J. Indian bot. Soc. Vol. 89 (3&4) 390-396

ANTI-BACTERIAL AND CLASTOGENIC PROPERTY OF NEEM *(Azadirachta indica* A Juss*)* EXTRACT

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In the present investigation, antimicrobial activities of crude, aqueous, acetone and methanol extracts of neem leaves were tested against twenty one strains of geocarposphere bacteria of peanut and results showed activity against four, two, four and five bacterial strains, respectively. The alcoholic extracts exhibited a higher activity when compared to that of the crude and aqueous extracts.

In vitro experiments on clastogenic property of neem extracts were performed in six-week old laboratory bred Swiss albino mice. The results indicate that the frequency of total abnormal cells increased in comparison to the control group of animals (4.3%) by about three fold (11.6%) three and a half fold (14.0%) and four fold (17.0%) after treatment with lowest (0.5 g/kg), middle (1.0 g/kg), and the highest (2.0 g/kg) doses respectively of the neem extract. The total chromosomes abnormalities increased significantly in lowest (12.3%) middle (15.0%) and highest (20.0%) doses, respectively in comparison to the control (4.6%).

Key words : Anti-bacterial, Clastogenic property, Neem extract.

The neem (*Azadirachta indica* A Juss, Family Meliaceae) is a versatile tree and has found multiple uses in medicine and agriculture (Subapriya and Nagini 2005). The leaf extract of neem contains a large proportion of azadirachtin ($C_{35}H_{44}O_{16}$), a steroid akin to tetranortriterpenoid, as well as other isoforms and compounds such as azadrichtanin, azadirachtol, azadirone, isoazadirolide, epoxyazadiradion, nimocinolide, isonimolide, epinimbin, nimbinene, nimocinol, nimbandiole, melianin A and B, melianone and many other unidentified constituents (Rastogi and Mehrotra 1995). Commercial preparations of neem leaf extract are used rather than the isolated azadirachtin as the active principle alone is seldom as effective as the whole extract. Because of the widespread utilization of neem extract in crude form, the human population is being frequently exposed to its various constituents. Of various toxic effects of neem histopathological changes have been reported very widely but their cytogenetic toxicity is less known (Awasthy and Chourasia 1995). Previous study on microbial inhibition of aflatoxin biosynthesis in peanut have reported that, of twenty one strains of geocarposphere bacteria screened against *Aspergillus flavus,* five and sixteen strains were found to accelerate and inhibit the aflatoxin producing potential of *A. Flavus,* respectively (Chourasia 1995). Since aflatoxin is a well known clastogen (Bose and Sinha 1994) and its production is enhanced by few bacterial strains, therefore, in the present investigation neem leaf extracts were tested for their toxicity against geocarposhphere bacteria of peanut and their clastogenic effects on laboratory mice, *Mus musculus.*

MATERIALS AND METHODS

A. Anti-bacterial activity of neem extract

Received on February 24, 2009 Accepted on June 30, 2010

Table 1 : Efficacy of different concentrations of crude and aqueous extracts of neem against geocarposphere bacteria of peanut (zone of inhibition in mm).

