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Is *Acanthus ilicifolius* a Potent Anticarcinogen against Hydrocortisone-Induced Genotoxicity? Study in Human Peripheral Lymphocytes Cultures

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ABSTRACT

Since it is difficult to avoid the use of steroids as drug, a potentially important area of research is the development of dietary strategies to prevent the genotoxic effects of Hydrocortisone. In this regard, the antigenotoxic actions of *Acanthus ilicifolius* extract were used against hydrocortisone induced genotoxicity. *In vitro* studies were carried on human lymphocyte culture, Chromosomal aberration (CA), Sister chromatid exchange(SCE) and Cell cycle kinetics (CCK), used as genetic markers and the parallel experiments were done applying with and without metabolic activation system. Four doses *viz.,* 30, 45, 65, 80 mg/ml of *Acanthus ilicifolius* extract were selected and found that *Acanthus* extract significantly reduced the frequencies of chromosomal aberration, sister chromatid exchanges and enhances Replication Index *in vitro* It was also noticed that the antigenotoxicity of *Accanthus* extract showed dose – response relationship. The data were collected at three durations *viz.,* 24, 48 and 72 hrs *in vitro*. The result suggested that *Acanthus* extract is highly effective to the cancer prevention and quench free oxygen radicals.

Key words: *Acanthus ilicifolius, antigenotoxicity, chromosomal aberration, sister chromatid exchange, replication index*

Acanthus ilicifolius, popularly known as *"Harkach Kanta"* belongs to the family Acanthaceae, has typical spinose margins on its evergreen leaves and stipular spine at stem nodes. The common name of the plant is Holy Leaved Acanthus. It is a gregarious, sparingly branched, evergreen shrub, 0.6-1.5 meters in height, common in the tidal swamps of creeks and rivers along the east and west coasts. It is a plant of marshy habitat distributed widely throughout the mangroves of India. The plant grows luxuriously by the side of the Ganges in Sunderbans, Hoogly, Howrah and 24 Parganas in West Bengal. The shrub is also planted as a sand-binder along the banks of tidal rivers and lakes. It is a folklore medicinal plant used mainly against anticancer, antitumor and asthma and snake-bites. A decoction of the plant with sugar candy and cumin is used in dyspepsia with acid eructation. In Goa, the leaves are employed as an emollient fomentation in rheumatism and neuralgia (Anand and Sridhar, 2002). The analgesic, anti-inflammatory (Kanchanapoom, *et. al.*, 2001) and leishmanicidal (Kapil, *et al*, 1994) properties of *A ilicifolius* have been documented, while Babu, *et. al.,* 2001 have reported the antioxidant and hepatoprotective properties of the plant. The propensity of cancer cells to show multiple genetic mutations underscores the concept that the carcinogenic process progresses by the accumulation of discrete genetic alterations. Evidence suggests that genomic instability may provide the driving force behind the genetic plasticity characteristic of cancer cells resulting in DNA damage, gene mutation, sister-chromatid exchanges (Stagos, *et. al.*, 2005) , chromosomal aberrations (CAs), and cellular transformation (Lindahl,1993; Morgan, *et. al.*, 1996).

Natural products and related drugs are used to treat 87% of all categorized human diseases including bacterial infection, cancer and immunological disorders (Newman and Cragg, 2007).About 25% of prescribed drugs in the world originate from plants and over 3000 species of plants have been reported to have anticancer properties (Graham, *et. al.*, 2000). About 80% of the populations in developing countries rely on traditional plant based medicines for their primary health care needs (FAO, United Nations, 2004). India has a rich and prestigious heritage of mangrove forest oriented medicines among the South Asian countries. However, the majority of these plants have not yet undergone chemical, pharmacological and toxicological studies to investigate their bioactive compounds (Singh, *et. al.,* 2009; Krause and Scherer, 2001)). The aim of the present study is to evaluate the anticancer activity in *in vitro* method on human lymphocyte culture.

MATERIALS AND METHODS

Hydrocortisone was dissolved in dimethylsulfoxide (DMSO 5mg/ml; E.Merck, Mumbai, India) at final concenteration of 50mg/ml; *Acanthus ilicifolius* (West coast, India) extract was prepared by dissolving their compounds in DDH_2O at four concentrations viz. 30, 45, 65, and 80mg/ml.

