Antibacterial Effects of *Azadirachta indica* Leaf and Bark Extracts in Clinical Isolates of Diabetic Patients

Ms. P. Chaturvedi*, Dr. A. Bag, Dr. V. Rawat***, Dr. N. S. Jyala****, Dr. V. Satyavali*****, Dr. P. K. Jha******

*Student, ** Assistant Professor, Department of Biochemistry, *** Assistant Professor, Department of Microbiology, **** Professor and Head, Department of Biochemistry, Govt. Medical College, Haldwani, ***** Assistant Professor, Department of Medicine, ****** Assistant Professor, Department of Surgery, Dr. Susheela Tiwari Govt Hospital, Haldwani

Abstract: Background: Antibacterial activities of crude *Azadirachta indica* (neem) bark and leaf extracts were investigated in bacterial species isolated from clinical samples of diabetic individuals. Methods and Material: Nine different dilutions of methanolic bark and leaf extracts were tested for this purpose in agar well diffusion method. Results: Both the extracts were active against Gram positive as well as Gram negative strains. Zones of inhibition produced by different bacteria for different concentrations were summarized by linear regression. Highest activities were exhibited for coagulase negative Staphylococcus (CONS) by both bark and leaf extracts, Y = 16.95 + 0.19X and Y = 18.90 – 0.70X, respectively. Conclusions: Results indicate that exhaustive studies involving identification of specific compounds in neem extracts and testing their activities in diabetic samples would be worthwhile considering steep emergence of multidrug resistant species in diabetic infections and infections in general.

Key words: Antibacterial activity, *Azadirachta indica*, bark extract, diabetes, leaf extract

Corresponding Author: Dr. A. Bag, Assistant Professor, Department of Biochemistry, Govt. Medical College, Haldwani , e-mail : arundhatis5@rediffmail.com

INTRODUCTION: Almost every part of a neem tree, *Azadirachta indica* (Meliaceae), is known for its therapeutic values and has been in use as traditional medicine to treat a wide range of human disorders since ancient times. It is an evergreen tree indigenous to south Asia and in most parts of Indian subcontinent[1]. Antimicrobial activities of neem have widely been recognized. While neem leaf, bark extracts, and neem oil are known to suppress several pathogenic bacterial species, its antiviral activities against vaccinia, chikungunya, measles virus and Coxsackie B viruses and antifungal activities against several human fungi have been established[2]. Antimicrobial properties of neem can be attributed to several bioactive compounds found in different parts of this tree, which are categorised into two major classes, isoprenoids and non- isoprenoids. Very a few compounds, however, could be studied for their specific bioactivities[2].

Although every part of neem tree is known to have medicinal properties, extracts from neem leaf, bark, twigs, fruits and oils are most commonly documented in literature for their antibacterial effects. From previous studies it is found that different neem extracts have antibacterial activities against a moderate range of bacterial species[3-5]. In this article we report on antibacterial effects of neem leaf and bark extracts in bacterial isolates collected from diabetic patients in a tertiary care hospital. Clinical isolates of diabetic patients are mostly polymicrobial with *Staphylococcus aureus* (including methicillin resistant *S. aureus*, MRSA), *Klebsiella, Streptococcus, Eschericia. coli, Pseudomonas aeruginosa, Acinetobacter* etc. as commonly present species[6,7].

MATERIAL AND METHODS: Fresh leaves and bark of neem (*A. indica*) were collected locally at Haldwani, Nainital. Collected leaves and bark were air dried separately in shade and then were coarsely

powdered. Hundred gram of each of the plant materials was dipped in 100 ml of methanol for 4 hrs and filtered thereafter in soxhlet apparatus. This was followed by evaporation of plant materials (leaves and bark) under reduced pressure using Rota- vapour. Filtration and extraction were carried out in Central Institute of Medicinal and Aromatic plants (CIMAP), Pantnagar. Then the extracts were dissolved in dimethyl- sulphoxide (DMSO) to make 20% stock solution of bark extract and 60% stock solution for leaf extract.

