

Anti-Bacterial Potential of Azadirachta Indica Extract Against Staphylococcus Aureus

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ABSTRACT

Background: Various parts of the Azadirachta indica (Neem) tree have shown effects against microbes of wide variety. Cheaper & newer antimicrobial agents with greater efficacy and safety may be developed by screening this medicinal plant for bioactive compounds.

Aim: To evaluate the in vitro antibacterial activity of Neem leaves extract (Azadirachta indica extract) against clinical isolates of Staphylococcus aureus & methicillin resistant Staphylococcus aureus by Agar Dilution Method.

Method: The in vitro activity of Neem leaves extract against Staphylococcus aureus (n=33); including 17 methicillin resistant Staphylococcus aureus clinical isolates was determined. Standard agar dilution method was used for the determination of Minimum inhibitory concentration of Neem leaves extract with different dilutions of Neem. Standard control strains ATCC 22922 Staphylococcus aureus and ATCC 33591 Staphylococcus aureus were used.

Results: Strong antimicrobial activity was exhibited by Azadirachta indica leaf extract antagonist to all bacterial species under study at all the concentrations tested. The range of different dilutions of Neem used against Staphylococcus aureus and MRSA showed that for Staphylococcus aureus the dilution range is 4-8mg/ml and for MRSA this value ranges between 2-4 mg/ml. MIC₅₀ of Neem leaves extract was comparable for staphylococci and methicillin resistant S. Aureus (8mg/L); however at MIC₉₀Neem leaves extract was twofold more active against methicillin resistant S.aureus(4mg/L) than staphylococci (8mg/L)

Conclusion: Azadirachta. indica (Neem) leaf extract exhibited stronger antibacterial activity against methicillin resistant Staphylococcus aureus strains (MRSA) than Staphylococcus aureus.

Keywords: Azadirachta indica, Staphylococcus aureus, methicillin resistant Staphylococcus aureus (MRSA)

INTRODUCTION

Staphylococcus aureus is one of the most common source of life threatening bacterial infections resulting in significant despair and mortality. Therapeutic agents of choice for S. aureus infections are semi-synthetic penicillin's. Rapid spread of strains resistant to the semisynthetic penicillin's, have become a global problem and newer antibacterial compounds to control MRSA are needed.¹ Increased resistance to bacterial isolates has led to the development of alternatives to available antibiotics for disease treatment¹.

A great number of plants are useful as pharmaceuticals as they accumulate organic substances in sufficient quantities. Plants have served as a rich source of medicines as they yield wide variety of bioactive molecules, many of which might have emerged as chemical warfare to ward off preys & infection².

Use of herbs as traditional medicine & their ability to cure is well reported since ancestral times. Plants grown in this region have not been systematically tested for their biological and antimicrobial activity.

It has been customary to use different parts of neem tree as a remedy against various human diseases since ancient times. One of the active principles present in Neem is Azadirachtin³ Azadirachtin is one of the many biologically active compounds obtained from Neem plant. It is an admixture of isomeric compounds out of which azadirachtin E has found to be more effective⁴

Neem has been used in alternative medicine extensively and is becoming an essence of present day

medicine, in Pakistan, India, Australia, China, and in Asia. It has been used in traditional medicine. It exhibited prominent antimicrobial activity against MRSA & Staph .aureus⁵.

The study aimed to check antibacterial activity (in vitro) of Neem leaves extract (Azadirachta indica extract) against clinical isolates of Staphylococcus aureus & MRSA by Agar Dilution Method.

MATERIALS & METHOD

The plant selected for study was Neem plant (Azadirachta indica). 100gm of dried Neem leaves were purchased from local market at Rawalpindi, identified by phytologist and taxonomist at National University of Sciences and Technology, Islamabad.

Leaf extract: The leaves were washed thoroughly with sterile distilled water and were then sun-dried. The sun dried leaves were crushed by pestle and mortar to smaller size and finely grated in blender and for successive extraction with methanol and ethanol was processed in soxlet extractor. Liquid extracts obtained were evaporated followed by concentration in vacuum at 40°C. Evaporation to dryness was done followed by storage in air tight bottle at 4°C.

Ethanol Extract

Dessicated & powdered leaf extract (50 gram) of Azadirachta indica was taken in a container, to which 250 ml of ethanol was added. It was kept for twenty-four hours & was regularly shaken followed by collection of filtrate.

Repetition of this procedure was done thrice. The collected filtrates were pooled.

