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Anticancer Effect of *Terminalia cattapa* against the Aflatoxin B1-induced genotoxicity in human peripheral lymphocytes in vitro.

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ABSTRACT

Terminalia catappa is found in the wormer part of India.It is known as Indian Almonds,Malabar Almond and Tropical Almond.It posses anticancer ,antioxidant as well as anticlastogenic characteristics.Terminalia catappa for disease such as diarrhea,gonorrhea and skin ailments including scabies..The antigenotoxic potential of *Terminalia catappa extract* (TC) was demonstrated on the Aflatoxin B1- induced genotoxicity. *In vitro* studies were carried on human lymphocyte culture. We have used chromosomal aberration (CA), sister chromatid exchange (SCE) and cell cycle kinetics (CCK) with and without S9 mix. as markers in this experiments. Four doses viz., 100, 150, 200, 250 µl/ml per culture were selected and found that *Terminalia catappa extract* significantly reduces the frequencies of chromosomal aberration, sister chromatid exchanges and enhances RI *in vitro* .It was also noticed that the antigenotoxic potential of (TC) shows dose – response relationship. The results suggest that TC was a potent anticarcinogen may contribute to the cancer prevention.

Key words: *Terminalia catappa extract, chromosomal aberration, Sister Chromatid Exchange,cell cycle kinetics,Aflatoxin B1 genotoxicity.*

INTRODUCTION

Terminalia catappa (also known as ornamental tree) belonging to the family Combretaceae, has been used since time immemorial in the folklore and traditional systems of medicine in India, to treat several diseases anticancer, antioxidant as well as anti clastogenic (Charang *et al.*, 2002). Common names of *Terminalia catappa* are badam, the timber is moderately easy to saw and work, polishes well as a good constructional timber (Inbaraj *et al* 2006).

The plant is grown in the wormer part of India.It is also known as Indian Almond, Malabar Almond and Tropical Almond growing up to 9.0 feet tall.Terminalia is a genus of combretaceous plants widely distributed in tropical and subtropical regions.In several Asian countries physicians have used the leaves,bark,and fruit of Terminalia catappa to treat dermatitis and pyresis.The leaves are used in the treatment of leprosy and for reducing travel nausea,treat eye problems,for w ound and to stop bleeding during teeth extraction (Untwal et al,2006).The leaves are also used in the treatment of different types of cancer and to treat skin diseases (Goun et al,2003).It is considered to

have aphrodisiac and antibacterial properties in Taiwan (Christian et al,2006).Terminalia leaves were believed to be responsible for the anti oxidant activities of the aqueous and methanolic extracts (Chyau et al,2006).

The plant is reported to have anti-mutagenic effect. The antioxidant activity was evaluated by determination of their ability to prevent lipid peroxidation,inhibit superoxide formation and by their free radical scavenging activity (Lin et al, 2001).Terminalia catappa extract ability to inhibit TPA induction of hydrogen peroxide formation and to scavenging of oxygen free radicals in human leucocytes (Liu et al,1996).The tannins isolated from leaves include coliragin and ellagic acid (Rayudu et al,1996), terflavins A and B,tergallagin, tercatain punicalin,punicalagin,geranin,granatin and desgalloyleugenin (Tanaka et al,1986).

Terminalia catappa leaf extracts exert has a range of biological effects on cells,including antioxidant and hepatoprotective activity on hepatocyte and liver mitochondria, and preventive activity against hepatocyte apoptosis (Kinoshita et al, 2007;Tang et al,2004).Terminalia exhibited significant reversal of altered lipid levels near to normal values in rats with experimentally induced fibrosarcoma,(Naitik et al,2012).

