Differential Genotoxicity of the Crude Leaf Extract of a Medicinal Plant, *Casearia tomentosa*

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The genotoxic potentiality of the crude leaf extract of *Casearia tomentosa*, a medicinal preparation, has been evaluated in Swiss albino mice. The extract significantly induced the division-disruptive chromosomal changes in bone marrow cells as well as in primary spermatocytes; the latter also exhibited marked increase in synaptic disruptions. A significant decrease in sperm count was noted. The incidence of structural damages in chromosomes, however, remained within the range of control level frequency. This herbal preparation, therefore, appears to be primarily spindle-poisoning in its action, but not clastogenic. The probable mechanism of this differential genotoxicity is discussed.

INTRODUCTION

Many herbal drugs are frequently used in their "crude" form by a large number of people in India. Toxic effects are, therefore, likely to be produced at one or the other level (krenzelok, 1995). Scant attention has been paid to the genotoxicity of these drugs; it is, therefore, highly pertinent to assess the mutagenic potentials of herbal preparations for hazard identification.

Casearia tomentosa Roxb. (Syn. *C. elliptica* Willd.), belonging to the family Flacourtiaceae/Samydaceae, is a small tree or shrub that grows wildly throughout India in forests and adjoining regions. It is most common in waste-grounds and river valleys; particularly on laterite in scrub-jungles. Its common name in the entire North India including Patna (Bihar) is *Churchu*, which is actually a name given by Santhal tribals living in Bihar. Various preparations of this plant have wide medicinal uses. The pulp from fruits has diuretic property and the powdered bark is applied externally in dropsy, fever and snake bite (Ambastha, 1986). The bark suspension is prescribed as a tonic for anemic conditions and the leaf paste is used as an antihelminthic while the decoction of its root (trade name "saptrangi") is reported to be a remedy for diabetes. Besides many other medicinal used, it has also been reported to have anticancer properties (Krishnamurthi, 1992). The present work is an attempt to evaluate the genotoxic potentiality, if any, of the crude leaf extract of *C. tomentosa* in Swiss albino mice.

MATERIALS AND METHODS

Mature leaves of *C. tomentosa*, collected during the flowering season (late March), were washed in running tap water, dried in an oven (at 60℃), coarsely powdered in glass mortar and then soxhlated with 80% ethanol (v/v). Extract so obtained was made ethanol

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0895-3988/2000
CN 11-2914

free under vaccorotatory evaporator and finally dried on water bath (at 60℃) to convert it in the form of a powder. A homogeneous suspension of this powder was then prepared by using 10% aqueous solution of gum-acacia (Loyd 's Pharma, Delhi) as its vehicle.

Experiments were performed in 6—8 weeks old healthy laboratory-inbred Swiss albino mice (S-cdri, seed colony supplied by Central Drug Research Institute, Lucknow, India). Animals were stratified into five experimental groups, comprising three treated groups for three different doses of the extract and two controls. A description of the experimental groups and the treatment protocol is given in Table 1. Long-term treatment with plant extract was preferred to the short-term treatment, because it affects a larger proportion of the stem cell population (Salvadori *et al.*, 1988). Treatments, given orally to animals once daily by intubation, were continued for seven days.

TABLE 1

Experimental Groups and Treatment Protocol

Experimental Groups	Treatment	Doses[a] (per kg body wt/day)
Solvent control : SC	Distilled water	20 ml
Vehicle control : VC[b]	Gum acacia	2.0 g
Treated groups : T_1	Leaf extract	0.5 g (1/4 MTD)[c]
T_2	"	1.0 g (1/2 MTD)
T_3	"	2.0 g (MTD)

[a] Dilution of aqueous solution/suspension of gum acacia/leaf extract was adjusted to keep administered volume equal (i.e., 20 ml/kg body wt/day) in all groups.

[b] homogeneous suspension of leaf extract was prepared by using 10% aqueous solution of gum-acacia as its vehicle; therefore besides a solvent control (received distilled water only), an experimental group of vehicle control (received 10% aqueous solution of gum-acacia) was also set aside to know the effect of gum-acacia, if any.

[c] MTD (maximum tolerated dose) : Maximum dose of the leaf extract suspension well tolerated by animals without showing any signs of clinical toxicity, estimated by subjecting several groups of animals to a series of different doses.

