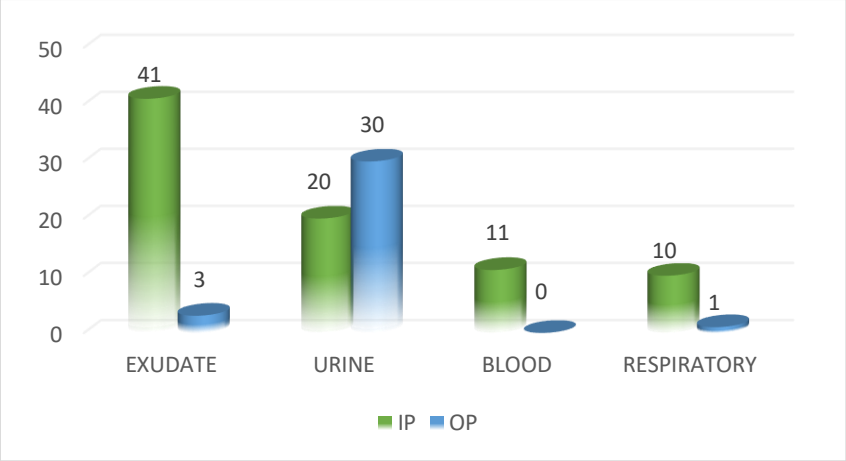


Figure- 05 AGAR DILUTION

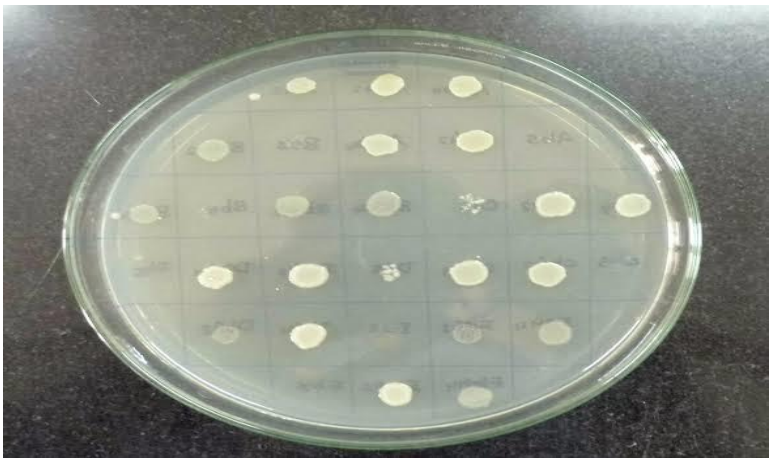
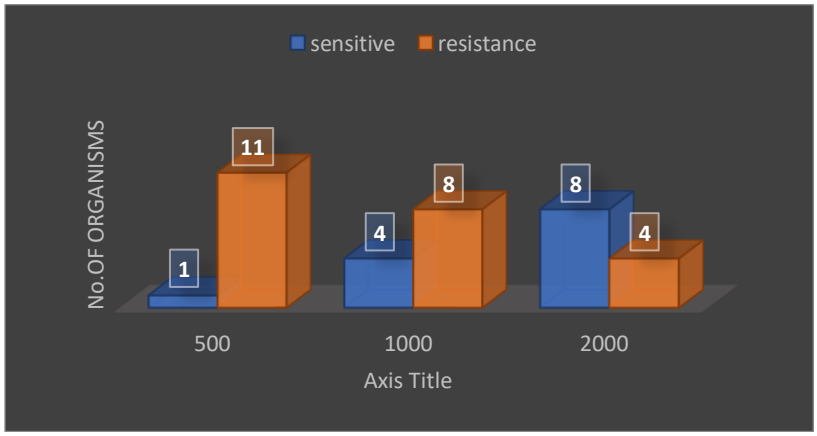