MATERIALS AND METHODS

Experiment was performed using the technique of Moorehead *et.al* (1960), for metaphase chromosome analysis and for detection of chromosomal aberration analysis (CAs). Human lymphocyte cultures were set by adding 0.5 ml of whole blood (from two adult and healthy donors, occupationally not exposed to mutagens) to 4.5 ml of RPMI 1640 (Gibco, USA), antibiotics (Penicillin and streptomycin 100 IU/ml each; Hoechst) and L. Glutamine (1 mM; Gibco, USA). Lymphocytes were stimulated to divide by adding 0.1 ml of phytohaemagglutinin– M (PHA– M, Gibco). The cultures were incubated at 37oC with 5% CO2 for 72 hours in dark. Aflatoxin B1 at a final concentration of 50 µg was added at 0 hour and kept for 24, 48 and 72 hours of duration, which served as positive control. Subsequently, desired test chemical were added along with aflatoxin B1, and the cultures were kept for 24, 48 and 72 hours.Terminalia catappa and Aflatoxin B1 were prepared in DMSO. In the metabolic activation experiments cultures were treated with S9 mix (0.8 ml.), the S9 mix was freshly prepared as per the standard procedures of Maron and Ames (1983). The S9 fraction was complemented by the addition of 5 µM NADP and 10 µM glucose –6-phosphate just before use. Colchicines (0.20 µg/ml, Micro lab) were added to the cultures 2.5 hours prior to harvesting. The cells were collected by centrifugation (10 min, 1200 rpm), hypotonic treatment (0.075M KCl) was given for 10-12 min at 370C and the recollected cells after centrifugation were fixed in methanol: acetic acid (3:1). DMSO and MMS were uses as negative and positive controls, respectively. Preparation of slides, staining and scanning was done under code. A total of 200 well - spread metaphases were analyzed per treatment per duration for all types of chromatid and chromosome type of aberrations. Aberrations were scored as per Hundal, *et al* (1997). Analysis of SCE was carried out following the fluorescent plus Giemsa technique of Perry and Wolfs (1974). The cells in the cultures were exposes to 5-bromo-2-de oxyuridine (BrdU 2 µg/ml; Sigma) after 24 hours of initiation of culture. The test compounds with same concentrations as in the case of CA analysis were added together with the BrdU. To minimize photolysis of BrdU another 48 hours cultures were maintained in the dark. After 90 min. of this pulse treatment the cells were spun down and the supernatant discarded. The cells were washed twice to remove any traces of the drug, phytoproducts and the liver metabolites. Finally the cell pellets were re-suspended in fresh medium supplemented with fetal calf serum, antibiotics and

BrdU, and kept for another 24 hours in the dark at 37oC. One day old slides were stained in Hoechst 33258 stain (Sigma 0.5 µg/ml), exposed to UV lamp (254 nm) for 30 min. and incubated in 2X SSC (0.3 M NaCl, 0.03M Sodium citrate; pH 7.0) at 60oC for 90 min and stain for sister chromatid. The slides were coded prior to scoring and 50 well- spread metaphase cells were scanned per concentration and the number of exchanges scored (Hundal, *et al*., 1997). Cells undergoing 1st (M1), 2nd (M2) and 3rd (M3) metaphase divisions were detected with BrdU – Harlequin technique for differential staining of metaphase chromosome by studying 200 metaphases for each combination and duration. The replication index (RI), an indirect measure of studying cell cycle progression, was calculated by applying the following formula (Tice, *et al*., 1976).

$$RI = \frac{M1x1+M2x2+M3\ x3}{100}$$

RESULT AND DISCUSSION

IN VITRO RESULT

In this experiments shows treatment with Aflatoxin B1, results in clastogenic abnormalities as observed in percent metaphase aberration, types of aberrations and aberration per cell were 39.75, 67.00, 69.50 percent or 0.40, 0.67 & 0.70 aberration per cell, whereas control the normal & DMSO plus Terminalia extract 04.00, 04.50 per cell at single standard dosage and three various durations viz, 24, 48 and 72 h. Terminalia extract bring down aberrations from 39.75 % to 32.50 ,28.75, 26.25 and 24.00 percent with four consecutive dosages of Terminalia extract at 24 h of duration, whereas at 48 h, it lower from 67.00% to 50.50, 43.35, 49.00,and 45.00 percent by I^{ts} to 4^{th} concentrations of Terminalia extract respectively. Same trend were noticed, when the treatment durations was increased to 72 h. These values show linear increasing trend with dosages, but it does not dependant on durations. The maximum percentage reductions in the aberrations were 39.62 for 24 h and 32.83 & 38.48 for 48 and 72 h respectively (Table 1).

When culture was setup along with metabolic activation system (+S_9 mix), the effect of Aflatoxin B1, increased. Similarly the effects of Terminalia extract also lower the clastogenic activity of Aflatoxin B1. These values show linearly increasing trend with doses (Table 2). The maximum effective percentage reductions were 45.31, 44.46, and 38.34 percent for 24, 48 and 72 h respectively. The highest reduction on clastogeny of cells was noticed at 24h durations; though the other values were also statistically significant.

In another marker the experiment were conducted and sister chromatid exchanges were counted (Table 3, 4), the reduction was evident both in the absence as well as in the presence of metabolic activation; there being a lowering of the mean range and the total SCEs and SCE per cell from 07.70 to 04.30 and from 7.20 to 04.20. For the analysis of SCE, only 48 h of cultures were used and 50 metaphases were scored.

The effects of Terminalia extract on replication index (Table 5,6) show on elevated level when compared from the Aflatoxin B1, treatment i.e. from 1.44 to 1.68. Though lower than the normal level of 1.71. The effect, after treatment with metabolic activation system shows from 1.43 to 1.66 i.e., much effective than without metabolic activation system. Therefore, we observed that Terminalia extract has potent anti-clastogenic activities in these experiments.