Bacteria	Crude extract Concentration (%)					Aqueous extract Concentration (%)					
	100	75	50	25	10	1000	900	800	700	600	500
Alcaligenes paradoxus	-	-	-	-	-	14.50	13.00	11.50	-	-	-
Azobacter vinelandii	23.50	15.75	14.00	14.00	-	14.60	13.50	11.00			
Bacillus magaterium	-	-	-	-	-	15.00	14.50	14.00	-	-	-
B. laterosporus	-	-	-	-	-	14.50	12.00	10.00			
B. thuringiensis	-	-	-	-	-	15.00	14.00	12.00	-	-	-
B. pasteurii	-	-	-	-	-	14.50	13.50	-			
B. macerans	-	-	-	-	-	2.50	-	-	-	-	-
Clavibacter michiganense	15.75	15.00	14.00	12.50	-	17.20	15.65	13.00			
Comamonas testosteroni	18.00	16.50	13.60	10.25	-	16.25	14.40	12.35	-	-	-
Cellulomonas cartae	-	-	-	-	-	15.60	13.70	11.50			
Cytophaga johnsonae	21.44	19.70	18.22	16.80	10.60	14.50	14.00	13.50	11.40	11.00	10.50
Enterobacter cloacae	19.50	19.25	18.00	16.55	10.50	14.95	14.25	14.00	12.20	11.20	11.70
Flavimonas oryzihabitans	-	-	-	-	-	16.50	12.70	11.50	-	-	-
Flavobacterium indologenes	-	-	-	-	-	15.00	11.60	10.00	-	-	-
F. odoratum	-	-	-	-	-	15.50	12.50	11.00	-	-	-
F. balustinum	-	-	-	-	-	14.95	11.40	10.20	-	-	-
Klebsiella planticola	24.25	15.75	15.25	14.25	-	15.50	14.50	12.00	10.25	-	-
Pseudononas aurofaciens	22.00	17.00	16.00	15.50	14.00	14.45	14.00	12.10	10.40	-	-
P. syringae	25.40	22.00	20.15	16.72	14.81	15.60	14.65	11.90	11.50	-	-
Salmonella enteritidis	-	-	-	-	-	14.00	-	-	-	-	-
Xanthomonas campestris	-	-	-	-	-	15.50	14.00	-	-	-	-
CD (P=0.05)	3.25	2.72	2.60	2.66	0.96	1.91	2.17	1.96	0.85	-	-
CV (%)	17.03	16.50	16.35	15.90	19.02	20.70	17.20	18.90	17.50	-	-

Values were square root transformed before analysis

(i) Preparation of crude fresh neem leaf extract

Leaf extract of neem was prepared by following the method of Kumar and Sachan (1979). The extract was filter sterilized with Whatman No. I filter paper and used for further investigations. The antimicrobial activity of crude neem extract was tested against an array of 21 strains of geocarposphere bacteria by following agar well diffusion method (Perz *et al.* 1990).

(ii) Preparation of solvent extract of dried neem leaf powder

Required quantity of the leaves were made-dried at room temperature, powdered and stored in air-tight containers to protect against light and humidity. The dried leaf powder (20 gm) was extracted in a solvent extractor successively with 200 ml. methanol and 200 ml. acetone for 48 hours. Methanol and acetone extracts on concentration under reduced pressure yielded 0.97 gm and 0.50 gm weight which were of brownish and sap green colour, respectively (Srinivasan *et al* 2001).

Both the aqueous crude extracts and solvent extracts were screened for their antibacterial activity against all 21 bacterial strains. To test the antimicrobial activity, paper disc method was followed (Dubey and Maheshwari 2007).

iii) Isolation, identification and maintenance of pure culture of geocarposphere bacteria of peanut

Altogether 21bectarial stains were isolated from geocarposphere regions of subterranean peanut pods grown on Alabama Agricultural Experiment Station, Auburn University, Auburn USA. Six replicate plants were selected at random from throughout the field trial. Three individual pods, roots and soil samples were removed from each replicate plant. The samples were weighed, placed into 10 ml of 0.2 M phosphate buffer (pH 7.0) individually and agitated for 1 hour at 150 rpm on an orbital shaker. After agitation, root and pod samples were macerated with a sterile mortar and pestle, serially diluted in phosphate buffer to 10^{-4} and plated with an automated plating system (Spiral Systems Inc, Bethesda,

Table : 2 Efficacy of different concentrations of acetone and methanol extracts of neem against geocarposphere bacteria of peanut (zone of inhibition in mm).

