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Inhibition of Clastogenic Effect of Cyclophosphamide and Mitomycin C by Neem leaf-extract in Mice

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Azadirachta indica **commonly known as 'Neem' is well known for its medicinal properties in the indigenous Indian system of medicine. Almost every part of the tree has some beneficial use. The anticlastogenic activity of 'Neem' against cyclophosphamide (CP) and mitomycin C (MMC) was studied *in vivo* in bone marrow cells of mice. Aqueous leaf-extracts of *A. indica* were injected intraperitoneally at doses 3, 6, 12 and 24 mg/kg body weight. Simultaneously, two known clastogens CP (10 mg/kg) and MMC (1.5 mg/kg) were administered individually to animals treated with 6 and 12 mg/kg of the leaf-extract. The end-points screened were chromosomal aberrations and damaged (aberrant) cells. Neem leaf-extract *per se* was found to be a weak clastogen; 6 and 12 mg/kg of the leaf-extract inhibited the clastogenicity of CP and MMC. The extent of inhibition was different for the two clastogens. An ANOVA test showed that the reduction in the frequency of chromosomal aberrations was significantly less when the leaf-extract was given in combination with CP. MMC co-administered with the leaf-extract showed a trend that was not statistically significant. The difference may be attributed to the degree of modulation of bioactivation of cytochrome P-450 enzymes, or the repair of damaged DNA or a difference in detoxification of the reactive species of the two genotoxicants.** © **1998 John Wiley & Sons, Ltd.**

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INTRODUCTION

In recent years, there has been an upsurge in the clinical use of indigenous plants (Sharma, 1995). Plant derived products that represent a structurally diverse array of antimutagenic and anticarcinogenic substances include plant pigments, vitamins, polyphenols, flavonoids, aromatic isothiocynates, coumarins, sterols etc. (Hayatsu *et al.*, 1988).

Azadirachta indica popularly known as Neem (NM) is an evergreen tree found in most parts of India, Burma, Sri Lanka and Malaysia. In Indian systems of indigenous medicine NM is used as a remedy for a wide variety of human diseases (Chopra *et al.*, 1956; Kirtikar and Basu, 1975). The purpose of the present study was to determine whether and to what extent NM could modulate the genotoxic damage of standard clastogens—cyclophosphamide (CP) and mitomycin C (MMC). Mouse bone marrow cells were chosen as indicator cells for their high sensitivity to the clastogens.

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MATERIALS AND METHODS

Animals. Male Swiss albino mice, 8–10 weeks old and weighing 20–25 g were obtained from the Departmental Animal House, housed six/cage under standard husbandry and feeding schedules followed for clean conventional colonies (temperature 25° ± 2°C, relative humidity 60% ± 5%, 12 h light/dark photo-period). Animals were allowed access to *ad libitum* to standard rodent pellet diet (Gold Mohar, Lipton India, India) and water.

Chemicals and dose. Based on our preliminary studies and the studies reported by Sen *et al.* (1992) doses of NM were chosen for the present study. An extract of dried NM leaves was prepared as an aqueous suspension in distilled water.

Treatment groups. Experiments were carried in the following groups. Group I, animals treated with NM leaf-extract at doses of 3, 6, 12 and 24 mg/kg body weight. Group II, animals treated with cyclophosphamide (CP) at a dose 10 mg/kg body weight. Group III, animals treated with mitomycin C (MMC) at a dose 1.5 mg/kg body weight. Group IV, animals treated with NM at doses of (i) 6 and (ii) 12 mg/kg body weight were given CP (10 mg/kg body weight) or MMC (1.5 mg/kg body weight).

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Table 1. Data on chromosomal aberrations in bone marrow cells of mice following treatment with different doses of Neem leaf extract (NM)

	Chromosomal aberration data						
Treatment (mg/kg b.w.)	G′	G″	B′	B″	RR	% DC $\overline{X} \pm$ SD	CA/Cell $\overline{X} \pm$ SD
Control	1	—	4	—	—	1.33 ± 1.15	0.026 ± 0.23
NM (3)	2	1	8	—	—	3.33 ± 2.30	0.05 ± 0.01
NM (6)	2	—	5	1	—	4.00 ± 3.46	0.04 ± 0.03
NM (12)	1	—	7	4	—	6.66 ± 3.05	0.06 ± 0.04
NM (24)	6	2	13	1	—	8.67 ± 2.31	0.09 ± 0.03
Trend test Z value						2.87[a]	4.14[a]

G′,G″, chromatid and chromosome gaps; B′,B″, chromatid and chromosome breaks; RR, rearrangements; DC, damaged cell; CA, chromosomal aberrations, $\overline{X}$, mean of 4 animals for single treatment (50 metaphase/animal); SD, standard deviation of the mean.
[a] Significant at $p < 0.05$ Cochran–Armitage trend test.

Table 2. Data on chromosomal aberrations in bone marrow cells of mice following treatment with mitomycin C (MMC) and cyclophosphamide (CP) singly and in combination with different doses of Neem leaf extract (NM)

	Chromosomal aberration data						
Treatment (mg/kg b.w.)	G′	G″	B′	B″	RR	% DC $\overline{X} \pm$ SD	CA/Cell $\overline{X} \pm$ SD
CP (10)	6	1	67	10	5	20.00 ± 4.00	0.54 ± 0.040
MMC (1.5)	2	1	54	19	2	29.33 ± 1.15	0.50 ± 0.072
NM 6 + CP 10	1	—	19	5	0	13.33 ± 6.11	0.48 ± 0.080
NM 12 + CP 10	4	1	16	7	4	11.33 ± 7.57	0.18 ± 0.140
NM 6 + MMC (1.5)	2	1	58	8	5	28.66 ± 9.01	0.47 ± 0.213
NM 12 + MMC (1.5)	9	4	29	17	2	20.67 ± 10.26	0.32 ± 0.160

G′,G″, chromatid and chromosome gaps; B′,B″, chromatid and chromosome breaks; RR, rearrangements; DC, damaged cell; CA, chromosomal aberrations; $\overline{X}$, Mean of 4 animals for single treatment (50 metaphase/animal); SD, Standard deviation of the mean.