Antioxidants also play an important role in cancer prevention. Cancer cells are "immortal" i.e. they have lost their growth restraining mechanisms and so multiply out of control.

This results from alteration of cellular DNA or genetic material, which can be an inherited defect. It was found that free radical damage is the cause of these genetic mutations. When DNA or genetic material is involved in free radical reactions, mutations or genetic alteration can result. Free radical chain reactions are stopped by the action of antioxidants.An 85% ethanolic bark extract of Terminalia showed antitumor and radiation sensitising activity against a mouse transplantable tumor and is cytotoxic to human tumour cell lines.Terminalia also significantly reduced cell proliferation activity of colonic mucosal epithelium as the proliferating cell nuclear antigen index was lower than of the control.The protection afforded by Terminalia against colon carcinogenesis was postulated to be related to its antioxidants activity.(Morioka et al, 2005). 2006).

The hot water extract of Terminalia catappa showed potent short term chemopreventive action on biomarkers of colon carcinogenesis.Colon cancer was induced in 6 weeks old male F344 rats by weekly subcutaneous injections of azoxymethane for 2 weeks.The rats were fed a diet containing 0.02 and 0.1% Terminalia for 5 weeks beginning a week before the first injection of azoxymethane.Terminalia significantly reduced the number of aberrant crypt foci (ACF)/colon/rat and b-catenin accumulated crypts/cm2/rat when compared to the control.ACF is well accepted as visible preneoplastic lesions that develop in the colonic mucosa of rats treated with azoxymethane,thus it is a useful biomarker for colon carcinogenesis, (Morioka et al,2005).

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TABLES

TABLE 1. Analysis of Chromosomal aberrations after treatment with Methylmethanesulphonate along with *Alstonia scholaris* extract *in vitro* in the absence of -S_9 mix.

Treatments	Durations (h)	Metaphase scored	Percent aberration metaphase		Types of Aberration (%)			Aberration/ Cell ± SE
			Including gap	Excluding gap	Chromatid	Chromo Some	Total	
Aflatoxin B1	24	200	25.00	23.50	27.50	12.25	39.75	0.40 ± 0.04
	48	200	40.25	36.25	43.75	23.25	67.00	0.67 ± 0.06
	72	200	42.75	37.35	48.25	21.25	69.50	0.70 ± 0.06
AF B1+TC_1	24	200	20.70	15.00	21.00	11.50	32.50	0.33 ± 0.03
	48	200	30.25	27.50	33.25	17.25	50.50	0.51 ± 0.05
	72	200	33.50	29.70	36.50	19.50	56.00	0.56 ± 0.05
AF B1 +TC_2	24	200	16.50	14.25	18.50	10.25	28.75	0.29 ± 0.03
	48	200	26.75	24.25	28.00	15.35	43.35	0.43 ± 0.04
	72	200	30.00	27.50	33.25	18.25	51.50	0.52 ± 0.05
AF B1+TC_3	24	200	15.75	14.00	16.50	9.75	26.25	0.26 ± 0.03
	48	200	24.20	22.30	24.25	14.75	49.00	0.49 ± 0.04
	72	200	28.00	24.50	31.50	16.25	47.75	0.48 ± 0.04
AFB1 + TC_4	24	200	14.50	13.50	14.50	9.50	24.00	0.24 ± 0.03
	48	200	23.00	21.50	22.25	12.75	45.00	0.45 ± 0.04
	72	200	26.50	22.75	28.50	14.25	42.75	0.43 ± 0.04
Control								
Normal	72	200	3.50	1.50	2.50	1.50	4.00	0.04 ± 0.01
DMSO+TC_2	72	200	4.50	1.70	3.00	1.50	4.50	0.05 ± 0.01

TABLE 2. Analysis of Chromosomal aberrations after treatment with Aflatoxin B1 along with *Terminalia catappa* extract *in vitro* in the presence of +S_9 mix.