Bacteria	Acetone extract concentration (mg)										Methanol extract concentration (mg)									
	1000	900	800	700	600	500	400	300	200	100	1000	900	800	700	600	500	400	300	200	100
Alcaligenes paradoxus	15.00	11.50	10.50	-	-	-	-	-	-	-	10.00	17.00	16.50	16.00	15.00	15.00	14.50	14.00	13.00	-
Azobacter vinelandii	16.50	15.00	13.50	12.00	12.50	12.50	12.50	12.00	-	-	17.00	17.00	16.50	14.00	13.00	14.50	12.50	12.00	-	-
Bacillus magaterium	15.00	11.00	11.00	-	-	-	-	-	-	-	15.50	15.00	15.00	14.50	14.00	13.70	13.50	13.00	-	-
B. laterosporus	14.00	13.50	12.50	-	-	-	-	-	-	-	15.00	14.80	13.50	-	-	-	-	-	-	-
B. thuringiensis	16.50	16.50	16.00	15.00	14.00	14.00	12.00	11.25	-	-	18.00	.15.00	14.80	14.00	13.50	12.50	11.50	11.00	-	-
B. pasteurii	14.50	13.00	12.00	-	-	-	-	-	-	-	15.00	14.70	13.40	-	-	-	-	-	-	-
B. macerans	14.00	13.50	11.00	-	-	-	-	-	-	-	15.00	14.50	-	-	15.00	15.00	-	-	-	-
Clavibacter michiganense	17.00	16.50	15.50	14.50	14.00	13.50	13.50	12.50	-	-	26.00	21.00	18.00	15.50	14.00	14.00	15.00	15.00	13.50	-
Comamonas testosteroni	17.50	16.50	16.00	15.50	15.00	15.00	13.50	13.00	-	-	18.00	17.00	16.00	14.00	13.50	13.00	14.00	14.00	14.00	-
Cellulomonas cartae	16.00	13.50	12.50	11.80	11.40	11.00	-	-	-	-	16.00	15.00	14.50	14.00	14.00	14.00	13.00	13.00	-	-
Cytophaga johnsonae	14.50	14.00	13.50	12.00	10.70	-	-	-	-	-	17.00	16.00	15.50	15.00	15.00	15.00	14.00	14.00	13.50	-
Enterobacter cloacae	17.50	15.50	14.80	11.90	-	-	-	-	-	-	19.50	17.50	16.70	16.50	14.00	13.50	15.00	15.00	13.70	-
Flavimonas oryzihabitans	17.40	15.00	14.00	11.00	12.80	11.50	10.70	-	-	-	19.70	17.00	15.50	14.70	12.50	12.00	1350	-	-	-
Flavobacterium indologenes	15.50	14.20	13.60	12.50	11.50	11.20	10.50	-	-	-	16.50	15.50	15.00	14.50	12.00	12.00	12.00	12.00	-	-
F. odoratum	16.50	14.70	12.80	11.40	11.00	10.40	-	-	-	-	18.70	16.00	15.50	14.70	12.00	12.00	12.00	12.00	-	-
F. balustinum	16.40	15.50	15.00	15.00	14.30	12.00	-	-	-	-	17.00	16.00	15.50	14.50	11.50	11.00	11.00	12.00	-	-
Klebsiella planticola	16.60	14.50	14.50	13.00	13.00	-	-	-	-	-	16.50	15.40	14.50	12.00	11.00	11.00	11.00	11.00	-	-
Pseudononas aurofaciens	15.50	15.00	15.00	13.50	13.00	12.50	--	-	-	-	16.70	15.50	14.50	12.40	11.00	11.00	11.00	11.00	-	-
P. syringae	14.50	13.50	12.50	11.00	10.50	10.50	-	-	-	-	16.50	15.00	14.00	12.00	-	-	-	11.00	-	-
Salmonella enteritidis	14.20	13.00	11.50	-	-	-	-	-	-	-	15.50	14.60	-	-	-	-	-	-	-	-
Xanthomonas campestris	14.70	11.00	11.00	-	-	-	-	-	-	-	15.70	14.70	-	-	-	-	-	-	-	
CD (P=0.05)	2.29	2.10	2.40	1.35	2.65	1.92	1.75	1.66	-	-	3.11	2.96	2.70	2.65	2.70	3.05	2.41	2.30	1.90	-
CV (%)	20.50	22.11	21.50	25.00	21.66	24.91	23.90	23.10	-	-	17.50	20.22	20.91	20.85	20.82	19.02	18.20	19.90	24.21	-

Values were square root transformed before analysis

Maryland) onto 5% TSA (Tryptic Soya Agar) medium. Plates were incubated at 28°C for 24-96 hours, emerging colonies were transferred onto fresh 5% TSA medium, identified with bacterial enumeration softwere system and all pure culture of geocarposphere bacteria were maintained on nutrient agar slopes at 28°C (Chourasia 1995).

