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Original Article

Antibacterial effect of neem (*Azadirachta indica*) oil on multidrug resistant bacteria isolated from human infections

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ABSTRACT

Aim: The aim of the present study was to determine the inhibitory and killing effect of neem (*Azadirachta indica*) oil on multidrug resistant bacteria isolated from human infections. Methods: Twenty five strains of multidrug resistant Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa isolated from different clinical specimens were used in the study. Time kill assay and broth macrodilution methods for determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were used to study the inhibitory and bactericidal effect of neem oil on these multidrug resistant bacteria. Results: Undiluted neem oil killed all strains of S. aureus within 8 h of exposure, whereas neem oil at concentration 500 µl/ml took 18 h to kill S. aureus. Undiluted neem oil killed E. coli and *P.aeruginosa* within 18 h of exposure. The MIC of neem oil was 500 µl/ml. Conclusion: Neem oil showed bactericidal effect on both gram-positive (*S. aureus*) and gram negative (*E. coli and P. aeruginosa*) bacteria. The anti-bacterial effect of neem oil was concentration and time dependent. *S. aureus* was more susceptible to neem oil than *E. coli* and *P. aeruginosa.*

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1. Introduction

In recent years, there has been an increase in the resistance of pathogenic bacteria to antibiotics. The emergence of multidrug resistant bacteria is a matter of concern. The global scenario is now changing towards the use of non toxic plant products having medicinal values. Neem (Azadirachta indica), the versatile medicinal plant is the source of several compounds having diverse chemical structure and biological effects[1]. A significant amount of research has already been carried out during the past to understand the chemistry and medicinal uses of different parts of neem. Several therapeutically and industrially useful preparations have been marketed.

Medicinal plants and herbal medicines are a part and parcel of human society to combat both infectious and non-infectious diseases. Neem is well known in India and other countries for more than 2000 years for its medicinal values[1]. Neem is an evergreen tree cultivated in various parts of Indian subcontinent. The Sanskrit name of neem is 'Arishtha' meaning 'reliever of sicknesses.

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More than 135 compounds have been isolated from neem. The compounds have been divided into 2 major groups- isoprenoids and others[1]. The isoprenoids include deterpenoids, azadirone, gedunin, nimbin, salanin and azadirachtin. The non-isoprenoids include proteins, carbohydrates, sulphurous compounds, polyphenoles, such as flavonoids and aliphatic compounds. Researchers have detected several medicinal effects of neem including antidiabetic effect, antifertility effect, antitumour effect, antiulcer effect, antimalarial effect and antipyretic effect[2-8]. Previous studies have shown that neem has antibacterial activity[9,10]. Previous studies have shown the effect of neem oil on dermatophytes[11,12].

Review of literature did not reveal studies on the effect of neem oil on multidrug resistant bacteria. The objective of the present study was to determine the growth inhibitory and bactericidal activities of neem oil on multidrug resistant bacteria.

2.Material and Methods

Bacterial strains

Twenty five multidrug resistant strains of S. aureus, E. coli, and P. aeruginosa isolated from chemical specimens in the Department of Microbiology, Kasturba Medical College, Mangalore were used in the study. The bacteria that were resistant to 2 or more antibiotics were considered multidrug resistant. Among 25 strains of S. aureus, 15 were methicillin resistant S. aureus(MRSA). S. aureus ATCC 25923 and E.coli ATCC 25922 were used as controls.

Neem oil

Neem oil manufactured by Oom Laboratories, Shimoga, India was used.

Preparation of bacterial inoculum

The bacteria were inoculated on blood agar and incubated at 37ºC for 24 hours. A single colony was picked using a sterile inoculating wire and inoculated into peptone water and incubated at 37ºC for 4-6 h. The turbidity of the peptone water culture was matched with Mc Farland 0.5 Standard (approximately 1.5 x 108 bacteria/ ml). The bacterial concentration was confirmed by surface plate method. All culture media were purchased from Hi Media Laboratories Pvt. Limited, Mumbai.

Time kill assay

The kill kinetics of neem oil was determined by time-kill assay[13,14]. For each bacterium, two concentrations of neem oil-undiluted and 500µl/ml neem oil in Mueller-Hinton broth were used. These were taken in volume of 2 ml in separate test tubes and inoculated with 20 µl of bacterial suspension and incubated at 37ºC. Initial control counts of the bacteria were obtained by serial dilution and spread plating of 0.01ml of the inoculums on nutrient agar just before incubation. Subsequently, 0.01 ml of serially diluted samples was spread plated at intervals of 2, 4, 8 and 18 h. The plates were incubated at 37ºC for 24 h and viable bacterial count was determined.