Chromosomal aberration (CA) analysis

Following the technique of Moorehead, *et. al.,* 1960, human lymphocytes cultures were done for metaphase chromosome analysis. 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 37°C with 5% CO_2 for 72 hours in dark.

Hydrocortisone at a final concentration of 50 mg/ml was added at 0 hour and kept for 24, 48 and 72 hours of duration, which served as positive control. Subsequently, *Acanthus* extract were added along with Hydrocortisone and the cultures were kept for 24, 48 and 72 hours. *Acanthus* extract and Hydrocortisone were prepared in DMSO. In the metabolic activation experiments cultures were treated with S_9 mix (0.8 ml.), the S9 mix was freshly prepared as per the standard procedures of Maron and Ames 1983. The S_9 fraction was complemented by the addition of 5 mM NADP and 10 mM glucose –6- phosphate just before use. Colchicines (0.20 mg/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 37^{0}C and the recollected cells after centrifugation were fixed in methanol: acetic acid (3:1). Preparation of slides, staining and scanning will be done under code. A total of 300 well - spread metaphases were analyzed per treatment per duration for all types of chromatid and chromosome type of aberrations. Aberrations were scored as per standard protocol.

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Sister chromatid exchange

Analysis of SCE was carried out following the fluorescent plus Giemsa technique Perry and Wolfs 1974. The cells in the cultures were exposes to 5-bromo-2 – de oxyuridine (BrdU 2 mg/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. *Acanthus* extract and DMSO were used as positive and negative controls respectively and incubated at 37°C. After 90min. 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 37°C.

One day old slides were stained in Hoechst 33258 stain (Sigma 0.5 mg/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 60°C 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.

Cell cycle kinetics

Cells undergoing 1st (M_1), 2nd (M_2) and 3rd (M_3) metaphase divisions were detected with BrdU – Harlequin technique for differential staining of metaphase chromosome, by studying 200 metaphases for each combination and

Table1. Analysis of C.A. after treatment of Hydrocortisone and *Acanthus* extract in presence of S9 - mix *in vitro*

Treatments	Duration (h)	Metaphase scanned	Percent aberrations Metaphase: Including gap	Percent aberrations Metaphase: Excluding gap	Types of Aberrations (%): Chromatid	Types of Aberrations (%): Chromosome	Types of Aberrations (%): Total	Aberrations / Cell ± SE
	24	200	22.50	19.25	23.50	8.75	32.25	0.32 ± 0.03
Hyd	48	200	39.25	37.50	42.50	20.25	62.75	0.63 ± 0.06
	72	200	42.50	41.75	49.00	27.25	76.25	0.76 ± 0.09
Hyd +A0	24	200	18.50	15.25	19.50	7.25	26.75	0.27 ± 0.04
	48	200	35.25	32.00	38.50	18.75	57.25	0.57 ± 0.05
	72	200	38.75	39.50	45.25	23.25	68.50	0.69 ± 0.07
Hyd +A1	24	200	17.50	14.50	18.25	6.50	24.75	0.25 ± 0.04
	48	200	32.75	29.75	35.50	16.25	51.75	0.52 ± 0.09
	72	200	34.25	37.25	42.25	20.25	62.50	0.63 ± 0.07
Hyd + A2	24	200	16.25	12.75	17.50	5.75	23.25	0.23 ± 0.03
	48	200	28.50	20.25	32.00	13.50	45.50	0.46 ± 0.04
	72	200	31.25	30.75	39.75	19.25	58.00	0.58 ± 0.06
	24	200	14.50	10.25	15.25	4.50	19.75	0.20 ± 0.03
Hyd + A3	48	200	24.25	21.50	28.50	11.75	40.25	0.40 ± 0.04
	72	200	27.50	26.75	33.75	17.50	51.25	0.51 ± 0.05
Control								
Normal	72	200	3.50	1.25	2.50	1.50	4.00	0.04 ± 0.01
DMSO+A2	72	200	4.50	1.25	3.25	1.25	4.50	0.05 ± 0.01

duration. The replication index (RI), an indirect measure of studying cell cycle progression, was calculated by applying the following formula (Tice, *et. al.*, 1976)

$$RI = M_{1x1}+M_{2x2}+M_{3x3}/100$$

Statistical analysis

Student's two-tailed "t" uses for calculating the statistical significance in CAs and SCEs, where as 2x3 chi-square test ($\div^2$) were use to analyze the cell cycle kinetics. The level of significance was tested from standard statistical tables of Fisher and Yates, 1963.