Pus, urine and sputum of 35 diabetic patients were taken from clinical samples collected for microbiological testing at Dr. Sushila Tewari Govt. Hospital and Mehta Charitable Memorial Hospital, Haldwani. Bacterial identification was carried out by conventional biochemical methods according to standard microbiological techniques[8]. Stock cultures of bacteria were maintained in 1% nutrient agar and were stored in 4^0C temperature.

Antibacterial activities of neem bark and leaf extracts were tested following agar well diffusion method in Muller Hinton agar. Stock bacterial solutions were thawed and suspended in peptone water. After 2-3 hrs, as the solution got turbid inoculum was spread on the top of the previously prepared petriplates with solidified media and was allowed to dry for 10 minutes. Wells of 10 mm diameter were punched using sterile borer. To test antibacterial properties of neem bark, serial dilutions 1, 2, 5, 10, 15, 20, 40, 60, 80 mg/ml were prepared from stock solution using DMSO. The wells were filled with 0.1 ml of different dilutions. The plates were then kept for 1 hour at room temperature to allow diffusion of the extract into the medium and then incubated aerobically at 37^0C for overnight. The solvent was checked for its antibacterial activity. The zones of inhibition were measured across the diameter.

For each concentration the zones of inhibitions produced by different strains of a species were averaged. Minimum Inhibitory Concentration (MIC) was determined by similar well diffusion method in Muller Hinton agar. It was carried out for each species by further preparation and applications of extracts of intermediate dilutions. Antibacterial properties and MIC of neem leaf extracts were tested following similar kind of experiments. All the chemicals and reagents used were purchased from Himedia.

RESULTS: The most commonly found pathogenic organisms were *Escherichia* (17 strains) followed by *S. aureus* and *Pseudomonas* (9 strains each). Single isolate of each of *Morgenella* and *Acinetobactor* could be collected. Table 1 and 2 represent average zone of inhibition (in mm) for increasing concentrations of *A. indica* bark and leaf extracts, respectively. We calculated the average of zone of inhibition produced by different strains of each bacterial species. Overall increase of zone of inhibition was noticed for increased concentrations of bark extract (Table 1).

Table 1. Zones of inhibition (in mm) for different concentrations of *Azadirachta indica* bark extract

Bacterial Species (Total strains)	Concentrations (mg/ml) of bark extract								
	1	2	5	10	15	20	40	60	80
S. aureus (9)	11.25	14.25	12.86	14.44	15.33	16.11	17.56	18.33	18.56
CONS (4)	21	17	15	16	16	17.25	17.25	18.75	18.75
Enterococcus (3)	0	11	11	11	11	12.5	13.33	15	16
Escherichia (17)	0	11	11	12.5	12.33	12.25	13.4	13.8	14.5
Pseudomonas (9)	13	12.75	13.44	14.11	14.67	15.22	16.43	16.71	16.28
Klebsiella (7)	0	0	12	12	13.4	13	13.5	14.5	15

However, no such trend was observed for leaf extracts except in *S. aureus* where highest concentration (80 mg/ml) showed higher inhibition zone (16mm) than that of the lowest concentration (11mm) (Table 2).

Table 2. Zones of inhibition (in mm) for different concentrations of *Azadirachta indica* leaf extract

Bacterial Species (Total strains)	Concentrations (mg/ml) of leaf extract								
	1	2	5	10	15	20	40	60	80
S. aureus (9)	11	12.5	12	11.5	11.5	12.5	13.88	14.88	16
CONS (4)	18	19	18	21.5	18.33	18.67	10.33	14.33	15.67
Enterococcus 3)	0	0	0	11	11	12	12.5	11	0
Escherichia (17)	14	14.67	14	13.5	14.25	15	13	13.2	12.83
Pseudomonas (9)	12.5	12.75	13.6	14.2	14.6	13.67	13.67	13.25	13
Klebsiella (7)	12.5	13.67	13.25	14.5	15.33	15	13	12.33	12

Higher concentrations of both the extracts were found to have increasing antibacterial activities against *Morgenella* and *Acinetobactor* also (not included in table). We calculated linear regression of the values presented in Tables 1 and 2, considering concentration of extract as independent variable (Table 3).

