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Antibacterial effect of Neem oil on Methicillin resistant *Staphylococcus aureus*

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Abstract
Methicillin resistant *Staphylococcus aureus* (MRSA) is an important hospital acquired pathogen, usually resistant to many antibiotics. This study was aimed at determination of antibacterial effect of Neem oil (*Azadirachta indica*, N.O. *Meliaceae*) on MRSA. Antibacterial effect of Neem oil (Medinova chemicals, Bangalore) was studied using 107 strains of MRSA isolated from different clinical specimens. Time kill assay (undiluted Neem oil & 10^6 bacteria/ml) was performed and the surviving bacteria were detected by culture on blood agar. Minimum Inhibitory Concentration was determined by agar dilution method (10% to 50% concentration of Neem oil in 20 ml of molten nutrient agar by inoculation of 10^4 CFU /spot on MHA. Out of 107 strains of MRSA, 56 (52.33%) were killed after 1hr exposure to Neem oil, 22(20.56%) were killed after 2 hr exposure, 19(17.75%) were killed after 4 hr & remaining 10(9.34%) were killed after 12 hr exposure to Neem oil. All MRSA strains were killed by Neem oil. On agar dilution, 90 (84.11%) of strains were inhibited at 10% concentration of Neem oil, 14 (13.08%) at 20% and remaining 3(2.80%) were inhibited at 30% concentration of Neem oil. The results are suggestive of antibacterial effect of Neem oil on multidrug resistant MRSA. Since Neem oil exhibited bactericidal effect on MRSA, it may find clinical application as a topical antibiotic for MRSA infections.

Keywords: Antibacterial activity, MRSA, MIC, Neem Oil.

1. Introduction
Staphylococcus aureus continues to be an important pathogen due to its versatility, types of diseases caused, virulence factors and drug resistance. Methicillin resistant *S. aureus* (MRSA) emerged in the 1960s, making *S. aureus* resistant to many antibiotics. MRSA is a significant pathogen causing both health care-associated (HA-MRSA) and community-acquired (CA-MRSA) infections worldwide including India [1].
Overall the rate of methicillin-resistance among large hospitals in India with *S.aureus* is nearly 32%. According to a study from Yenopoya Medical College, Mangalore out of total of 237 isolates of *S.aureus* were studied, 69 (29.1%) were found to be methicillin-resistant [2].
Recent increase of methicillin-resistant and multiple-resistant strains started to pose great difficulty in selecting antimicrobial agents for the management of the infections they cause at large hospitals. Cephalosporins and other beta-lactam antibiotics have been shown to be clinically ineffective even though certain vitro tests such as the standard disk diffusion test would suggest that the strains are susceptible [3]. Heterogeneous resistance to the beta-lactam antibiotics and cephalosporins is also responsible for the problems encountered in detecting MRSA [4, 5]. Resistance to erythromycin, clindamycin, tetracycline, aminoglycosides, and chloramphenicol has also been reported with MRSA strains [4, 5].
Presently, emperical vancomycin therapy is strongly considered for serious *S. aureus* infections and for patients with significant risk factors for previously-acquired nosocomial MRSA. But the higher price of vancomycin, its unavailability in many parts of the country, and also the possibility of emergence of resistance to the drug should at least make the clinicians look into the alternatives. Hence it becomes important to search for potential alternative antibacterial agent against MRSA.
Nimbidin, a major crude bitter principle extracted from the oil of seed kernels of *Azadirachta indica* has antifungal, antibacterial, antimalarial, antiviral, antifertility, anticarcinogenic and hepatoprotective properties [6]. Neerja M *et al*, have discussed the known/novel pathways that are involved in the synthesis of biologically active compounds from Neem oil by Next generation sequencing [7]. Oil from Neem leaves possess antibacterial activity against a wide spectrum of Gram negative and Gram positive microorganisms, including *M. tuberculosis* and Streptomycin-resistant strains [8, 9, 10]. A study from Manipal assessed that Neem mouthwash

inhibited *Streptococcus mutans* in saliva [11].

