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Oroxylum indicum has Immense Potential to Ameliorate Genotoxicity Induced by Xenobiotics in Human Lymphocyte Culture

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A B S T R A C T

Oroxylum indicum is commonly known as Sonapatha, belongs to family *Bignoniaceae* and is widely distributed throughout India. It is an important medicinal plant used in Ayurvedic preparations because it possesses wide range of biological and pharmacological activities. Parts like seed, fruit, root bark and stem bark are used by tribal people of India incuring diseases like fever, respiratory disorder, stomach disorder, pneumonia and cancer. This plantis the reservoir of alkaloids, flavonoids, tannins, terpenoids, carotenoids and anthocyanin. In this study, we have used alcoholic extracts of barks of stem and root as anti-genotoxic agents in four increasing concentrations of 100, 150, 200 and 300 µg/ml. The methodology adopted is human lymphocytes culture and the biomarkers are Chromosomal aberrations (CA, Sister Chromatid Exchange (SCE) and Replication indices (RI). The studies were performed in the absence as well as presence of Liver S_9 fractions. Culture was set up for 24-hour, 48 hour and 72 hours i.e., three durations. It was found that *Oroxylum* extracts reduces CA significantly in both conditions viz: in the absence as well as presence of metabolic activation system but the effects was more prominent in the presence of S_9-mix. Similarly, it also reduces SCE from 960 due to Aflatoxin B1 to 695 per 100 cells in the presence of S_9-mix. RI also enhanced significantly in both experiments i.e., from 1.23 to 1.43 and 1.27 to 1.70. The effects were seeming to be dose dependent.

***Key words*:** *Oroxylum* extracts, Antigenotoxicity, Chromosomal aberrations, Sister chromatid exchange, Replication index, Lymphocytes

India is considered to be a rich reservoir of medicinal plants that were used for treatments of various ailments due to preventive and curative properties. *Oroxylum indicum* is one such plant, used in several Ayurvedic preparations. *Oroxylum indicum* (Bignoniaceae), commonly known as Midnight horror, is a deciduous tree well known among ethnic communities of South Asia including India for its medicinal property. The tree was distributed throughout the greater part of India but now the existence of *Oroxylum indicum* in natural population is highly threatened and has been categorized as vulnerable [1]. Roots of *Oroxylum indicum* are used as one of the ingredients in Ayurvedic formulations. The plant is claimed to possess anti-inflammatory, diuretic, anti-arthritic, antifungal and antibacterial activity. Mao [2] reported that the bark is taken for curing gastric ulcer and a paste made of the bark powder is used for treating mouth cancer, scabies and other diseases. The traditional knowledge of Maram Naga village of Senapati district, Manipur, reveals that the decoction of *Oroxylum indicum* bark can be used as a potent anticancer medicine, especially against nasopharyngeal cancer [2]. It was also reported to possess anticancer properties [3]. The plant was reported to possess various pharmacological activities, which may be due to its antioxidant potential.

Several studies have progressively accumulated knowledge on biological potential of extracts as antioxidant [4], and is exhibiting cytotoxic activity against B-16, HCT-8, CEM and HL-60 (leukemia) tumor cell lines [5] and HeLa cells [6]. Furthermore, very little is known about the possible mechanisms of cytotoxicity induced. We carried out a detailed investigation on antimutagenic and anticarcinogenic aspects of bark extracts of *Oroxylum* in human lymphocyte culture *in vitro*.

MATERIALS AND METHODS

The bark of stem and root were shade dried at room temperature. Then the shade dried samples were powdered, 60g of coarse powder was defatted with petroleum ether and extracted exhaustively with 95% of methanol at temperature of 60°C. The extract was air dried by vacuum evaporator. Methanol extract of *Oroxylum indicum* was dissolved in DMSO to prepare different optimum concentrations for studies.

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In vitro lymphocytes culture Method

Most of the cytogenetic studies being carried out involve the examination of metaphase chromosomes. The evaluation of chromosomal damage at metaphase stage gives more precise and detailed picture of the clastogenic agent than those at anaphase or telophase stage. Human peripheral blood lymphocytes are extremely sensitive indicators of the *in vitro* assay system. The chromosomal changes (numerical and structural) were utilized for investigation of the genotoxic as well as antigenotoxic potentiality of test chemicals. The parameters studied included chromosomal aberrations (CA), sister chromatid exchanges (SCEs) and cell growth kinetics (RI) both in the presence as well as in the absence of exogenous metabolic activation system.

Preparation of S_9 liver / microsome fraction.

