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# ANTIBACTERIAL EFFECT OF METHANOLIC NEEM LEAF EXTRACT ON ESBL, AMPC AND MBL PRODUCERS FROM SKIN AND SOFT TISSUE INFECTIONS.

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**Abstract:** The most common types of beta lactamases produced by Gram negative bacteria causing skin and soft tissue infections (SSTIs) are ESBL, AmpC and MBL. Clinical management of severe skin infections caused by the beta lactamase producer has become very complicated due to the lack of effective antibiotics. Natural products, both as pure compounds or as standardized plant extracts, provide unmatched availability of chemical diversity and hence can serve as a potent antimicrobial agent. The aim of the present study was to evaluate the antibacterial activity of *Azadirachta indica* methanolic leaf extract against ESBL, AmpC and MBL producers, isolated from SSTIs. The isolates were confirmed for ESBL, AmpC and MBL production by phenotypic confirmatory disc diffusion test and E-test. All the selected 50 isolates were also found to be multi drug resistant. The screening for antibacterial activity of *Azadirachta indica* methanolic leaf extract (NLM) was performed using disc diffusion technique. The minimum inhibitory concentration (MIC) of NLM extract was determined using agar dilution technique and was estimated to be in the range of 0.5 - 8% (5 - 80 mg/ml) against the selected isolates. The NLM extract was found to be more effective against the AmpC producers.

**Keywords:** ESBL, AmpC, MBL, *Azadirachta indica,* SSTIs, E-test

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## INTRODUCTION

Infections caused by microorganisms resistant to commonly used antimicrobials have become a major healthcare problem worldwide in the 21st century. Restricted usage of antibiotics, antibiotic cycling, use of combination antibiotic therapy and search for new antimicrobials have been suggested as options to reduce resistance [1]. Skin or tissue infections, skin structure and deep seated soft tissue infections, are general terms used for infections of the entire skin layer, including the subcutaneous and muscle tissue layers and their respective fascia structures [2].

Few years ago, concern related to bacterial SSTIs was centered on Gram-positive bacteria, particularly methicillin-resistant *Staphylococcus aureus, Streptococcus pyogenes* and Vancomycin-resistant *Enterococcus spp.* However, now it has been observed that complicated infections frequently involve Gram negative bacilli and anaerobic bacteria [3, 4].

The Beta lactam (β-lactam) antibiotics are the most commonly used drug for treatment of various Skin and Soft Tissue infections (SSTIs). Resistance to beta lactams is seen most commonly by production of Beta lactamases [5].

Extended Spectrum Beta Lactamases (ESBLs) are typically plasmid mediated clavulanate susceptible enzymes that hydrolyze penicillins, expanded-spectrum cephalosporins, monobactams and are commonly inhibited by beta lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam [6]. AmpC class beta lactamases are cephalosporinases that are poorly inhibited by clavulanic acid. They can be differentiated from ESBLs by their ability to hydrolyse cephamycins as well as other extended-spectrum cephalosporins. Metallo beta lactamases (MBLs) are a type of carbapenemase, which are characterized by the ability to hydrolyse carbapenems and are inhibited by EDTA, chelators of $Zn^{2+}$. They lack inhibition by the commercially available beta lactam inhibitors; Clavulanic acid, Sulbactam, and Tazobactam [7].

Increasing resistance to beta lactam group of antibiotics by the Gram negative pathogens has led to the search of new antimicrobials, from natural products and secondary metabolites of the medicinal plants. *Azadirachta indica* (neem) belongs to Meliaceae family, and is a very important medicinal plant which is traditionally used to treat different diseases. Almost every part of the tree has been used in folklore and traditional system of medicine for the treatment of a variety of human ailments, particularly against diseases of bacterial and fungal origin [8].

Neem leaf has been used to heal wounds, chronic diabetic foot and gangrene developing conditions [9]. Neem elaborates a vast array of biologically active compounds that are chemically diverse and structurally complex. More than 140 compounds have been isolated from different parts of neem. The antibacterial activity of neem might be due to presence of triterpenoids - bitters, phenolic compounds, carotenoids, steroids, and tetratriterpenoids-

azadirachtin [10]. Previous studies indicate that the methanolic extracts of the neem leaves possessed significant antibacterial and antifungal activity [11, 12].

The aim of the present study was to evaluate the antibacterial activity of methanolic extract of the neem leaves on ESBL, AmpC and MBL producers, isolated from skin and soft tissue infections. The study would analyze whether neem leaves could be used as an alternative therapeutic agent against beta lactam resistant organisms.