While testing non water soluble antimicrobials such as essential oils, it is necessary to incorporate an emulsifier or solvent into the test medium to ensure the contact between the test organism and the agent for the duration of the experiment. However, this may have certain problems like changes in the physicochemical properties of the test system resulting in either enhancement or reduction in the antimicrobial activity and separation of oil-water mixture during incubation period [12]. The incorporation of low concentrations of bacteriological agar as a stabilization of oil- water mixture has been suggested as an alternative which overcomes these disadvantages [13].

Literature search gave very few studies attempting to study antibacterial effect of Neem oil. Hence the objective of the study was to determine the antibacterial effect of Neem oil on MRSA and the use of agar based dilution method to determine MIC of Neem oil.

2. Material and Methods

Bacterial strains: One hundred and seven MRSA strains isolated from various samples in exudates section (84 from pus, 15 from catheters and 8 from synovial fluid) for culture and sensitivity in Department of Microbiology, over a period over one year from February 2012 to March 2013 were included in the study.

Susceptibility testing: Clinical Laboratory Standards Institute (CLSI) recommends disk diffusion test using cefoxitin (30 µg) disc for identifying methicillin resistance as it is a better inducer of mecA gene. MRSA were confirmed by the 30 µg cefoxitin disc by the Kirby Bauer's disc diffusion method [14]. Mueller –Hinton broth culture of the test organism adjusted to McFarland 0.5 standard was used as the inoculum. Swabbing was done on Mueller – Hinton agar plate followed by testing with cefoxitin (30 µg), Amoxycilin-clavulinic acid (30 µg), Vancomycin (30 µg), Amikacin (30 µg), Doxycycline (30 µg), Ciprofloxacin (5 µg), Chloramphenicol (30 µg), Azithromycin (15 µg), Clindamicin (2 µg), Trimethoprim-sulphamethoxazole (25 µg) and incubated for 35 ºC for 24 hours. Standard strain of *Staphylococcus aureus* ATCC25923 was used. Zone diameter > 22 mm was interpreted as sensitive and < 21 mm as resistant.

Time kill studies: Bactericidal activity of Neem oil on MRSA was assessed by performance of in vitro time kill assay [15]. Neem oil marketed from Medinova Chemicals, Bangalore was used. Undiluted Neem oil (1 ml) was inoculated with 10^6 Colony Forming Units (CFU) of MRSA/ml and incubated at 37 ºC. A growth control tube containing only Methicillin resistant *S. aureus* inoculum was also taken. Subculture was done on blood agar and nutrient broth at 1, 2, 4, and 18 h. The number of bacteria remaining after each time interval was noted down to determine rate of killing by Neem oil. A three or more log10 reduction in bacterial counts as compared to growth control was taken as adequate bactericidal response.

Agar dilution method: The agar dilution method was used for determining antimicrobial susceptibility for the MRSA isolates [16, 17].

Preparation of agar dilution plates: A series of plates were prepared in triplicate starting from 10% to 50% concentration of Neem oil in 20 ml of molten Mueller - Hinton agar {0.2% (w/v)}. For 10% concentration, 2ml (one part) of the 100X Neem oil was added to 18 ml (nine parts) of molten agar. After equilibration in a water bath to 45 to 50 °C the agar, solution was mixed thoroughly and poured immediately into Petri dishes on a level surface to result in an agar depth of 3 to 4 mm. The plates were stored in sealed plastic bags at 2 to 8 °C after solidification at room temperature. Two growth control plates (no Neem oil) were prepared for each dilution.

Preparation of inoculum and inoculation: A standardized inoculum was prepared in nutrient broth for the agar dilution method by growing *S. aureus* to the turbidity of the 0.5 McFarland standard. Cultures adjusted to the 0.5 McFarland standard contain approximately 1.5×10^8 CFU/mL. This was diluted to achieve a concentration of $4\text{-}5\times 10^6$ CFU mL (0.75 ml 0.5 Mc Farland added to 25 ml water diluent, 1:33 dilution). An aliquot of diluted inoculum (0.01 mL) was applied to the surface of agar medium with standardized loop with the final inoculum of 10^4 CFU per spot of 5 to 8 mm in diameter. First growth control plate was inoculated at the beginning and second at the end to ensure there was no contamination or significant antimicrobial carryover during the inoculation. A sample of inoculum was plated on Nutrient agar plate and incubated overnight to detect mixed cultures and to provide freshly isolated colonies in case of retesting.