For preparing S_9 fraction, the standard procedures as were followed and adopted by Ahmad *et al*. [7]. The S_9 mix from S_9 fraction was prepared fresh every time for use in the culture. The S_9 fraction was complemented with 8 µM of NADP, 100 µM of Na_2HPO_4 buffer with 7.4 pH, 0.8 ml of S_9 mix was added every time along with the test chemicals in the cultures.

Chromosomal aberrations

Preparation of culture media

Tissue culture medium RPMI–1640 (flow Laboratories) with L-glutamine and Hepes buffer without $NaHCO_3$ was prepared in advance and stored at 4°C following the method of Ahmad *et al*. [7].

Collection of blood samples

Peripheral blood from the healthy donors was taken fresh every time through veinal puncture under aseptic conditions (disposable needle and disposable syringes, Unitech) and Heparin (500 IU/ ml; Micro Lab) was used as anticoagulant. The tightly capped glass vials were gently mixed and stored at 4^0C for half an hour to separate blood cells from plasma.

Setting of the cultures

Lymphocyte culture was carried out by adding 0.8 ml of plasma containing white blood cells (WBC) in 4.5 ml of culture medium supplemented with 0.1 ml phytohaemagglutinin–P (PHA –P, Micro lab) and 15% fetal calf serum (Gibco). The culture vials were then tightly capped to avoid loss CO_2 of and after gently mixing, culture tubes were incubated at 37°C in dark and colchicine was added 2 hours prior to harvesting for arresting the cells at metaphase stage. Harvesting of the cultures, Slide preparation and staining were following the methods of Ahmad *et al*. [7].

Analysis of the cells

In order to avoid the bias in scoring of the chromosomal anomalies before and after treatment of different test chemicals all slides were coded prior to scoring. A total of 150 well spread metaphase were analyze for each concentration of the test chemicals and for each time duration to analyzed various chromosome and chromatid type aberrations by using the method as described by Evans [8].

Sister chromatid exchange analysis

Sister chromatid exchange is a sensitive rapid and objective method of observing reciprocal exchange between sisters chromatid. This method depends upon the phenomenon of 5-bromo-2-deoxyuridine (BrdU) incorporation into DNA in place of thymidine. After two rounds of cell division, the chromatids were labeled with Brd U and consequently differentially stained with Hoechst stain. The BrdU incorporation quenches the fluorescence of 33258 Hoechst. Therefore, the light energy is absorbed but not emitted by such dyes, which results in the reduced staining of chromatid with Giemsa. Labeling of chromosomes with BrdU, slide preparation and staining processes were done following the methods of Ahmad *et al*. [7].

Analysis of the cells

All slides were coded prior to scoring so as to avoid any ambiguity. Around 100 metaphases (50 metaphases/ donor) with differentially stained chromatid were scored for each test chemical treatment in absence of S_9 mix and 100 metaphases were scored for each treatment in the presence of S_9 mix. The interstitial exchanges between two sister chromatid were scored as two exchange and the terminal exchanges were scored as a single exchange. Student's 't' test was applied for calculating the significance of difference between the treated and the controls.

Cell cycle kinetics analysis

The cells undergoing first (M_1) second (M_2) and third (M_3) divisions were detected by studying the BrdU labeled differentially stained chromosomes, following the method of Crossen and Morgan [9] The cells with both the chromatids being darkly stained were scored as M_1 cells, those with one dark and one lightly stained chromatid as M_2 cells and those having mixture of both the differentially stained and uniformly stained chromatids were scored as M_3 metaphase. Around 100 well spread metaphase were scored for each concentration and each treatment durations from each donor in the absence as well as in the presence of S_9 mix. The replication index (RI) was calculated according to the formula of Tice [10] as given below. The deviation from the controls was determined by using Chi-square (x^2) test.

$$\text{R. I} = \frac{(M_1X1)+(M_2X2)+(M_3X3)}{100}$$

Statistical analysis

Standard deviation (SD) and standard error (SE) were calculated, 2 x 3 Chi-Square test χ^2 for homogeneity test of variance was used to analyses the cell growth kinetics exchange with the normal control. The level of significance was tested from standard statistical tables of Fisher and Yates. Student two-tailed 't' test was used for calculating the statistical significance in SCE and chromosomal aberration by comparing the effect induced by different test chemicals with the respective control. The statistical significance was calculated from Fisher and Yates table at $(n_1 + n_2 – 2)$ degree of freedom (df) at 0.05% level of significance.