Treatments	Durations (h)	Metaphase scored	Percent aberration metaphase		Types of Aberration (%)			Aberration/ Cell ± SE
			Including gap	Excluding gap	Chromatid	Chromosome	Total	
Aflatoxin B1	24	200	22.00	19.00	23.50	8.50	32.00	0.32 ± 0.04
	48	200	37.50	32.25	40.00	20.50	60.50	0.61 ± 0.06
	72	200	39.25	33.00	45.25	21.25	66.50	0.67 ± 0.06
AF B1+TC_1	24	200	15.50	12.75	18.25	7.25	25.50	0.26 ± 0.03
	48	200	27.75	25.25	31.50	15.00	46.50	0.47 ± 0.04
	72	200	30.25	27.50	35.00	17.50	52.50	0.53 ± 0.05
AF B1 +TC_2	24	200	14.25	12.50	16.50	7.00	23.50	0.24 ± 0.03
	48	200	23.50	22.25	25.50	13.25	38.75	0.39 ± 0.04
	72	200	26.25	23.50	31.25	15.50	46.75	0.47 ± 0.04
AF B1+TC_3	24	200	14.50	13.25	14.25	6.50	20.75	0.21 ± 0.03
	48	200	22.50	21.35	21.50	12.75	34.25	0.34 ± 0.04
	72	200	25.75	21.50	30.25	14.75	45.00	0.45 ± 0.04
AF B1 +TC_4	24	200	13.75	12.35	11.50	6.00	17.50	0.18 ± 0.03
	48	200	21.50	20.00	22.35	11.25	33.60	0.34 ± 0.04
	72	200	24.50	19.75	27.50	13.50	41.00	0.41 ± 0.04
Control								
Normal	72	200	2.30	1.80	1.75	1.50	3.25	0.03 ± 0.01
DMSO+TC_2	72	200	3.50	1.50	2.00	1.50	3.50	0.04 ± 0.01

TABLE 3. Analysis of sister chromatid exchange after treatment with Aflatoxin B1, along with *Terminalia catappa* extract *in vitro*, in the absence of -S_9 mix.

Treatment	Duration (h)	METAPHASE SCC	Total	Range	SCE /Cell ± SE
AF B1	48	50	385	1 — 11	7.70 ± 1.50
AF B1 + TC_1	48	50	330	1 — 11	6.60 ± 1.50
AF B1 + TC_2	48	50	275	1 — 10	5.50 ± 1.50
AF B1 + TC_3	48	50	245	1 — 10	4.90 ± 1.50
AF B1 + TC_4	48	50	215	1 — 10	4.30 ± 1.50
Control					
Normal	48	50	91	0 — 4	1.82 ± 1.00
DMSO	48	50	94	0 — 5	1.88 ± 1.00
DMSO+TC_2	48	50	90	0 — 4	1.80 ± 1.00

TABLE 4. Analysis of Sister chromatid exchange after treatment with Aflatoxin B1 along with *Terminalia catappa* extract *in vitro*, in the presence of +S_9 mix.

Treatment	Duration (h)	METAPHASE SCC	Total	Range	SCE /Cell ± SE
AF B1	48	50	360	3 — 12	7.20 ±1.50
AF B1 + TC_1	48	50	310	1 — 11	6.20 ±1.50
AF B1 + TC_2	48	50	270	2 — 11	5.40± 1.50
AF B1 + TC_3	48	50	250	1 — 10	5.00 ± 1.50
AF B1 + TC_4	48	50	155	1 — 11	4.20 ± 1.50
Control					
Normal	48	50	95	0 — 5	1.90 ± 1.00
DMSO	48	50	94	0 — 5	1.88± 1.00
DMSO+TC_2	48	50	97	0 — 5	1.94 ± 1.00

TABLE 5. Analysis of cell cycle kinetics after treatment with Aflatoxin B1, along with *Terminalia catappa* extract *in vitro*, in the absence of -S_9mix.

Treatment	Cell scored	(%) cell in			Replication Index	2×3 chi square Test
		M_1	M_2	M_3		
AF B1	200	61	34	05	1.44	
AF B1 + TC_1	200	58	36	06	1.48	
AF B1 + TC_2	200	54	35	11	1.57	Significant
AF B1 + TC_3	200	50	38	12	1.62	Significant
AF B1 + TC_4	200	49	40	13	1.68	Significant
CONTROL						
Normal	200	44	41	15	1.71	
DMSO	200	41	45	14	1.73	
DMSO +TC_2	200	38	46	16	1.78	

TABLE 6. Analysis of cell cycle kinetics after treatment with Aflatoxin B1, along with Terminalia catappa extract***,*** *in vitro*, in the presence of +S_9mix.

Treatment	Cell scored	(%) cell in			Replication Index	2×3 chi square Test
		M_1	M_2	M_3		
AF B1	200	61	35	04	1.43	
AF B1 + TC_1	200	60	34	06	1.46	
AF B1 + TC_2	200	57	32	11	1.54	Significant
AF B1 + TC_3	200	53	34	13	1.60	Significant
AF B1 + TC_4	200	49	36	15	1.66	Significant
CONTROL						
Normal	200	47	37	16	1.69	
DMSO	200	46	38	16	1.70	
DMSO +TC_2	200	43	40	17	1.74	