## Materials and Methods

### Bacterial strains

Gram negative isolates from Skin and soft tissue infections of patients from a tertiary care hospital in Mumbai were identified by using standard Microbiological methods.

### Plant Extract

Fresh *Azadirachta indica* leaves were purchased from local market. The leaves were separated, washed and dried in shade. Ten grams of dried and grounded leaves were transferred into a flask containing 150 ml of methanol and Soxhlet extraction was carried out. The extract was filtered and the solvent was evaporated. The dried powder obtained after solvent evaporation was dissolved in 50% Dimethyl sulfoxide (DMSO) to obtain a concentration of 500 mg/ml. The prepared extract was stored at 4°C for further use in the study after sterility testing of the extract [13].

### Antimicrobial susceptibility Test (AST)

The antimicrobial susceptibility was determined by Kirby-Bauer disk diffusion method in accordance with CLSI guidelines using commercially available antimicrobial discs (HiMedia, Mumbai). The following antibiotics were used- Ampicillin (10 µg), Amikacin (10 µg), Ciprofloxacin (5µg), Gentamicin (10 µg), Amoxyclav (30 µg), Ceftazidime (30 µg), Cefoxitin (30 µg), Imipenem (10 µg), Meropenem (10 µg), Piperacillin (10 µg), Piperacillin-Tazobactam (100/10). Susceptibility testing was performed on Mueller-Hinton agar using culture suspensions having turbidity equivalent to McFarland 0.5 standard.

All isolates which were resistant to Ceftazidime/Cefoxitin/Imipenem as per the CLSI (2011) susceptible breakpoints were screened for beta lactamase production by the confirmatory tests [14].

**Confirmatory Phenotypic Disc diffusion test**

Confirmatory phenotypic disc diffusion test was performed on Mueller-Hinton agar using culture suspensions having turbidity equivalent to McFarland 0.5 standard.

For ESBL producers - Ceftazidime (CAZ, 30µg) - Ceftazidime/Clavulanic acid (CAC 30/10, HiMedia) were used for ESBL detection. If there was ≥ 5 mm increase in the inhibition zone diameter of Ceftazidime/Clavulanic acid versus Ceftazidime alone, the isolate was considered as an ESBL producer [14].

For AmpC producers - Cefoxitin (CX 30µg) - Cefoxitin/Cloxacillin (30/200) discs (HiMedia) are used for AmpC detection. An increase of ≥4 mm in the inhibition zone diameter of Cefoxitin/Cloxacillin acid versus Cefoxitin alone indicated AmpC production [15].

For MBL producers - Imipenem–EDTA (10-750 µg) disc was used for detection of Metallo-β-lactamases producers. An increase of ≥7 mm in the inhibition zone diameter of Imipenem/EDTA versus Imipenem alone indicated MBL production [16].

**E-test**

ESBL and AmpC detection Ezy MIC™ strips (EM081, HiMedia, Mumbai) are drug-impregnated strips in which upper half contains a concentration gradient of 4 antibiotics; Ceftazidime, Cefotaxime, Cefepime and Cloxacillin plus Clavulanic acid and Tazobactam (MIX + Clav-TZ) and lower half contains of Ceftazidime, Cefotaxime, Cefepime and Cloxacillin in a concentration gradient in a reverse direction. The isolates were reported and confirmed as ESBL and AmpC beta lactamase producer as per the application sheet supplied by the manufacturer. These strips are to be used along with pure ESBL detection strips (EM079 HiMedia, Mumbai) to avoid false positive results.

The phenotypic MBL detection strip is coated with mixture of Imipenem+ EDTA and Imipenem on a single strip in a concentration gradient manner. The upper half has Imipenem + EDTA with highest concentration tapering downwards, whereas lower half is similarly coated with Imipenem in a concentration gradient in reverse direction. The isolates were reported and confirmed as Metallo beta lactamase producer as per the application sheet supplied by the manufacturer.

E-test was performed on Mueller-Hinton agar using culture suspensions having turbidity equivalent to McFarland 0.5 standard [14]. These phenotypically confirmed ESBL, AmpC and MBL producers were further used in the study

A standard reference strain of *Escherichia coli* (ATCC 25922), susceptible to all antimicrobial drugs tested, and positive control strain *Klebsiella pneumoniae* ATCC 700603 were used as a quality control for antimicrobial susceptibility test, confirmatory phenotypic disc diffusion test and the E-test, as per CLSI(2011) guidelines.