Three separate set-up of experimental animals, each with five groups of animals, were used for three different tests to analyze the effect of leaf extract. These were mitotic chromosome test in bone marrow cells, meiotic chromosome test in primary spermatocytes, and sperm toxicity test.

Mitotic Chromosome Test

All the experimental animals were injected colchicine intraperitoneally (4 mg/kg body wt) 24 h after the last treatment and 2 h prior to their sacrifice by cervical dislocation. Bone marrow from both femora was flushed out in hypotonic KCl solution (0.075 mol/L) and slides were prepared by the standard aceto-alcohol-flame drying-Giemsa staining technique (Preston *et al.*, 1987). 600 well spread metaphases per experimental group of six animals (three males and three females) were selected randomly (at about 100 metaphases per animal) from coded slides which were screened simultaneously for the detection of chromosomal abnormalities. The abnormalities detected were put into two categories namely structural damages (chromatid/chromosome break, gap, acentric fragment) and division-disruptions (aneuploidy, polyploidy, centric fusion, C-mitosis, precocious separation, stickiness). The data obtained from each animal were pooled in their respective group and expressed as mean frequency per 100 cells. An equality of proportion test (Z-test) using $P<0.05$

as criterion of significance as well as ANOVA were applied for statistical evaluation of the data (Downie and Heath, 1970).

Meiotic Chromosome Test

Germ cells exposed to various treatments during a week-long period at their differentiating spermatogonial stage entered into meiotic (reduction) division after a further lapse of 6—7 days (Adler, 1982). Accordingly, animals were sacrificed on fifteenth day from the start of treatment for harvesting the treated cells as primary spermatocytes. Animals were injected colchicine intraperitoneally (at 4 mg/kg body wt) 1.5 h prior to their sacrifice. Slides were prepared from the testicular cell-suspension by the standard aceto-alcohol-flame drying-Giemsa staining schedule (Das and Nayak, 1988).

A total of 500 first metaphases for each experimental group of five animals (males only) were selected randomly from coded slides and about 100 metaphases were screened per animal. The chromosomal abnormalities detected were put into three categories, namely structural damages (chromosome/chromatid breaks, fragments), division-disruptions (aneuploidy, polyploidy) and synaptic disturbances (univalents of autosomes and/or sex-chromosomes). The data obtained from each animal were pooled and subjected to the equality of proportion test (Z-test) with $P<0.05$ as the crite-rion of significance and ANOVA (Downie and Heath, 1970) for statistical evaluation.

Sperm Toxicity Test

Variations in sperm head morphology as well as in sperm counts were assayed by sacrificing the animals on the 28th day from the termination of week-long treatment period. The sperms thus screened were derived from the differentiating spermatogonial cells exposed to various treatments (Wyrobek *et al.*, 1983, 1984).

For sperm count, the two caput epididymides were taken out from each animal. They were minced in 2 ml of distilled water (Meistrich, 1989) and counting of sperms was made from their suspension with the aid of RBC counting chamber of Neubauers hemocytometer at 400× magnification (Rastogi and Levin, 1987). Average sperm counts from randomly marked five animals in each group were pooled and expressed as mean sperm count per ml of the suspension. The significance of differences between the mean sperm counts of various groups was determined by Student's t-test (at 5% level), whereas the effects of three doses were analyzed by ANOVA followed by Scheff's test (Downie and Heath, 1970).

For the analysis of gross morphology of sperm heads, the two cauda epididymides along with their corpuses from each mouse were minced together in 2 ml of distilled water (Khan and Sinha, 1996) to squeeze-out sperms. After filtration through a metallic net of 80 μ mesh size, smears of sperm suspension were prepared on clean slides, air dried and fixed in absolute methanol for 10 min. Slides were stained after 24 h in 1% aqueous Eosin-Y for 1 h (Rastogi and Levin, 1987), rinsed in running tap water and then air dried. A total of 3000 sperms from each group of 5 animals, selected randomly from coded slides at about 600 sperms per animal, were examined at 1000× magnification (Wyrobek, Watchmaker and Gordon, 1984). Sperms with gross abnormalities in their head shape, head size, head number and head spine were categorized separately (Khan and Sinha, 1996). The results, expressed as percent occurrence of abnormal sperms, were evaluated by the equality of proportion test (Z-test at 5% level) and ANOVA (Downie and Heath, 1970).