Incubation of Agar Dilution Plates: The inoculated plates were allowed to stand at room temperature until the moisture in the inoculum spots has been absorbed into the agar, i.e., the spots are dry, but no more than 30 minutes. After that the plates were inverted and incubated at 35±2 °C for 16 to 20 hours.

Determination of Agar Dilution End Points: After placing the plates on a dark, nonreflecting surface the MIC was recorded as the lowest concentration of antimicrobial agent that completely inhibits growth in two of the three plates, disregarding a single colony or a faint haze caused by the inoculum. The end point was read as the concentration in which there is 80% or greater reduction in growth as compared to the control as antagonists in the medium may allow some slight growth.

3. Results

A total of 107 MRSA strains were studied, out of which 57.94% of patients were less than 30 years and 42.05% more than 30 years of age. The mean age of the subjects was 28 years and males dominated with a male: female ratio of 1.37:1. 73.83% of patients presented with a previous history of broad spectrum antibiotic.

Out of 107 strains of MRSA 56(52.33%) were killed after 1hr exposure to Neem oil, 22(20.56%) were killed after 2 hr exposure, 19(17.75%) were killed after 4 hr & remaining 10 (9.34%) were killed after 12 hr exposure to Neem oil (Table 1).

Table 1: Inhibitory effect of Neem oil on MRSA

No of strains inhibited	Time of exposure(in hours)	Percentage
56	1	52.33%
22	2	20.56%
19	4	17.75%
10	12	9.34%

Minimum Inhibitory concentration as obtained by agar dilution method was found to be 10% for maximum number of strains (84.11%). 14 (13.08%) MRSA strains had MIC of 20% and 3 (2.80%) strains had MIC of 30% of Neem oil (Table 2).

Table 2: Minimum Inhibitory concentration by agar dilution method

Neem oil (pure) (ml)	Agar (ml)	No of Strains inhibited (n=107)
2 (10%)	18	90 (84.11%)
4 (20%)	16	90 (84.11%) +14 (13.08%)
6 (30%)	14	90 (84.11%) +14 (13.08%) +3 (2.80%)
8 (40%)	12	107 (100%)
10 (50%)	10	107(100%)

4. Discussion

Our study showed 100% susceptibility to Neem oil with MIC of 10% - 30% concentration of Neem oil. However another study by Rao *et al* showed 92% susceptibility with MIC varying between 1/4 to 1/64 dilutions [18]. According to a study in Bhubaneswar in 2001, the antibacterial activity of Neem seed oil in vitro was assessed against fourteen strains of pathogenic bacteria. Using the tube dilution technique, it was observed that 21.42% of the pathogens were inhibited at 500 µl/ml; 71.42% at 125 µl/ml; and 7.14% at 250 µl/ml of Neem oils, respectively [19]. The activity with the oil was bactericidal and independent of temperature and energy and was due to the inhibition of cell-membrane synthesis in the bacteria. The results of our study are concurrent with other studies in India and in other parts of the world.

Time kill studies were performed with undiluted Neem oil due to limitations and 10% concentration of Neem oil was used as the starting point for agar dilution due to initial stages of our experiment but varying concentrations of Neem oil should also be tested for bactericidal effect.

Patel *et al*. used 0.1, 0.15, 0.2.....0.5 per cent v/v concentrations of Neem oil and found 0.3 and 0.4 percent to be effective against Micrococcus pyogens var. aureus and *S. typhosa* respectively [10]. As per CLSI guidelines two fold serial dilutions of antimicrobial agent (particularly essential oils) starting from 2% (v/v) to 0.01% (v/v) is recommended [20, 12]. So, future studies with lower concentrations of may prove helpful in determining MIC of Neem oil. The initial data appears to prove the antibacterial effect of Neem oil. Hence it can be used as a topical antibiotic as an alternate to expensive, harmful antibiotics against MRSA. However long term studies with larger number of samples are necessary to study the toxicity in order to set an appropriate formulation for clinical use.

5. Conclusion

The results of present study are suggestive of antibacterial effect of Neem oil on multidrug resistant MRSA. Since Neem oil exhibited bactericidal effect on MRSA, it may find clinical application as topical antibacterial agent.

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