Table of chemical concentration

(A) Control

Positive and negative control	Concentrations
Aflatoxin B1	5 µg/ml
Dimethyl sulfoxide (DMSO)	5 µg/ml

(B) *In vitro cconcentrations of phyto-chemicals*

Phyto-products	1st Dose	2nd Dose	3rd Dose	4th Dose
Alcoholic extracts of *Oroxylum indicum in vitro* (µg/ml)	OIE_1 100	OIE_2 150	OIE_3 200	OIE_4 300

RESULTS AND DISCUSSION

In vitro effects

Treatment with Aflatoxin B1 results in clastogenic abnormalities as observed in percent metaphase aberration, types of aberrations and aberration per cell viz., 34.40, 64.80, 75.5 percent or 0.34, 0.65 & 0.76 aberration per cell, whereas the control the normal and the DMSO plus *Oroxylum* extract values are 03.00, 04.00 per cell at single standard dosage and for three various durations are 24, 48 and 72 h. *Oroxylum* extract bring down aberrations from 34.40% to 31.00, 28.30, 25.5 and 26.00 percent with four consecutive dosages of *Oroxylum* extract at 24 h of duration, whereas at 48 h, it is lowered from 64.80% to 63.75, 56.20, 54.27 and 57.25 percent respectively by I^{ts} to 4^{th} concentrations of *Oroxylum* extract. Similar trend was noticed, when the treatment durations was increased to 72 h. These values showing linear increasing trend with dosages, but it does not depend on doses and durations of treatments. The maximum percentage reductions in the aberrations were 28.87 for 24 h which were 16.28 and 13.77 for 48 and 72 h respectively (Table 1).

Table 1 Effects on chromosomal aberration after treatment with Aflatoxin B_1 along with *Oroxylum indicum* extract (OIE) *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	Chromosome	Total	
Aflatoxin	24	150	25.1	22	27.4	7	34.4	0.34 ± 0.03
	48	150	42	38.17	43.9	20.9	64.8	0.64± 0.05
B_1	72	150	48.3	43.1	49	26.5	75.5	0.75 ± 0.08
AFB_1+OIE1	24	150	20.21	17.31	23	8	31	0.31 ± 0.04
	48	150	43.32	35.61	40.15	23.6	63.75	0.63 ± 0.06
	72	150	43.18	38.21	48.78	22	70.78	0.70 ± 0.09
AFB_1+OIE_2	24	150	21.32	18.32	21	7.3	28.3	0.28 ± 0.03
	48	150	38.73	34	40.3	15.9	56.2	0.56 ± 0.06
	72	150	41	36.67	49	22	71	0.71 ± 0.08
AFB_1+OIE_3	24	150	20	16	19.5	6	25.5	0.25 ± 0.04
	48	150	37.65	32.38	38.8	15.47	54.27	0.54 ± 0.05
	72	150	39	34.77	45	20.1	65.1	0.65 ± 0.09
AFB_1+OIE_4	24	150	19.12	15.12	19	7	26	0.26 ± 0.03
	48	150	37	31.89	42.45	14.8	57.25	0.57 ± 0.06
	72	150	35.1	35.55	43.2	22.6	65.8	0.65 ± 0.06
				Control				
Normal	72	150	2.98	2.34	2.67	0.56	3.23	0.03 ± 0.01
DMSO+OIE_2	72	150	4.76	2.45	2.3	2	4.3	0.04 ± 0.01

Table 2 Effects on chromosomal aberration after treatment with Aflatoxin B_1 along with *Oroxylum indicum* extract (OIE) 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	Chromosome	Total	
Aflatoxin	24	150	28.63	22.45	27.22	07.83	35.05	0.35± 0.04
	48	150	43	45.67	51.89	23.00	74.89	0.74± 0.08
B_1	72	150	50.55	43.25	53.5	28.31	81.81	0.81 ± 0.09
AFB_1+OIE1	24	150	25	22	24	7.30	31.3	0.31 ± 0.04
	48	150	40	36.5	45.6	20.67	66.27	0.65 ± 0.06
	72	150	46.66	42	49.1	26.00	75.1	0.75 ± 0.09
AFB_1+OIE_2	24	150	24	20.88	21.77	7.00	28.77	0.28 ± 0.03
	48	150	36	33	42.34	17.35	59.69	0.59 ± 0.06
	72	150	43.99	39	46	24.45	70.45	0.70 ± 0.08
AFB_1+OIE_3	24	150	21.32	20.32	22.67	6	28.67	0.28 ± 0.04
	48	150	36.12	32.22	38	17.45	55.45	0.55 ± 0.05
	72	150	41.63	36.5	45.12	21.89	67.01	0.67 ± 0.09
AFB_1+OIE_4	24	150	21	19	21.7	4.17	25.87	0.25 ± 0.03
	48	150	34	29.79	36.5	16.8	53.3	0.53 ± 0.06
	72	150	37.65	32	45.15	25.52	70.67	0.70 ± 0.06
				Control				
Normal	72	150	3.34	4	2.46	1	3.46	0.03 ± 0.01
DMSO+OIE_2	72	150	2.55	1.4	2.45	2.8	5.25	0.05 ± 0.01