Bacterial strains: Total fifty (50) clinical isolates of *Staphylococcus aureus* and MRSA were used and collected during September 2010 to November 2011, obtained from the Microbiology department of Armed forces Institute of Pathology (AFIP), Rawalpindi. The isolates were grown on nutrient agar and golden yellow colonies were observed after 24 hrs. On mannitol salt agar, they changed the colour of medium from red to yellow. These bacteria were stored in microbank and served as test pathogens for antibacterial activity assay.

Antimicrobial susceptibility testing: Modified Kirby-Bauer disk diffusion method was used as per recommendation of Clinical Laboratory Standards Institute (CLSI) (Oxoid, Basingstoke, U.K) for Antimicrobial susceptibility testing⁶

Antibacterial activity assay

Preparation of sterile glass bottles & petri dishes for agar dilution method:-

Mueller-Hinton agar was used for agar dilution method.

Dissolution of 38grams of base powder was done in 1 liter of distilled water followed by autoclaving. After autoclaving the agar was allowed to cool and was poured in sterile glass bottles on a leveled surface; each bottle contained 20 ml of the media. The bottles were solidified at room temperature.

Prior to use, the bottles were kept in water bath at 50°C & then micropipette was used to pour the extract into bottle, followed by mixing and pouring into petri dishes. Finally plates with different concentration of the extract (0.25, 0.5, 1, 2, 4, 8, 16 mg/ml) were prepared. At room temperature, these were allowed to solidify. Prior to use, the bottles were placed for five minutes in hot air oven so that no moisture remains on the surface.

Agar dilution method: One strain of *Staphylococcus aureus* was selected for screening the inhibitory effects of Neem leaves extract on *Staphylococcus aureus*. This was done to observe the zones of inhibition at different concentrations that would help to perform MIC later.

Inoculum preparation: Nutrient Broth was made according to manufacturer's details. It was autoclaved for 30 minutes and then under aseptic conditions was allowed to cool down to 45°C. It was poured in autoclaved petri dishes. With the help of wire loop, heated under flame, 2-3 colonies of organism were scraped and inoculated into the nutrient broth containing dishes. The tubes were sealed and placed in incubator for 24 hours under 37°C temperatures, adjusting to 0.5 McFarland Standards. Sterile normal saline was used to dilute until the turbidity was comparable to standard.

Procedure: Neem leaves extracts were mixed with autoclaved Muller Hilton agar at 50°C, vigorously vortexed and placed into Petri dishes (Greiner bio-one, Austria) having 90mm diameter. Drying of poured plates was done at 45°C for about 10 to 15 minutes.

Separated colonies were emulsified from overnight blood agar (4-5), which was done in 5 ml of sterile distilled water according to 0.5 McFarland's standard. Multipoint inoculator (Denley, U.K) was used to inoculate the extract incorporated plates. Plates were incubated at 37°C for 18 hours and observed for growth⁷.

Drying of the poured plates was done at 45°C for about 10 to 15 minutes. Three control plates were instituted in likewise manner at the same instance.

1. For the confirmation of culture viability, MH agar which was inoculated with various strains was employed.
2. Second control plate consisted of only the medium.
3. For checking the sterility of the medium & the extract a third medium with extract was used

Determination of MIC: Blood agar was used to subculture isolates of *Staphylococci* & then overnight incubation at 35 °C was done. Sterile isotonic saline was used for the emulsification of morphologically similar colonies (3-5) & calibration of suspension was done according to 0.5 McFarland standards (10⁸ CFU/ml).

Dilution of suspension was taken 1:10 in sterile saline. Concentration of inoculate was 10⁷CFU/ml. Mueller Hilton agar plates were spot inoculated with inoculums which contained successive dilutions in duplicates from 0.015 to 8.0 mg/L of Neem leaves extract with a multipoint inoculator .

After 18-24 hours of incubation, MIC reading was taken against non-reflecting & dark surface as the initial antibiotic concentration that checks the evolvement of the organism completely. The faint haze presence caused by the colony or inoculum was ignored as growth. Interpretation of the results of MIC were in accordance to the breakpoints given by CLSI 2007.

ATCC 22922, ATCE-33591 of *S. aureus* were used as reference strain. To make sure about the replicability of the results experiment was performed in triplicate.

RESULTS

MIC of Neem ranges between 4-8mg/ml, and at concentration of 8mg/ml maximum bacteria are inhibited i.e 23 out of 32 isolates of *Staphylococcus aureus*. MRSA shows MIC range of 2-8µg/ml and out of 17 MRSA, 4 are inhibited at 2mg/ml, 10 at concentration of 4 mg/ml, and 3 isolates are inhibited at 8 mg/ml which shows that they are resistant at concentration range between 0.25 –1 mg/ml. At MIC of 16 mg/ml, no growth was observed as MIC ranges from 4-8 mg/ml. Table 1 shows the MIC of Neem against *Staphylococcus aureus* and MRSA.