**Antibacterial activity of *Azadirachta indica* leaves**

Antibacterial activity of neem leaf extract (NLM) was carried out by disc diffusion method using Mueller Hinton agar. The turbidity of the culture was adjusted to 0.5 McFarland standards. Sterile paper discs (6 mm, HiMedia, Mumbai) were impregnated with 20 $\mu$l of the 500mg/ml NLM extract and placed on the inoculated agar. For the positive control, a disc of Imipenem (10µg) and for negative control, disc impregnated with DMSO were placed on the inoculated Mueller Hinton agar. The plate was incubated at 37°C for 24 hours. The experiment was performed in triplicate [14].

**Minimum Inhibitory Concentration (MIC)**

The MIC of NLM extract was determined by agar dilution method. For MIC of NLM, dilutions were prepared by mixing NLM extract with sterile Mueller Hinton agar to get final concentrations ranging between 0.25% - 8% (2.5 -80 mg/ml). A plate of Mueller Hinton agar with DMSO served as a control. These plates were seeded with bacterial suspensions and were incubated at 37°C for 24 hr. The MIC was recorded as the lowest concentration of NLM extract at which visible bacterial growth was completely inhibited. The experiment was performed in triplicate [17].

**Statistical analysis**

Using the parametric and non-parametric tests, significance of difference between the mean/median of minimum inhibitory concentrations of the NLM extracts between the three beta lactamase groups were determined. If the p value was ≤ 0.05, then the difference was considered to be statistically significant [18].

**Results**

In-vitro resistance of the isolates to Ceftazidime, Imipenem and Cefoxitin during the AST studies indicated the production of ESBL, MBL and AmpC beta lactamase, respectively. This was confirmed by phenotypic confirmatory disc test and E-test.

Out of the 50 isolates positive for production of beta lactamases, 34, 9 and 7 isolates were found to be ESBL, AmpC and MBL producers, respectively. 50% of ESBL producers (17/34) and

44% of AmpC producers (4/9) were found to be *E.coli*, whereas 86% (6/7) of MBL producers were *Acinetobacter* spp. isolates (figure 1).

The AST studies revealed that carbapenems and aminoglycosides were the most effective antibiotics i.e. the isolates showed least resistance towards them in comparison with other antibiotics. None of the ESBL and AmpC producers exhibited resistance towards carbapenems. The aminoglycosides and the beta lactam inhibitor group were more potent against ESBL producers in comparison to AmpC and MBL producers. All the three groups exhibited a higher resistance rate towards quinolones in comparison with carbapenems and aminoglycosides (figure 2).

The MBL producers were observed to be exhibiting 100% resistance against carbapenems, quinolones and beta lactam inhibitor. This indicates that infection caused by MBL producing bacteria would be the most difficult to treat as it is resistant to most of the antibiotics used. Amongst the two beta lactamase inhibitors, Piperacillin – tazobactam was more effective than Amoxyclav against all the clinical isolates. All the 50 isolates were found to be multi drug resistant i.e. they were resistant to 3 or more than 3 groups of antibiotics.

Primary screening for in-vitro antibacterial activity of NLM was carried out by disc diffusion method. The extract showed activity against all the 50 isolates from SSTIs producing ESBL, AmpC and MBL. The range of mean ZOI for NLM was 9 mm-13 mm. No zone of inhibition was seen for DMSO used as control.

The mean ZOI for ESBL producers was 9.8 ± 1.9 mm, for AmpC producer it was 12.4 ± 3.2 mm and for MBL producer it was 12.2 ± 3.6 mm. The MIC range of the NLM extract against all beta lactamase producers was found to be 0.5% - 8% (5.0 -80 mg/ml). The average MIC of NLM extract for all isolates was 6.7% (67 mg/ml). Growth of all isolates was observed on DMSO control plates (figure 3).

The difference in antibacterial activity of NLM between ESBL and AmpC group was statistically significant ($p < 0.05$). However, there was no statistically significant difference in the antibacterial activity between the other groups ($p > 0.05$). Thus the antibacterial activity of NLM extract was found to be more enhanced for AmpC group of beta lactamase producers.