B. Clastogenic effect of crude neem extract

Healthy leaves of mature neem plant were thoroughly washed and oven dried at 60°C for 3-4 days. Coarsely powder leaves were soxhleted with 80% ethanol (v/v) and the extract was reduced under vacuum at 60°C on a water bath (Padma *et al* 1989). The extract finally obtained was in the form of a water insolube latex. Therefore, an aqueous suspension of gum acacia in sterile distilled water was used has vehicle for the extract-latex.

The experiments were performed in 6-week-old (body weight, 22 ± 3 g) laboratory bred Swiss albino mice (2n = 40), matched for sex in five groups of ten mice each group. The first solvent control group were given glass double distilled water at the rate of 0.2 m1/10g body weight/day. The second vehicle control group received the vehicle (gum-acacia) solution at a similar rate. The remaining three groups were fed orally with at the rate of 0.5g, 1.0g and 2.0g/kg body weight for seven consecutive days. These doses of the neem extract are in fact, quarter-half and full maximum tolerated dose at which the animals apparently do not exhibit any perceptible change in their general physical profile and behaviour. All the experimental animals were raised in standard laboratory conditions.

The animals were sacrificed by cervical dislocation on the eighth day, bone marrow cells of control and treated animals were taken out from the animals pre-treated with colchicine and slides were prepared by following the standard technique of Preston *et al*. (1987). The cytogenetic effect was assessed in mitotically dividing bone marrow cells by random screening of 300 Giemsa-stained well

Table : 3 Number of abnormal cells and the frequencies of different types of abnormalities scored in the bone marrow cells of mice fed with leaf extract of neem.

Amount ↑	Abnormal cells#		Individual type abnormality		Gross type abnormality		Grand Total	
	No.	%± SE	No.	%± SE	No.	%± SE	No.	%± SE
Cont. A	14	4.6 +_1.2	8	2.6 +0.9	6	2.0 ±0.8	14	4.6 ± 1.2
Cont. B	13	4.3 ± 1.2	8	2.6 ± 0.9	5	1.6 ± 0.7	13	4.3 ± 1.2
0.5 gm	35	11.6 ± 1.8*	8	2.6 ± 0.9	29	9.6 ±1.7*	37	12.3 ± 1.8*
1.0 gm	42	14.0 ± 1.9*	12	4.0 ± 1.1	33	11.0 ± 1.8*	45	15.0 ± 2.1*
2.0 gm	51	17.0 ± 2.2*	27	9.0 ± 1.7*	35	11.6 ± 1.8*	62	20.6 ± 2.3*

Cont. A (Control A) = No treatment. Cont. B (Control B) = Gum-acacia only. = gm /kg b.wt. of extract administered per animal (10 animals in each group). # = total no. of metaphases screended in each group = 300* =Indicates significant difference (at 0.1% level) with the corresponding value in the control.

spread metaphase plates selected at the rate of 30 metaphase/animal in each group.

Data were analyzed by least significant difference test at probability of 0.05 to identify significant effect of a treatment (Gomez and Gomez 1984).

RESULTS AND DISCUSSION

The crude extract of neem extended maximum zone of inhibition for *Pseudomanas syringe* (14.81 mm) (Table-1). Aqueous extract of neem showed activity upto 500 mg against *Cytophaga johnsonae* (10.50mm) and *Enterobacter cloaceae* (11.70mm). Acetone extract exhibited activity upto 300 mg against *Azobacter vinelandi* (12.00 mm), *Bacillus thuringiensis* (11.25 mm), *Clavibacter michigannse* (12.50 mm), and *Comananas testosterone* (13.00 mm). The methanol extract of neem showed activity upto 200 mg against five bacterial strains, maximum zone of inhibition was recorded for *Enterobacter cloacae* (13.70 mm).