RESULTS

Effect on Mitotic Chromosomes

The Treatments with the crude leaf extract of *C. tomentosa* produced no significant increase in the frequency of structural damages or in sum total of all abnormalities in bone marrow chromosomes compared to the corresponding values in solvent control or vehicle control (Table 2). These remained almost in the same range (structural damages: 2.33% to 2.64%; sum total of all abnormalities: 4.33% to 8%). However, the frequency of division-disruptions in the extract treated groups (T_1: 4.67%; T_2: 5.17%; T_3: 5.67%) increased significantly compared to the control (SC: 2%; VC: 1.83%).

TABLE 2

Frequency of Chromosomal Abnormalities in the Bone Marrow Cells of Mice From Various Experimental Groups[a]

Experimental Groups	Abnormalities in Chromosomes[b]		Sum Total
	Structural damages (Ⅰ) % ± SE	Division-disruptions (Ⅱ) % ± SE	Ⅰ + Ⅱ % ± SE
SC	2.33 ± 0.61	2.00 ± 0.57	4.33 ± 0.83
VC	2.67 ± 0.66	1.83 ± 0.55	4.50 ± 0.85
T_1	2.67 ± 0.66	4.67 ± 0.86[cd]	7.33 ± 1.06
T_2	2.50 ± 0.64	5.17 ± 0.90[cd]	7.67 ± 1.09
T_3	2.33 ± 0.61	5.67 ± 0.94[cd]	8.00 ± 1.10

[a] Among 600 metaphases screened in each experimental group of 6 animals at about 100 metaphases per animal; abnormalities scored in metaphases of all the six animals in each group were pooled and expressed as mean (percent) occurrence of individual abnormality or sum total of all abnormalities.

[b] Abnormalities scored: chromosome/chromatid breaks/gaps/fragments as strucutral damages; numerical variations in chromosomes as division-disruptive changes.

[cd] Significant differences (at 5% level) compared to both SC and VC (*Z*-test).

The results did not reveal dose-dependency for any abnormality type and the one-way ANOVA failed to indicate difference in mean variance for sum total of all abnormalities.

Effect on Meiotic Chromosomes

The frequency of all abnormalities in groups T_1 (9.2%), T_2 (10.4%) and T_3 (11.6%) was significantly higher than the 3% abnormality frequency in vehicle control (Table 3). Of the total abnormality in the T_1 dose group, structural damages, division-disruptions and synaptic disturbances accounted for 0.6%, 1.0% and 7.6% respectively. Structural damages were also minimal (0.6%) while the division-disruptions and synaptic disturbances were 1.6% and 8.2% respectively in the T_2 dose group. T_3 dose group exhibited 1.6% structural damages, 1.2% division-disruptions and 8.8% synaptic impairments. Thus, the significant increase in the total of all abnormalities was due to marked increase in synaptic disturbances since no significant increase was observed in the incidence of structural or division-disruptive changes at any dose level.

TABLE 3

Frequency of Chromosomal Abnormalities in the Primary Spermatocytes of Male Mice From Various Experimental Groups[a]

Experimental Groups	Abnormalities in Chromosomes[b]			Sum Total
	Structural damages (Ⅰ) % ± SE	Division-disruptions (Ⅱ) % ± SE	Synaptic disturbances (Ⅲ) % ± SE	Ⅰ + Ⅱ + Ⅲ % ± SE
SC	0.6 ± 0.35	1.2 ± 0.49	2.4 ± 0.68	4.2 ± 0.90
VC	0.6 ± 0.35	0.8 ± 0.40	1.6 ± 0.56	3.0 ± 0.76
T_1	0.6 ± 0.35	1.0 ± 0.44	7.6 ± 1.19[cd]	9.2 ± 1.29[cd]
T_2	0.6 ± 0.35	1.6 ± 0.56	8.6 ± 1.25[cd]	10.4 ± 1.37[cd]
T_3	1.6 ± 0.56	1.2 ± 0.49	8.8 ± 1.27[cd]	11.6 ± 1.43[cd]

[a] Among 500 metaphases screened in each experimental group of 5 animals at about 100 metaphases per animal; abnormalities scored in metaphases of all the five animals in each group were pooled and expressed as mean (percent) occurrence of individual abnormality or sum total of all abnormalities.

[b] Abnormalities scored: chromosome/chromatid breaks/gaps/fragments as structural damages; numerical variations as division-disruptive changes; univalent formations as synaptic disturbances.