**Figure 1- Incidence of beta lactamase producers**

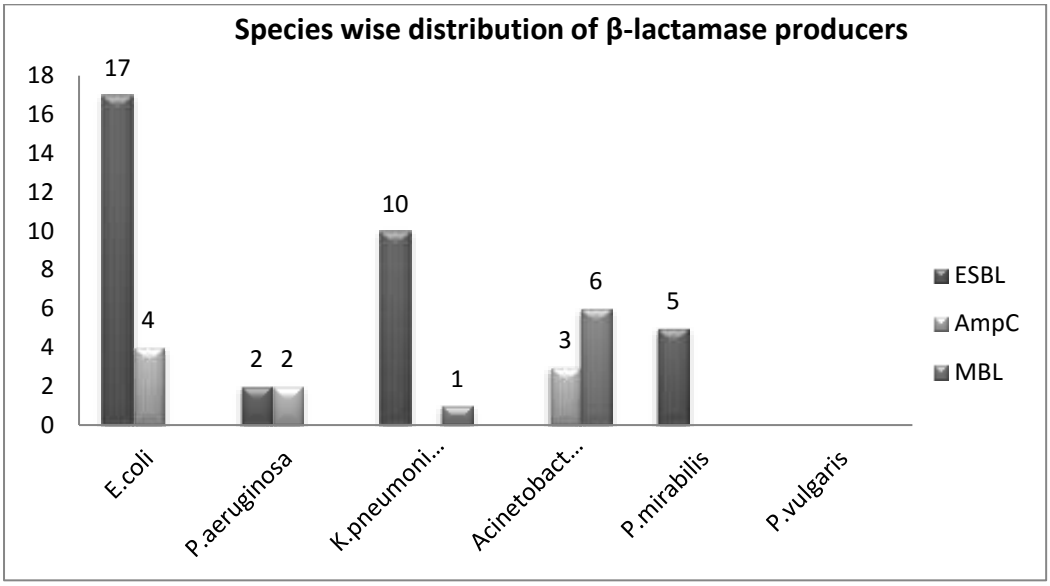



**Figure 2- Rate of antibiotic resistance amongst beta lactamase producers**

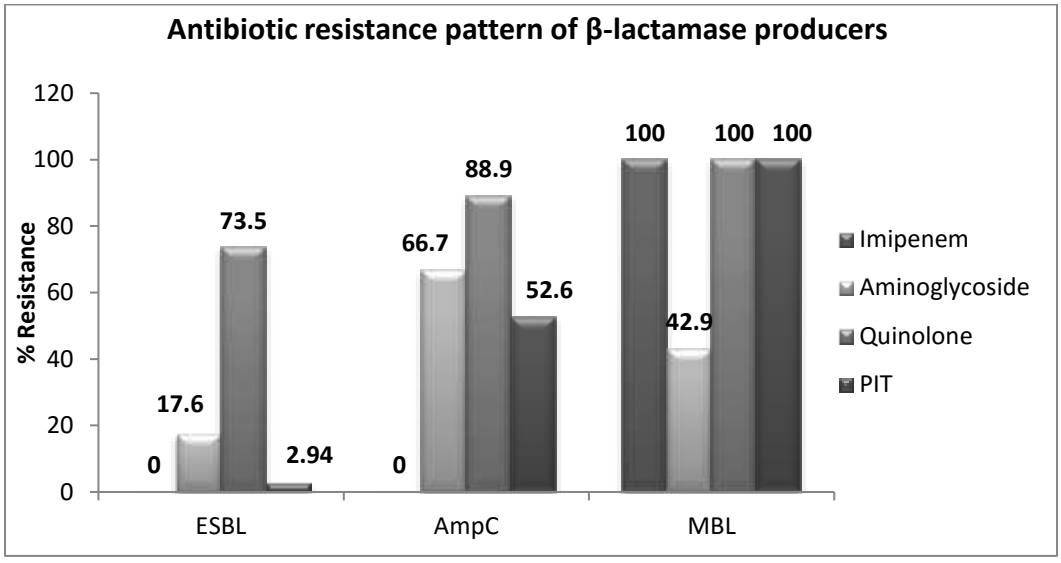
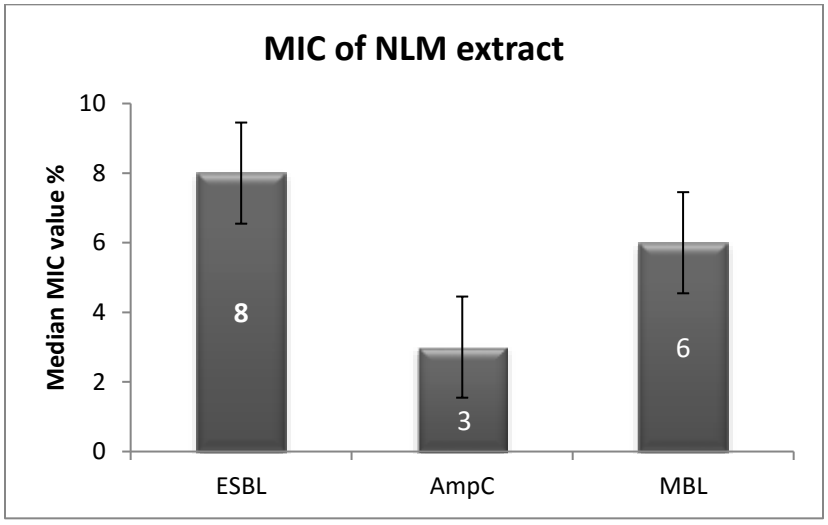