Table 3. Duncan's multiple range test showing significant differences (if any) among different treatment groups

A. Number of chromosomal abnormalities

Doses: NM 6, CP 10			
Experimental sets	Neem	Neem + CP	CP
Sample means	2	8	27.33
Doses: NM 12, CP 10			
Experimental sets	Neem	Neem + CP	CP
Sample means	3.66	9	27.33
Doses: NM 6, MMC			
Experimental sets	Neem	Neem + MMC	MMC
Sample means	2	23.66	25
Doses: NM 12, MMC			
Experimental sets	Neem	Neem + MMC	MMC
Sample means	3.66	16	25

B. Number of damaged cells

Doses: NM 6, CP 10			
Experimental sets	Neem	Neem + CP	CP
Sample means	2	6.66	10
Doses: NM 12, CP 10			
Experimental sets	Neem	Neem + CP	CP
Sample means	3.33	5.66	10
Doses: NM 6, MMC			
Experimental sets	Neem	Neem + CP	CP
Sample means	2	14.3	14.6
Doses: NM 12, MMC			
Experimental sets	Neem	Neem + MMC	MMC
Sample means	3.33	10.3	14.6

The blocks which are underlined together are not significantly different (at 5% level) among each other.

Group V, animals treated with distilled water (vehicle) only.

In all cases, treatments were done by intraperitoneal (i.p.) injections in a volume of 10 mL/kg body weight. The animals were killed by cervical dislocation 18 h after administration (McFee and Tice, 1990). Three animals were used for each group of treatment/control. At 90 min prior to killing the animals were injected i.p. with 0.04% colchicine.

Chromosomal aberration assay (CA). Bone marrow cells were flushed in 0.075 M KCl, incubated at 37°C for 30 min, and fixed in cold 1:3 glacial acetic acid–methanol. Slides were prepared by flame drying and stained in Giemsa (Preston *et al.*, 1987). Fifty well spread metaphase plates were scanned per animal per treatment. The types of aberrations were scored according to the method of Tice *et al.* (1987). Thus the percentage of aberrant cells (% DC) and chromosomal aberration/cell (CA/cell) were calculated. In all cases, gaps were scored but not included for calculation.

Statistical analyses. For statistical analyses, the Cochran–Armitage one-tailed trend test (Margolin *et al.*, 1986) was used to determine if a treatment-related increase occurred. A two-way ANOVA test followed by Duncan's multiple range test (Sokal and Rohlf, 1981) was carried out to observe significant differences between the individual groups. For all statistical analyses, the level of significance was established at $p < 0.05$.

RESULTS

The clastogenic effects of the NM leaf-extract are summarized in Table 1. The incidence of metaphase with chromosomal aberrations was proportional to the doses tested. A Cochran–Armitage trend test showed a significant positive dose-response for the frequencies of aberrant cells as well as for CAs per cell. Table 2 shows the observations made after the exposure to CP or MMC alone and in combination with the two doses of NM leaf-extract. The aberrations scored were mainly chromatid and chromosome type aberrations. In general, the frequency of damaged cells and aberrations per cell was lower than in the animals given the clastogens (CP and MMC) alone.

Statistical analysis using one-way ANOVA followed by Duncan's new multiple range test was used to compare the inhibitory effect of the NM leaf-extract on CP and MMC-induced clastogenesis (Table 3). Doses of 6 and 12 mg of NM leaf-extract were much more effective in antagonizing the clastogenic potentiality of CP. The values of % DC and CA per cell in the group treated with MMC in combination with the NM leaf-extract decreased marginally and the reduction was not significant when compared with MMC-induced clastogenesis.

DISCUSSION

The principal constituents of the NM leaf-extract are the alkaloid margosine, margosic acid, nimbidin, nimbin, nimbolide, meliacin in addition to other compounds with sulphur moieties (The Merck Index, 1983). There is substantial information available on the antihelmintic, antibacterial and antifertility properties of Neem (Riar *et al.*, 1991; Agomo *et al.*, 1992; Chattopadhyay *et al.*, 1992). Information on the modulatory action of Neem leaves against clastogenicity or mutagenicity is not well documented. The present work is a part of a series of investigations which are being carried out in our laboratory to study the protection afforded by a series of crude extracts of plant parts and related compounds against known clastogens (Mukherjee *et al.*, 1991; De *et al.*, 1995; Sharma, 1995; Sen *et al.*, 1996).

In the present communication we report that the NM leaf-extract *per se* is a weak clastogen and could reduce CP and MMC-induced clastogenicity in mouse bone marrow cells. This reduction was statistically significant for the NM leaf-extract given in combination with CP, while in the other set given in combination with MMC it was not. It is possible that NM might inhibit cytochrome P450 enzymes involved in CP and MMC metabolism, *in vivo*. However, the extent to which MMC activity was inhibited by NM was either not sufficient to elicit complete suppression of MMC-clastogenicity to a significant level, or the repair of damaged DNA or detoxification of reactive species predominated over NM-induced inhibition of clastogen activation.

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