It is clear from the result that the leaves of neem are effective in controlling bacterial pathogens. Hamsaveni and Prushothaman (1986) have reported the control of *E. coli* with the leaves of *A. indica.* Rao and Satyanarayana (1977) reported antibacterial activity of neem against *B. anthracis, S. albus, B. pumili*s and *Pseudomonas* sp. In the present study, crude extract of neem leaves was found active against *C. johnsonae* and *E. cloacae. P. aurofaciens and P. syringae* even at 10% concentration. Aqueous extract was effectively controlled by *C. johnsonae* and *E. cloacae.* the crude extract of neem did not control *B. thuringiensis, C. cartae, F. oryzihabitans, F. indologenes, F. odoratum, F. balustinum,* whereas the alcoholic extracts of neem were extremely effective in controlling these bacterial strains (Table-2). The maximum of inhibition against *Clavibactrer michiganense* was recorded with methanol extract (26.00mm) followed by acetone extract (17.50mm). The alcoholic extracts of neem leaves showed a higher antibacterial activity when compared to the of the crude and aqueous extracts. The action may be synergistic and not due to that efficacy of one single substance.

The frequency of total abnormal cells increased in comparison to the control group of animals (4.3%) by about three fold (11.6%), three and a half fold (14.0%) and four fold

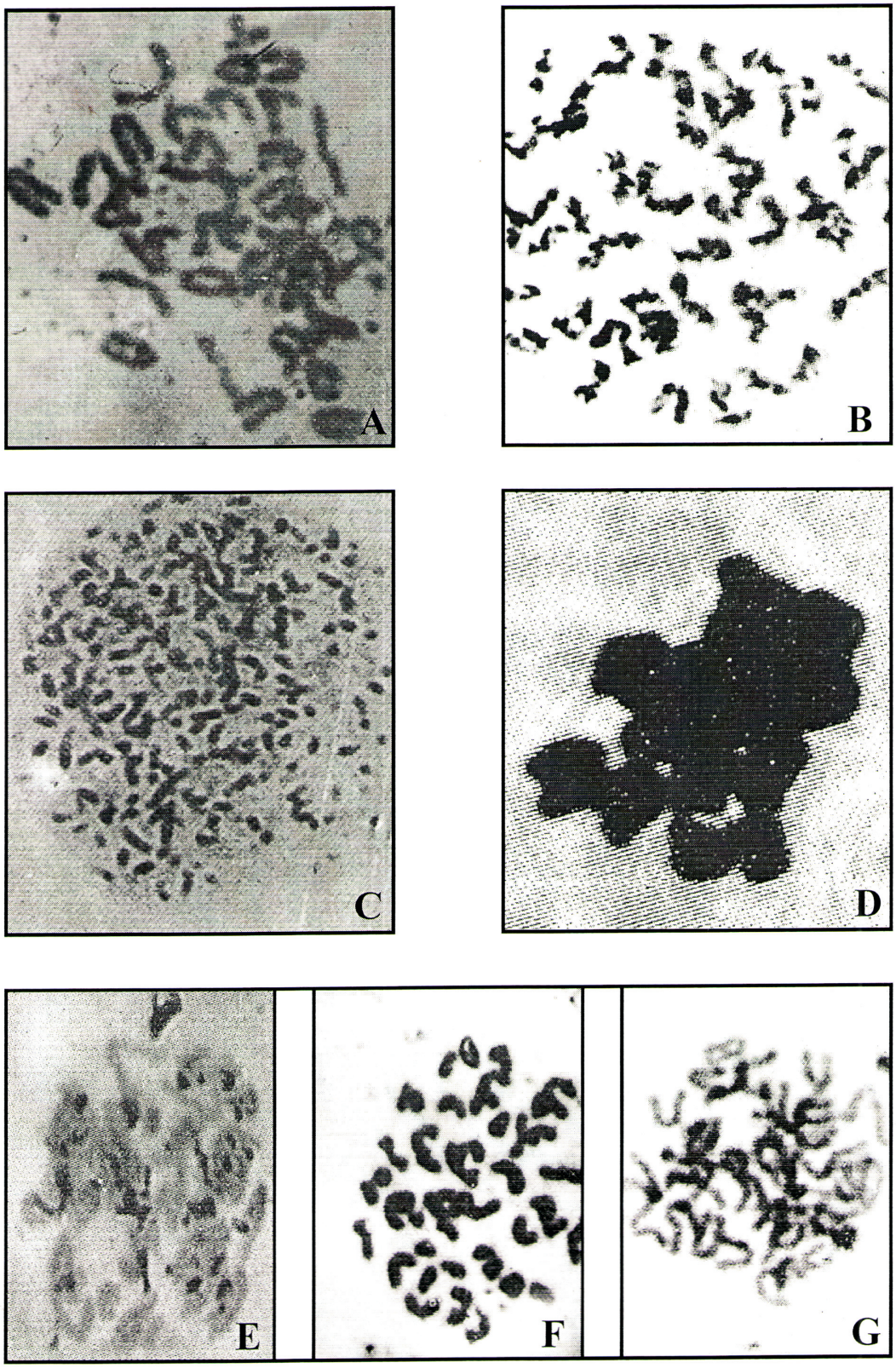

Plate - 1. Figures A - G : A - Ring, B - Pulvirisation, C - Fragmentation, D - Clumping, E - Stickness, F - Precocious Chromatid Separation (PCS), G - Picnotic.

(17.0%) after treatment with lowest (0.5/g/kg), middle (1.0g/kg) and the highest (2.0 g/kg) doses, respectively of the neem extract (Table-3). The alcoholic leaf extract induced both gross and individual types of chromosome abnormalities in each treatment group. The changes of the latter type were higher than the former. The individual type comprised 76% of the total abnormal plates which included breaks, gaps and fragmentation of the chromosomes. The gross type abnormalities showed stickiness, clumping, hypoploidy, polyploidy and pulverisation of chromosmes. Plate-1shows some of the common abnormalities. The frequency of total chromosomal abnormalitiess mal increased significantly in lowest (12.3%), middle (15.0%) and highest (20%) doses, respectively in comparison to the control (4.6%). Thus the increase in abnormal cells and chromosome abnormalities were dose dependent i.e. increase gradually with the increase of doses.

