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Synergistic effect of (*Azadirachta indica*) Neem extract on multi drug-resistant gram-negative isolates from clinical samples at a tertiary care center

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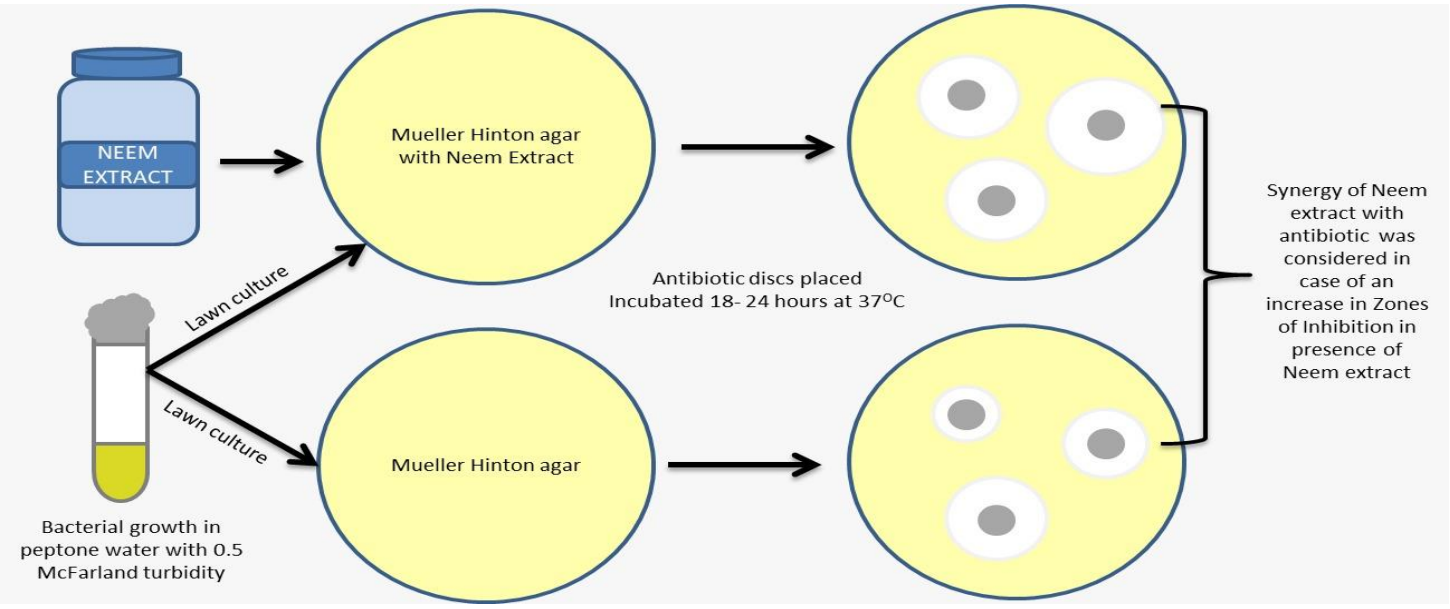
ABSTRACT

The indiscriminate usage of antibiotics in treatment and animal feed has led to the development of pan and multi drug resistance. The developing resistance in addition to the mortality had skewed the minds of researchers in search of newer antimicrobial agents. Among the native plants, one with potent antibacterial activity is Neem (*Azadirachta indica*). The leaves, bark, flowers, fruits, seed, and gum of the neem tree are utilised for various beneficial effects. The clinical samples including pus, urine, aspirate, sputum was sent to the microbiology department. The Gram negative Multi drug resistant organisms were included in the study. Around 210 isolates were obtained during the study period out of which 66 (31.4%) isolates were MDR. The synergistic effect of Neem extract was evaluated which showed a significant change in the susceptibility as intermediate range to susceptible range, resistant to intermediate and resistant to sensitive. The Neem extract has better antibacterial effect however, multicentric studies must be carried out to identify the minimum inhibitory concentration against various isolates.



Keywords: Multi drug resistant, Neem extract, synergistic effect, Azadirachta indica, Synergistic effect.

INTRODUCTION

Antimicrobial resistance is a rising concern posing a threat for the treatment of potentially pathogenic infections. The indiscriminate usage of antibiotics in treatment and animal feed has led to the development of pan and multi drug resistance. The critical care physicians are left with meagre antibiotics to fight the infectious agents warranting urgent action plan to circumvent health burden [1]. As per the WHO reports the estimated global death due to antimicrobial resistance would range from 7, 00,000 to 10 million by the year 2050[2]. The developing resistance in addition to the mortality had skewed the minds of researchers in search of newer antimicrobial agents.