[cd] Significant differences (at 5% level) compared to both SC and VC (*Z*-test).

Variations in Mean Sperm Count

Significant decreases in mean sperm count were noticed in the T_1 (8.521×10^6/ml), T_2 (6.630×10^6/ml) and T_3 (5.934×10^6/ml) dose groups over the two controls (SC: 10.514×10^6/ml; VC: 10.636×10^6/ml). ANOVA confirmed the differences to be significant at 1% level; though the inter-animal variation was insignificant. The decrease in mean sperm count, however, did not show any dose dependency (rSC: +0.928; rVC: +0.927).

Effect on Sperm Head Morphology

The frequencies of all aberrant sperms were 4.07%, 4.87% and 5.57% in the T_1, T_2 and T_3 groups respectively, which indicate that significant increase over the frequency in solvent control (4.03%) occurred only at T_3 dose group. ANOVA confirmed the significant difference in variance at 1% level but with insignificant inter-animal variation. Correlation between the dose and rate of abnormality did not show dose-dependency with solvent control (at 1% level); however, the same was positive (rSC: +0.885; rVC: +0.982) with vehicle control (Table 4).

DISCUSSION

Synthetic drugs such as niclosamide (Ostrosky *et al*., 1986), hydroxyquinoline (Ghaskadbi *et al*., 1987), clofazimine (Das and Roy, 1990) and cyclophosphamide (Ghaskadbi *et al*., 1992) are potent clastogens. The presently used herbal extract, when compared with these synthetic drugs, was found to be less damaging genetically, since it produced no significant structural damages in the mitotic or meiotic chromosomes. The extract induced alterations in chromosomes were mainly confined to numerical variations (division-disruptions) or their synaptic impairments. However, subtle changes at the level of DNA induced by the extract cannot be ruled out as menifested by the significant increase in the frequency of abnormal sperms. Such sperms are primarily produced as a result of alterations in testicular DNA and sperm-

chromatin structure (Evenson *et al.*, 1986). Never-theless, the extract-inflicted physiological toxicity might be responsible for this adverse effect on sperm morphology.

TABLE 4

Frequency of Murine Sperms With Abnormal Head From Various Experimental Groups[a]

Expt. Groups	Sperms With Head Abnormalities				Sum Total
	Mis-shapen (Ⅰ) % ± SE	Spine-less(Ⅱ) % ± SE	Twin-headed (Ⅲ) % ± SE	Odd-sized (Ⅳ) % ± SE	Ⅰ + Ⅱ + Ⅲ + Ⅳ % ± SE
SC	2.87 ± 0.31	0.87 ± 0.17	0.0 ± 0.0	0.30 ± 0.09	4.03 ± 0.36
VC	3.30 ± 0.33	0.87 ± 0.17	0.0 ± 0.0	0.40 ± 0.12	4.57 ± 0.38
T_1	2.91 ± 0.31	0.73 ± 0.16	0.03 ± 0.03	0.37 ± 0.11	4.07 ± 0.36
T_2	3.37 ± 0.33	0.90 ± 0.17	0.0 ± 0.0	0.60 ± 0.14	4.87 ± 0.39
T_3	3.37 ± 0.35	1.00 ± 0.18	0.03 ± 0.03	0.80 ± 0.16[a]	5.57 ± 0.42[b]

[a] Among 3000 sperms screened randomly from each experimental group of 5 animals at about 600 sperms per animal and the results expressed as mean (percent) occurrence of abnormal sperms.

[b] Significant difference (at 5% level) compared to SC (*Z*-test).

The leaf extract, therefore, seems to interfere with or poison the functioning or assemblage of the spindle-apparatus of dividing cells, the mechanism of action proposed for spindle poisons (Au and Hsu, 1982). The damages in the proliferating cells due to the spindle poisoning activity of the extract might have led to suicidal self-killing (apoptosis) of the cells of succeeding stages, which is expressed in the present study as decreases in sperm count.

The present observation, thus suggesting the spindle poisoning nature of the crude leaf extract of *C. tomentosa*, is in full conformation with its reported anticancer properties (Krishnamurthi, 1992) and also with the effects of the crude leaf extract of another medicinal plant, Neem (*Azadirachta indica*), which preferentially induce division-disruptive changes in chromosomes over the structural ones (Awasthy *et al.*, 1995, 1999).

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(*Received May* 5, 1999 *Accepted December* 11, 1999)