RESULTS AND DISCUSSION

This study showed that *Accanthus ilicifolius* extract is a potent anticarcinogens towards Hydrocortisone induced genotoxicity. Ribose derivatives of benzoxazoline extracted from *Acanthus illicifolius*.is reported to be main bio-active component acts against cancer (Kapil, *et. al.*, 1994. *Accanthus* has long been used as antioxidant for combating various degenerative actions of free oxygen radicals and reactive oxygen species. Comparing with the controls, hydrocortisone treatment shows aberrations ranging from 19.46% to 42.0% with increasing durations. When acanthus extracts are used, it reduces aberrations from 25.75 to 50.0% at lower concentration and 19.0 to 42.0% at the highest concentration (Table 1.).When augmented with S9 mix, the percent aberrations are higher, and however these get reduced when acanthus extract are used concurrently with hydrocortisone. The least values go with control and A2(second dose).The range and mean values of SCE get increased with Hydrocortisone in the absence as well in the presence of metabolic activation. The total numbers of cells counted are 50, without S9 and with S9 treatment (Table 2). For cell cycle kinetics studies, 200 cells scored, the proportion of cell appearing in M1, M2 and M3 phase show-lowering trend due to hydrocortisone administration (63 to 03) in comparisons to normal control. When *Accanthus* extract are used along with hydrocortisone treatment, the replication indices get corrected and reach from 1.28 to 1.45, being nearly equal to control level of 1.56.The highest (A3) concentrations again shows much effective dose. The metabolic activation seems not to differ from earlier results (Table 3).

The present study demonstrates protective action of *Acanthus ilicifolius* against Hydrocortisone induced genotoxicity in the human lymphocytes. The extracts of the plant have demonstrated significant pharmacological activities

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Table 2. Analysis of SCE after treatment with Hydrocortisone along with *Acanthus* extract *in vitro*, in the presence of S_9-mix

Treatments	Duration (h)	Metaphase Scored	Total SCE	Range	$\frac{SCE}{Cell} \pm SE$
Hydrocortisone	48	50	560	3 — 12	11.20 ± 1.50
Hydt + A_0	48	50	500	3 — 12	10.00 ± 1.50
Hydt + A_1	48	50	490	2 — 12	9.80 ± 1.50
Hydt + A_2	48	50	479	1 — 11	9.58 ± 1.50
Hydt + A_3	48	50	471	1 — 11	9.42 ± 1.50
CONTROL					
NORMAL	48	50	239	0 — 6	4.72 ± 1.00
DMSO	48	50	251	0 — 6	5.02 ± 1.00
DMSO + A2	48	50	238	0 — 6	4.76 ± 1.00

Table 3. Analysis of cell cycle kinetics after treatment with Hydrocortisone along with *Acanthus* extract *in vitro,* in the presence of S9-mix

Treatments	Cell Scored	Percent Cell in			Replication Index	2 x 3 Chi-Square Test
		M_1	M_2	M_3		
Hydrocortisone	200	63	28	03	1.28	Significant
Hydt + A_0	200	57	31	04	1.31	,,
Hydt + A_1	200	52	32	06	1.34	,,
Hydt + A_2	200	50	34	09	1.45	,,
Hydt + A_3	200	48	37	10	1.52	,,
CONTROL						
Normal	200	33	48	11	1.62	----
DMSO	200	29	49	13	1.56	---
DMSO + A_2	200	31	50	13	1.70	---

like antioxidant, anticarcinogenic, anti-osteoporotic and hepato-protective. *Acanthus ilicifolius* represents a potential DNA-damaging reducer anticancer agent. Alcoholic extract of *A. ilicifolius* (250, 500 mg/kg b wt) was found to be effective against tumour progression and carcinogen induced skin papilloma formation in mice. The extract was found to be cytotoxic towards lung fibroblast (L-929) cells in 72 h MTT assay and the concentration required for 50% cell death was 18 ìg/ml. Oral administration of the extract (500 mg/kg b wt) reduced the tumour volume and administration of the same concentration increased the life span by 75% in ascites tumour harbouring animals. The extract also significantly delayed the onset of dimethylbenzanthrazene/Croton oil induced skin papilloma in mice in a dose dependent manner (Babu *et al*, 2002).

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