The traditional plants from the Indian subcontinent have antibacterial effect. They are used in tradition conventional medicine for treating infections. Their utility is often underestimated in the modern medicine field. Among the native plants, one with potent antibacterial activity is Neem (*Azadirachta indica*). The leaves, bark, flowers, fruits, seed, and gum of the Neem tree are utilized for various beneficial effects. The leaves are found to have antibacterial, antiviral, and anti-hyperglycaemic effect [3]. They are used in traditional medicine to treat respiratory disorders, constipation, dermatitis, helminthiasis, diabetes, and other diseases [4]. With paucity of armamentarium to treat infections in modern medicine. The antibacterial property of neem extract must be analysed and utilised for treatment. Hence the aim of the study is to identify the synergistic effect of Neem leaf extract on isolates from clinical samples at our tertiary care center.

MATERIALS AND METHODS

Study Design

A prospective cross-sectional study was conducted at the microbiology department MGMCRI. The study was approved by the institutional human ethical committee.

Sample Collection

The clinical samples including pus, urine, aspirate, sputum was sent to the microbiology department. The consecutive isolates grown from such samples were included in the study. The Gram negative Multi drug resistant organisms were included in the study. The repeat isolates which are obtained from the same patient will be excluded from the study.

Methodology

The samples were inoculated into blood agar, and Mac-conkey agar and identified by standard bacteriological procedure.

The identified isolates were subjected to antimicrobial susceptibility testing by Kirby Bauer disc diffusion method and the results were interpreted as per the CLSI guidelines 2023 [5].

Among the isolates obtained during the study period Gram negative Multi drug resistant (MDR) isolates were included in the study. The isolates which were resistant to three or more class of antimicrobial agents were termed as MDR [6].

The isolates were simultaneously inoculated into Muller Hinton agar which were prepared with concentration of Neem extract under sterile precautions. The Mueller Hinton agar was prepared from the commercially available Mueller Hinton base HIMEDIA. The preparation was autoclaved at 121° C for 15 minutes at 15 lbs pressure. The Mueller Hinton was allowed to cool at 40°C with constant shaking in the Biosafety cabinet. Around 50mg/ml of commercially available Neem leaf extract was added to the medium at a molten state and were poured into Petri dish.

The isolates were inoculated into peptone water and incubated at 37°C for 2 hours. The turbidity was matched with 0.5 Mc Farland standard. The antibiotic discs including gentamicin 10µg, amikacin 30µg, sulfamethoxazole and trimethoprim (1.25/23.75 µg), ciprofloxacin 5 µg, ceftriaxone 30 µg, piperacillin tazobactam 30/6 µg, cefoperazone sulbactam 75/30 µg, imipenem 10 µg, meropenem 10 µg, were placed on the Muller Hinton agar plates after making a lawn culture. The synergistic effect was compared as increase in zone size compared to the plain Muller Hinton agar plates.

RESULTS

Around 210 isolates were obtained during the study period out of which 66 (31.4%) isolates were MDR. The isolates which were resistant to more than 3 class of antibiotic were considered as MDR. Among the 66 MDR isolates majority of samples were from exudates 45.4%, respiratory 32% followed by urine 22.7% samples as shown in Table 1. Gender wise distribution showed majority of the MDR were obtained from male 77% (Table 2).

Table 1: Distribution of Sample and organisms

Row Labels	Exudate	Respiratory	Urine	Grand Total
Acinetobacter baumannii	1	10	-	11
Escherichia coli	4	-	4	8
Klebsiella oxytoca	1	1	-	2
Klebsiella pneumoniae	19	10	5	34
Non Fermenting Gram Negative Bacilli	3	-	3	6
Proteus vulgaris	1	-	1	2
Citrobacter. Spp	1	-	2	3
Grand Total	30	21	15	66

Table 2: Age wise and gender wise sample distribution

	Male	Female	Total
0-20 years	8	0	8
21-40 years	5	5	10
41-60 years	19	5	24
61-80 years	16	4	20
> 80 years	3	1	4
Grand Total	**51**	**15**	**66**

The common organism isolated were *Klebsiella pneumoniae* 51.5%, followed by *Acinetobacter baumannii* 16.6%, *Escherichia coli* 12%, Non-Fermenting Gram Negative Bacilli 9%, *Citrobacter spp* 4.5%,

Klebsiella oxytoca 3% and *Proteus vulgaris* 3% (Table 1).