Broth macrodilution test

Broth macrodilution method was used for the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of neem oil to multidrug resistant bacteria[15]. Neem oil was diluted 2 folds in Mueller- Hinton broth (500µl/ ml- 62.5µl/ ml). The diluted neem oil was taken in volume of one ml in sterile test tubes. Each tube was inoculated to achieve an initial concentration of 1.5 x 105 bacteria/ ml. Mueller- Hinton broth without neem oil was used as the growth control. The tubes were incubated at 37ºC fir 24 hours. The minimum concentration of neem oil that inhibited the bacterial growth was considered MIC. Subculture on blood agar was done taking material from tubes that did not show bacterial growth. The inoculated plates were incubated at 37ºC for 24 h and examined for bacterial growth. The minimum concentration of neem oil that did not grow bacteria was MBC.

3. Results

The initial bacterial inoculums used for time-kill assay was 1.5 x 106 cfu/ml. The mean readings obtained for time kill assay of neem oil on S. aureus, E.coli, and P. aeruginosa determined by time kill assay is shown in Tables 1-3.

It is clear that undiluted neem oil had better inhibitory effect compared with neem oil at concentration 500µl/ ml. The viable count of S. aureus decreased as the time advanced. Complete killing of S. aureus occurred within 18 h of exposure to undiluted neem oil and neem oil at concentration of 500 µl/ ml. All strains of S. aureus had similar response to neem oil.

In case of *E. coli* and *P. aeruginosa*, the results were different. Although neem oil reduced the viable count of these bacteria, only undiluted neem oil could destroy the bacteria completely after 18 h. Neem oil at concentration 500 µl/ ml could not completely kill E. coli and *P. aeruginosa* after 18 h of exposure. The viable counts of gram-negative bacteria were more at each time interval when compared to *S. aureus*. All strains of *E. coli* had similar kind of response to neem oil. It is clear from the results that *E. coli* was less susceptible to neem oil. The MIC of neem oil to multidrug resistant bacteria is shown in Table 4

Table 1. Time kill assay results of neem oil on *S. aureus*

Neem Oil	Viable Count(cfu/ ml)			
Concentration	2 hr	4hr	8 hr	18 hr
Undiluted	2×10^4	2×10^2	NG	NG
500µl/ ml	6×10^4	3×10^3	2×10^2	NG

NG= No Growth

Table 2. Time kill assay results of neem oil on *E. coli*

Neem Oil	Viable Count (cfu/ ml)			
Concentration	2 hr	4 hr	8 hr	18 hr
Undiluted	1×10^5	8×10^4	3×10^3	NG
500µl/ ml	1×10^6	8×10^5	5×10^4	2×10^2

NG= No Growth

Table 3. Time kill assay results of neem oil on *P. aeruginosa*

Neem Oil	Viable Count (cfu/ ml)			
Concentration	2 hr	4 hr	8 hr	18 hr
Undiluted	3×10^4	6×10^4	3×10^4	NG
500µl/ ml	8×10^4	5×10^4	3×10^3	2×10^2

NG= No Growth

Table 4. Minimum inhibitory concentration of neem oil to multidrug resistant bacteria

Neem Oil	MIC(µl/ ml)			
Bacteria	Number of Bacteria			
	500	250	125	62.5
S. aureus (n=25)	22	3	0	0
E. coli (n=25)	22	0	0	0
P. aeruginosa (n=25)	23	0	0	0

4.Discussion

The neem seed yields arid bitter greenish yellow oil. The medicinal properties of neem oil are attributed to the bitter principles and odorous compounds. Neem oil is used in the treatment of ulcers, leprosy, gum and dental diseases[16]. Studies have shown that neem has antibacterial, antifungal and antiviral effects[17]. The seed oil is considered to be non-mutagenic19. Neem preparations are used in mouthwashes also.

The present study showed that neem oil had antibacterial effect. The effect was bactericidal rather than bacteriostatic. The bactericidal effect of neem oil was concentration and time dependent. Neem oil did not show antibacterial effect at concentration lower than 500µl/ ml. It is interesting to note that S. aureus was more sensitive to neem oil than *E. coli* and *P. aeruginosa*. This could be due to difference in cell wall of gram positive and gram negative organisms. Although previous studies have shown antibacterial effect of neem oil,[9,18], the present study showed that neem oil could kill even multidrug resistant bacteria. Neem preparations are safely used in mouthwashes. It is possible that different extracts from various parts of neem can have different anti-microbial effects. NIM-76, a fraction of neem seed oil has been shown to have better anti-microbial action[19].

4. Conclusion

Neem oil showed bactericidal effect on both gram-positive (S. aureus) and gram negative (*E. coli and P. aeruginosa*) bacteria. The anti-bacterial effect of neem oil was concentration and time dependent. *S. aureus* was more susceptible to neem oil than E. coli and *P. aeruginosa*. Further in-vitro and in-vivo studies are required to understand the antibacterial effects of neem oil.

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