**Figure 3. Comparison of median of MIC of NLM against the three beta lactamases group**




**(Error bars with Standard error)**

## DISCUSSION

In the current study the isolates from SSTIs were resistant not only to beta lactam antibiotics but also were resistant to other group of antibiotics used in the study, indicating therapeutic failure, if treated with these groups of antibiotics. The isolates were more sensitive to carbapenems – Imipenem and Meropenem, justifying their usage in empirical therapy. The emergence of MBL producing bacteria is of significant concern due to its resistance to carbapenems and thus suggests judicious usage of carbapenems in therapeutics. The aminoglycosides like Amikacin and Gentamicin were effective under in-vitro conditions, against the beta lactamase producers.

In the current study, amongst the ESBL producers, *E.coli* and *K.pneumoniae* isolates had the highest incident rate. These results were comparable to a study carried out in tertiary care hospital in Mumbai, by Shinde et al. [19]. In this study, there were no ESBL producers amongst *Acinetobacter spp.*, but the production of carbapenemase was the highest amongst all other clinical isolates. This result was in accordance with Bandekar et al., [20] which also showed a predominance of *Acinetobacter spp*. in MBL production.

In the current study, incident rate of *P. aeruginosa* was lower than the rates obtained in the studies by Raifee et al. [21] and Khan et al. [22]. The low rates of β-lactamase production by *P.aeruginosa* isolates in our hospital setup may be due to acquiring other modes of resistance towards β-lactam antibiotics by the isolates, like modification of penicillin-binding-proteins (PBPs) or porin deficiency.

Antibacterial activity of NLM was studied using disc diffusion test and the extract inhibited all the strains indicating a potent antibacterial activity against resistant pathogens. This justifies the use of neem in healing of SSTIs since ancient days. Our results are in agreement with the findings of previous studies of Koona and Budida, [10] and Chaturvedi et al [23]. In the current study, the mean inhibition zone size for beta lactam resistant isolates by NLM was comparable with the study of Dahiya and Purkayastha [24] and Chaturvedi et al. [23].

The average MIC of NLM extract against the selected clinical isolates was determined to be 6.7% (67 mg/ml). A lower range of MIC value (1.56 – 12.5 mg/ml) was reported by Dahiya et al. [24], against isolates sensitive to beta lactams. Another study by Emmanuel et al. [25] also reported a comparatively lower MIC (12.5 - 25 mg/ml) against isolates sensitive to beta lactams. The higher MIC values in our study against the isolates may be attributed to β-lactamase production by the clinical isolates.

In the current study, based on mean ZOI and median MIC value, it was analyzed that NLM extract was more effective against the AmpC producers. This difference in antibacterial activity of NLM was statistically significant ($p < 0.05$). Thus it can be stated that NLM might have a selective activity against AmpC producers. There is a paucity of information on comparison of antibacterial activity of neem leaves against different types of β-lactamase producers. To the best of our knowledge comparative study of antibacterial activity of neem leaves against various types of beta lactamase producers has not been reported earlier.

**CONCLUSION**

The present findings add substantially to our understanding that new antimicrobial agents are required for controlling the rising rate of antibiotic resistant pathogens. The results of the in-vitro study suggest that the methanolic extract of neem leaves had significant antibacterial activity against the isolates resistant to beta lactam antibiotics. India is rich in its diversity of natural products. These products are easily available and cost effective. Natural products have multiple target sites; hence the development of resistance against them will be difficult. Natural products can provide lead molecules which can be used for drug development. Thus this study vindicates the usage of natural products for therapeutic purposes.

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**CONFLICT OF INTEREST STATEMENT**: The authors declare that they have no competing interest with whomsoever.

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