Table 3. Linear regression of zones of inhibition on different concentrations of A*zadirachta indica* extracts for each species

Bacterial species	Bark extract	Leaf extract
S. aureus	Y = 13.39 + 0.78X	Y = 11.39 + 0.57X
CONS	Y = 16.95 + 0.19X	Y = 18.90 – 0.70X
Enterococcus	Y= 8.25 + 1.14X	Y = 5.63 + 0.29X
Escherichia	Y= 8.97 + 0.86X	Y = 14.31 – 0.19X
Pseudomonas	Y = 13.52 + 0.47X	Y = 13.52 - 0.02X
Klebsiella	Y= 6.98 + 1.31X	Y = 14.09 – 0.23X

For both bark and leaf extracts the highest relationship has been found for CONS (Y = 16.95 + 0.19X and Y = 18.90 – 0.70X, respectively) whereas least effects of neem extracts were found for *Acinetobactor* species (Y = 2.37 + 1.96X for bark and Y = -1.27 + 1.07X for leaf). It was observed that bark extract was most effective against *Staphylococcus* (both *S. aureus* and CONS) and *Pseudomonas* (Table 3), the most commonly reported Gram positive and Gram negative bacteria in clinical samples of diabetic patients[7,9-11]. Bark extract was more effective than leaf extract against *S. aureus* and equally effective against *Pseudomonas* (Table 3). Among all tested species MIC was lowest (0.5 mg/ml) for both bark and leaf extracts in CONS (Table 4). Solvent had no antibacterial activity.

Table 4. MIC for methanolic leaf and bark extracts of A*zadirachta indica*

Bacterial species	Bark extract (mg/ml)	Leaf extract (mg/ml)
S. aureus	1	1
CONS	0.5	0.5
Enterococcus	2	10
Escherichia	2	1
Pseudomonas	1	0.5
Klebsiella	5	1

DISCUSSION: Sharma et al.[12] demonstrated pronounced activity of neem leaf methanolic extract against *S. aureus*. They found lower activity of leaf extract against *E. coli* and *Klebsiella*. For *Escherichia* and *Klebsiella* we found that leaf extract was more effective (Y= 14.31 - 0.19X and Y= 14.09 - 0.23X, respectively) than bark extract (Y= 8.97 + 0.86X and

Y= 6.98 + 1.31X, respectively). Okemo et al[4] estimated the kill kinetics (rate and extent of bacterial killing) of *S. aureus*, *E. coli* and *P. aeruginosa* for neem stem bark extract. They found that extract concentration of 0.5 mg/ml significantly reduced *S. aureus* inoculum after 24 hrs while extracts with increasing concentrations completely wiped out viable bacteria in lesser time. Earlier Fabry et al.[13] with same bark extract reported an MIC of 8 mg/ml for *E. coli* and *P. aeruginosa*. In the present experiment MIC values were quiet low, 2 mg/ml of bark extract for *Escherichia* and 1 mg/ml for *Pseudomonas*. This difference might be due to the strains present in the clinical samples taken in the present study.

While increasing incidence of MRSA are being reported in diabetes, high rates of antibiotic resistant methicillin sensitive *S. aureus* (MSSA) in diabetic foot ulcer and multidrug resistant methicillin resistant *S. epidermidis* (coagulase negative species) have also been found in diabetic patients[14]. Further, carbapenem resistant metallo- β- lactamase (MBL) producing *P. aeruginosa*, can be considered as the therapeutic challenge to the individuals with diabetes[15] as they can degrade higher generation cephalosporins. The present study emphasizes that neem extracts can be used to produce drugs, which can effectively combat multidrug resistant strains commonly reported in diabetic infections. Further, as present day antibiotics have several side effects, a combination of antibiotics with neem extracts can reduce these effects because in this case total exposure to antibiotic would be less. Thus safer, cost effective drugs could be prepared and marketed. However, all these require much more exhaustive studies involving identification and isolation of specific neem compounds active against a specific resistant strain colonizing in such types of infections.