Figure-06

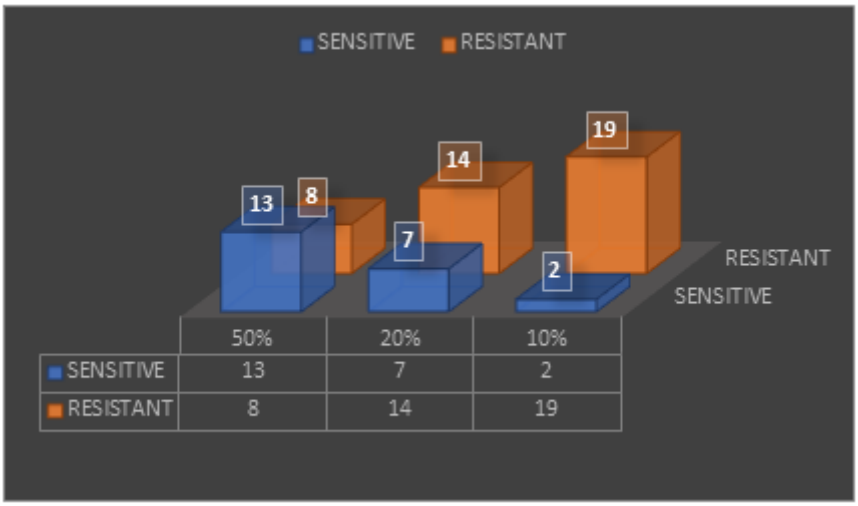


Neem leaf extract concentration(2000µg/ml) showed higher sensitivity to candida in the agar dilution method.
Figure- 07

Neemleaf extract (2000µg/ml) showed higher sensitivity to S. aureus in the agar dilution method.

ABST PATTERNS OF ORGANISMS' BROTH DILUTION

Figure- 08 represents the broth dilution showing different concentrations (50%,20%, and 10%) of neem leaf extractonS. aureus.



Neem leaf extract concentration (50%) showed higher sensitivity to S. aureus in the broth dilution method.

Figure-09:

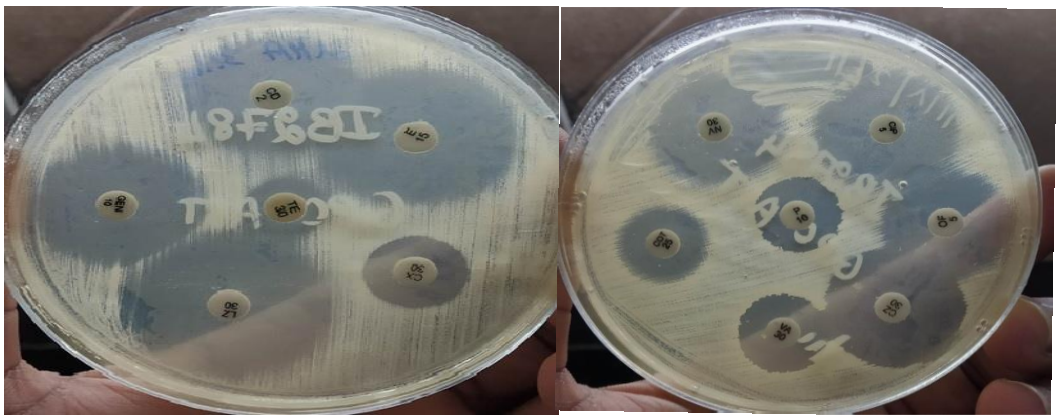
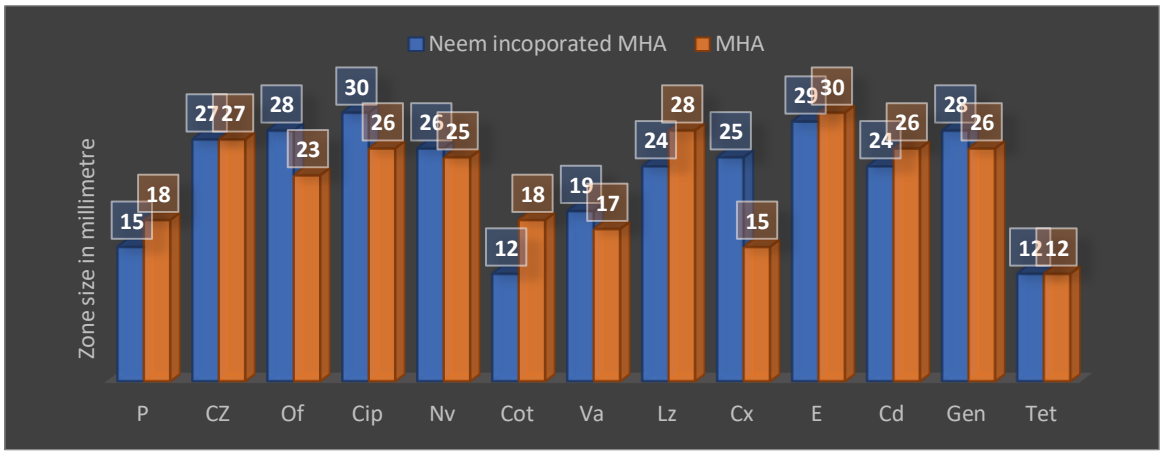