The gross and individual types of chromosomes however, differed at different doses of extract, gross type damages were significantly noticed with respect to the control at all the treatment doses but individual type of damages was only at the highest dose. Presence of gross type changes in treated with lower dose and of structural type only at highest dose of extract might be result of the damages occurring at two different levels by affecting first the internal milieu of cells at lower dose leading to meiotic poisoning, appearing as gross type of damages and by affecting the morphology of chromosomes at highest dose in latter stage leading to individual type of damages. In these quantities, the damages first occurred at the level of protein leading to gross cytotoxic effect. Production of ions and radicals during metabolism of leaf-extract might be interacting with the nucleophilic sites of DNA leading to the chromatid breaks and other related damages in the latter(Kolpman *et al.* 1985)

Biomutagens like ochratoxin A and aflatoxin (secondary fungal metabolites) were capable to induce significantly more gross type than that of individual type of damages, while individual type of damages were most frequent than the gross type at various lower and higher concentration of synthetic pesticides (Bose and Sinha 1994). It is therefore, clear that biomutagens are of meiotic poisoning leading to gross type of damages. By virtue of this property neem extract can be considered as less genotoxic than the synthetic pesticides. Its use should therefore, preferred to avoid injuries to the non-target organisms.

The antimicrobial action of neem has been exploited in retarding the decomposition of organic manures and in protecting the biodegradable insecticide like carbofuran under soil application (Morgan 1987). Most of the recent studies on antibacterial activity of neem has been done in laboratories because treating bacterial is relatively straight-forward. In test tubes, neem has been shown to have significant effect on both gram-positive and gram-negative organisms and other bacteria that cause a wide array of human and animal diseases (Subapriya and Nagini 2005).

Despite much availability of neem trees in India, most of the Indians do not know the significance and how to utilize them. It becomes necessary to take special effort to publicize these neglected booms of our backyards. The present investigation confirms the claim of antimicrobial activity of neem trees. The neem tree could be used as the best substitute for modern antibiotic because the later medicine has undesirable side effect and its long term use can develop resistance to pathogenic bacteria.

The senior author (HKC) is thankful to Government of India, Department of education, New Delhi for the award of Post-doctoral Fellowship Abroad and to Prof. Barry J. Jacobsen, Head, Department of Plant Pathology, Alabama Agricultural Experiment Station, Auburn University, USA, for

providing laboratory facilities.

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