ACKNOWLEDGEMENTS: Authors are grateful to Prof Y. P. S. Pangtey, Kumaun University for identifying the plant material and to Dr. Padalia, CIMAP, Pantnagar for his help in preparation of neem extracts. The authors also thank Dr. K. C. Lohni, Mehta Charitable Memorial Hospital, K. Belwal and A Srivastava for their support in collection of samples.

REFERENCES:

1. Govindachari TR. Chemical and biological investigations on *Azadirachta indica* (the neem tree). Curr Sci. 1993; 63: 117-22.

2. Biswas K, Chattopadhyay I, Banerjee RK, Bandyopadhyay U. Biological activities and medicinal properties of neem (*Azadirachta indica*). Curr Sci. 2002; 82: 1336- 45.

3. Wolinsky LE, Mania S, Nachnani S, Ling S. The inhibiting effect of aqueous *Azadirachta indica* (neem) extract upon bacterial properties influencing in vitro plaque formation. J Dent Res. 1996; 75: 816-22.

4. Okemo PO, Mwatha WE, Chhabra SC, Fabry W. The kill kinetics of *Azadirachta indica* A. Juss (Meliaceae) extracts on *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. Afr J Sci Technol. 2001; 2: 113-8.

5. Helmy WA, Amer H, EL- Shayeb NMA. Biological and anti- microbial activities of aqueous extracts from neem tree (*Azadirachta indica* A Juss, Meliaceae). J Appl Sci Res. 2007; 3: 1050-5.

6. Huang CR, Lu CH, Chang HW, Lee PY, Lin MW, Chang WN. Community- acquired spontaneous bacterial meningitis in adult diabetic patients: an analysis of clinical characteristics and prognosis factors. Infection 2002; 30: 346-50.

7. Bansal E, Garg A, Bhatia S, Attri AK, Chander J. Spectrum of microbial flora in diabetic foot ulcers. Indian J Pathol Microbiol. 2008; 51: 204-8.

8. Colle JG, Duguid JP, Fraser AG, Marmion BP, Simon A. Laboratory strategy in the diagnosis of infective syndrome. In: Colle JG, Fraser AG, Marmion BP, Simon A (Editors). Mackie and McCartney. Practical Medical Microbiology 14th ed. London: Churchill Livingstone 1996; p.84-90.

9. Armstrong DG, Lipsky BA. Diabetic foot infections: stepwise medical and surgical management. Int Wound J. 2004; 1: 123-32.

10. Sharma VK, Khadka PB, Joshi A, Sharma R. Common pathogens isolated in diabetic foot infection in Bir Hospital. Kathmandu Univ Med J. 2006; 4: 295-301.

11. Sun Y, Dowel SE, Smith E, Rhoads DD, Wolcott RD. In vitro multispecies Lubbock chronic wound

biofilm model. Wound Repair Regen. 2008; 16: 805-13.
12. Sharma D, Lavania AA, Sharma A. In vitro comparative screening of antibacterial and antifungal activities of some common plants and weeds extracts. Asian J Exp Sci. 2009; 23: 169-72.
13. Fabry W, Okemo PO, Ansorg R. Antibacterial activity of East African medicinal plants. J Ethnopharmacol. 1998; 60: 79-84.
14. Galkowska H, Podbielska A, Olszewski WL, Stelmach E, Luczak M, Rosinski G, Karnafel W. Epidemiology and prevalence of methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* in patients with diabetic foot ulcers: focus on the differences between species isolated for individuals with ischemic vs. neuropathic foor ulcers. Diabetes Res Clin Pract. 2009; 84: 187-93.
15. Varaiya A, Kulkarni M, Bhalekar P, Dogra J. Incidence of metallo- beta- lactamase- producing *Pseudomonas aeruginosa* in diabetes and cancer patients. Indian J of Pathol Microbiol. 2008; 51: 200-3.