Figure-10:



Figures- 9 and 9&10 represent the Comparison of the ABST pattern in MHA and Neem incorporated MHA, whichshows variations on S. aureus.

DISCUSSION

Antibiotic resistance is a major concern and the development of new agents from plants could be useful in meeting the demand for new antimicrobial agents with improved safety and efficacy [6]. (Srivastava et al 2000). In this study, we have shown that aqueous extracts of neem leaf exhibited the highest antimicrobial activity compared with the bark and seed. The difference in the antimicrobial efficacy could be due to the variable distribution of phytochemical compounds in different parts. Margolone, margolonone, and isomargolonone are tricyclic diterpenoids isolated from stem bark and are shown to exhibit antibacterial activity [7]. (Pennington et al 1981).Nimbidin and nimbolide from seed oil show antifungal, antimalarial, and antibacterial activity including inhibition of Mycobacterium tuberculosis [8].(Rojanpo et al 1985, Khalid et al 1989). However, the presence of high concentrations of azadirachtins, quercetin, and β- sitosterol in A. Indicaleaves might be responsible for strong antibacterial and antifungal activity compared with bark and seed [9]. (Subapriya R. Nagini S 2005. Although crude extracts from various parts of neem have had medicinal applications from time immemorial, very little work has been done on the biological activity and plausible medicinal applications of isolated compounds. Hence drug-development programs could be undertaken to investigate the bioactivity, mechanism of action, pharmacokinetics, and toxicity of com-pounds isolated from neem plants. Newer antimicrobials from plant extracts could also be useful in the food, dairy, and pharmaceutical industries to prevent contamination by limiting microbial growth. The tests performed in the current study compared the antimicrobial efficacy of aqueous extracts of neem leaf, bark, and seed which showed high, moderate, and low anti-microbial activities respectively [10].

CONCLUSION

Naturally herbal plants have shown a major role in disease curing and prevention through the enhancement of antioxidants and inhibition of bacterial growth. The plant-based therapeutic study was enthusiastically conducted due to their lesser side effects and cost constraints compared to Allopathy.In this study, we performed different methods to check the antibacterial and antifungal activity of Neem leaf (Azadirachtaindica).2000µg/ml concentration shows a high sensitivity against Candida species in the MHA agar dilution method and also S. aureusshows high sensitivity. 50% of neemleaf extract broth dilution shows high sensitivity against S. aureus. ABST pattern comparison determined that Neem leaf extract incorporated MHA has a higher sensitivity pattern than non-incorporatedMHA against S. aureus. Through this study, we conclude that neem leaf extract's higher concentration (2000µg/ml) has both antibacterial and anticandidal activity. Further in the future neem leaf extracts can be used in the treatment as incorporated tropical ointment againstS. aureusandCandidal skin and soft-tissue infections.

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