Table 2 summarizes the MIC 50 and MIC 90 of Neem against *Staphylococcus aureus* and MRSA. The MIC 50 of *Staphylococcus aureus* is 8 mg/ml, and that of MRSA is also 8mg/ml. The MIC 90 of *Staphylococcus aureus* is 8 mg/ml, and that of MRSA is 4mg/ml. For comparison purpose we have used geometric mean, which clearly showed that for *Staphylococcus aureus* it is 6.4 and for MRSA it is less i.e. 3.8 which showed its pronounced antibacterial activity with a MIC range of 2-8 mg/ml.

Regarding MIC range of Neem it can be interpreted from the fig 1, bar chart that 88% of *Staphylococcus aureus* are inhibited at MIC range of 4-8 mg/ml and 12% are inhibited at concentration range of 2-4mg/ml at the same time 76% are inhibited at 4-8 mg/ml. 12% of MRSA are inhibited at 2-4mg/ml. That concludes that MIC of Neem is same for *Staphylococcus aureus* and MRSA with a concentration range of 4-8 mg/ml.

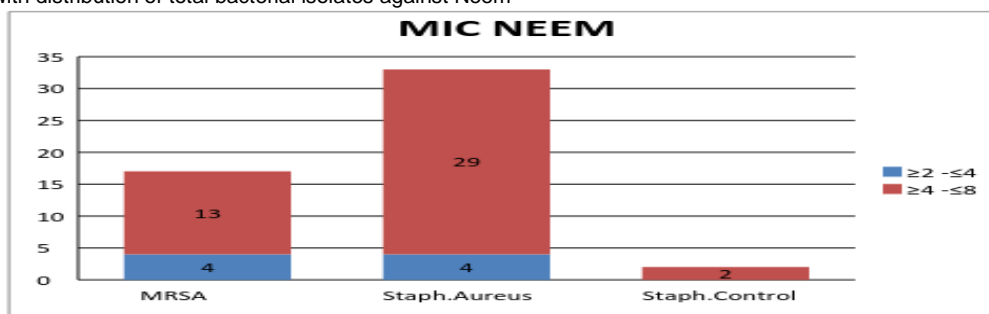
Table-1: Percentage of isolates susceptible at different MIC of Neem

Specimen (n=52)	Antimicrobial agent (Neem)	% of isolates susceptible at MIC (mg/ml)						
		0.25	0.5	1	2	4	8	16
Staph. Aureus (n=33)	Neem	0	0	0	0	10	23	0
MRSA (n=17)		0	0	0	4	10	3	0
Staph. Control (n=2)		0	0	0	0	1	1	0

Table 2: Summary for MIC Neem

Summary for MIC Neem				
Specimen	MIC 50	MIC 90	Geometric mean	MIC range
Staph. Aureus	8	8	6.48	4.8
MRSA	8	8	5.66	4.8
Staph Control	4	8	5.66	4.4

Fig 1: MIC range with distribution of total bacterial isolates against Neem



DISCUSSION

Newer drugs development on plant-based compounds causing minimal side-effects is required to meet the demands, as the existing synthetic drugs cause various side effects. A.indica leaves have activity against bacteria which promise the potential of bioactive compounds and justify usage of Neem plant in primary healthcare⁸.

Several researchers have investigated antibacterial efficacy of the neem leaves showing similar results. Our study showed antibacterial efficacy of Neem leaves extract against all tested bacterial strains

Subapriya and Nagini reported that the strong antibacterial and antifungal activity of A. indica was due to the high concentrations of azadirachtins, quercetin and β -sitosterol⁹. Maragathavalli et al. compared the antimicrobial action of ethanolic extracts of Neem & gentamycin against pathogenic bacteria. Maximum inhibition on Bacillus pumillus, Pseudomonas aeruginosa and Staphylococcus aureus was reported by ethanolic extract of Neem¹⁰.

On the other hand, Aslam F and co-workers⁹ checked the action of Neem extract on three bacterial strains: Staphylococcus aureus, Corynebacterium bovi and E.coli. They compared the antimicrobial activity of aqueous extracts of A. indica leaves against human pathogenic bacteria (Staphylococcus aureus, Enterococcus faecalis, Proteus mirabilis and Pseudomonas aeruginosa). Strong antimicrobial activity against these bacteria was exhibited by leaf extract at all the concentrations tested (500, 1000 and 2000 μ g/ml)¹¹.

CONCLUSION

A. indica (Neem) leaf extract possess antibacterial activity against different strains of disease causing bacteria . Bioactive compounds effective against bacterial strains

may be isolated & separated using advanced scientific techniques.

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