The highest resistance was shown to ceftriaxone 95.5%, followed by piperacillin tazobactam 93.9%, gentamicin (84.8%), amikacin (84.8%), imipenem (84.8%) and meropenem (83.3%). Among the urinary antibiotics 80% resistance was seen in nalidixic acid and nitrofurantoin.

Table 3a: Distribution of antibiotic susceptibility pattern

N=66	Gentamicin (GEN)	Amikacin (AK)	Sulfamethoxazole and trimethoprim (COT)	Ciprofloxacin (CIP)	Ceftriaxone (CTR)	Imipenem (IPM)	Meropenem (MRP)	Cefoperazone sulbactam (CFS)	Piperacillin tazobactam (PIT)
R	56	56	54	62	63	56	55	44	62
%	84.8	84.8	81.8	93.9	95.5	84.8	83.3	66.7	93.9
I	5	7	0	3	1	3	2	13	1
%	7.6	10.6	0.0	4.5	1.5	4.5	3.0	19.7	1.5
S	5	3	12	1	2	7	9	9	3
%	7.6	4.5	18.2	1.5	3.0	10.6	13.6	13.6	4.5

Table 3b: Distribution of Antibiotic susceptibility pattern of Urinary antibiotics

N=15	Nalidixic acid (NA)	Nitrofurantoin (NIT)	Fosfomycin (FOS)
R	12	12	3
%	80	80	20
I	1	1	3
%	6.7	6.7	20
S	2	2	9
%	13.3	13.3	60

The synergistic effect of Neem extract was evaluated which showed a significant change in the susceptibility as intermediate range to susceptible range, resistant to intermediate, and resistant to sensitive as shown in Tables 4a and 4b.

Table 4a: Change in Susceptibility status in presence of Neem extract

N=66		Gentamicin (GEN)	Amikacin (AK)	Sulfamethoxazole and trimethoprim (COT)	Ciprofloxacin (CIP)	Ceftriaxone (CTR)	Imipenem (IPM)	Meropenem (MRP)	Cefoperazone sulbactam (CFS)	Piperacillin tazobactam (PIT)
No change in susceptibility		53	53	63	59	60	59	55	53	63
Increased susceptibility	I-->S	5	6	-	1	-	1	-	6	1
	R-->I	1	4	-	4	3	4	5	6	
	R-->S	7	3	2	2	3	2	6	1	2
Decreased susceptibility (S-->I)		-	-	1	-	-	-	-	-	-

Table 4b: Change in Susceptibility status in presence of Neem extract of Urinary antibiotics for 15 urinary isolates

N=15		NA	NIT	FOS
No change in susceptibility		13	15	13
Increased susceptibility	I-->S	1	-	1
	R-->I	1	-	-
	R-->S	-	-	1

DISCUSSION

Although the plant based antimicrobial products were surpassed by the synthetic antibiotics, the rising global antimicrobial resistance merits alternative sources of antimicrobial agents. Among the medicinal plants Neem (*Azadirachta indica*) has been utilized for the therapeutic management of various diseases. One of the prime activities of the Neem extract being antibacterial, antiviral, antifungal, and antiparasitic. It has been identified that the phytochemicals of *Azadirachta indica* have potent activity against bacteria. The aqueous extracts of the Neem leaves have potential activity against the microorganisms.

In a study conducted by Uwimbabazi F and coworkers it is shown that the leaf extracts of *Azadirachta indica* had better zone of inhibition against *Staphylococcus aureus* and *Escherichia coli* [7]. Similar study conducted by Maragathavalli S and coworkers against opportunistic pathogens showed an increase in minimum inhibitory concentration for opportunistic pathogens [8]. In our study with a standard concentration of Neem leaf extract added to molten agar plates showed a change in the susceptibility pattern of the antibiotic discs against the MDR pathogens isolated from clinical specimens. The change in sensitivity pattern as shown in table 4 denotes that the Neem extract could have effective therapeutic outcome when utilized in combination with the antibiotics.

However, our study showed difference in the zone sizes among few isolates compared to most isolates which were tested in combination. The probable reasons could be the crude extract was utilized in our study. The extraction and utility of the phytochemicals from the Neem leaf could improve the results. Similarly, we utilized standard concentration of the Neem leaf extract which could have

altered the results. In addition, all the isolates utilized in our study were MDR isolates.

Hence the Neem extract has better antibacterial effect however, multicenter studies must be carried out to identify the minimum inhibitory concentration against various isolates. This could reduce the burden of antimicrobial resistance and provide addition to the armamentarium against the global infectious MDR pathogens.

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Conflict of interest
The authors declare that there is no conflict of interest.

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