When culture was setup along with metabolic activation system (+S_9 mix), the effect of Aflatoxin B1 increased. Similarly, the effects of *Oroxylum* extract also lowered the clastogenic activity of Aflatoxin B1. These values show linearly increasing trend with doses (Table 2). The maximum effective percentage reductions were 26.19, 28.82, and 18.09 percent for 24, 48 and 72 h respectively. The highest reduction on clastogeny of cells was noticed at 48h durations; though the other values were also statistically significant.

The experiment were conducted for sister chromatid exchanges assay (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 8.72 to 7.56 and from 9.60 to 6.95. For conducting SCE assay, only 48 h of cultures were done and 100 metaphases were scored for counting the number of exchanges.

Table 3 Effects on sister chromatic exchange after treatment with Aflatoxin B_1 together *Oroxylum indicum* (OIE) in vitro in the absences of $-S_9$ mix

Treatment	Duration (h)	Metaphase scored	Total	Range	SCE /Cell ± SE
Aflatoxin B_1	48	100	872	3- 12	08.72 ± 1.50
AF B_1 + OIE_1	48	100	800	3- 12	08.00 ± 1.50
AF B_1 + OIE_2	48	100	838	2- 12	08.38 ± 1.50
AF B_1 + OIE_3	48	100	824	1-11	08.24 ± 1.50
AF B_1 + OIE_4	48	100	756	1-11	07.56 ± 1.50
		Control			
Normal	48	100	400	0-6	04.00 ± 1.00
DMSO	48	100	406	0-6	04.06 ± 1.00
DMSO + OIE_3	48	100	412	0-6	04.12 ± 1.00

Table 3 Effects on sister chromatic exchange after treatment with Aflatoxin B_1 together *Oroxylum indicum* extract (OIE) in vitro in the presence of $+S_{9mix}$

Treatment	Duration (h)	Metaphase scored	Total	Range	SCE /Cell ± SE
Aflatoxin B_1	48	100	960	3- 12	09.60 ± 1.50
AF B_1 + OIE_1	48	100	850	3- 12	08.50 ± 1.50
AF B_1 + OIE_2	48	100	825	2- 12	08.25 ± 1.50
AF B_1 + OIE_3	48	100	808	1-11	08.08 ± 1.50
AF B_1 + OIE_4	48	100	695	1-11	06.95 ± 1.50
		Control			
Normal	48	100	440	0-6	04.40 ± 1.00
DMSO	48	100	435	0-6	04.35 ± 1.00
DMSO + OIE_3	48	100	430	0-6	04.30 ± 1.00

The effects of *Oroxylum* extract on replication index (Table 5-6) show an elevated level when compared with the Aflatoxin B1 treatment i.e., rising from 1.23 to 1.45, though still lower than the normal level of 1.69. The effect, after treatment with metabolic activation system shows to elevated from 1.27 to 1.70 i.e., being much effective than one without metabolic activation system. Therefore, we observed that *Oroxylum* extract has potent anti-clastogenic activities in these experiments.

Oroxylum indicum is an affluent source of active anticancer components. Apoptosis is a physiologic intracellular process, crucial to maintain metabolic equilibrium required to attain homeostasis in the human body [11]. Thus, apoptosis induction in cancerous cells is a circumstantial approach towards cancer therapeutics. Cytotoxic anticancerous compounds damage cells, particularly DNA. Aqueous and methanolic extracts of stem bark of *O. indicum* was evaluated for its anticancer potential against Hep3B (human hepatic carcinoma), MDA-MB-435S (human breast carcinoma) and PC-3 (human prostate cancer) cells lines and it was concluded that the phytoconstituents of the extract were competent to bring apoptosis in cancerous cells [12].

Table 5 Effects on cell kinetic after treatment with Aflatoxin B_1 along with *Oroxylum indicum* extract (OIE) *in vitro* in the absence of $+S_9$ mix

Treatment	Cell scored	(%) cell in			Replication Index	2 × 3 chi square test
		M_1	M_2	M_3		
Aflatoxin B_1	150	72	21	3	1.23	Significant
AFT B_1 + OIE_1	150	75	23	4	1.33	Significant
AFT B_1 + OIE_2	150	72	26	3	1.33	Significant
AFT B_1 + OIE_3	150	72	24	5	1.35	Significant
AFT B_1 + OIE_4	150	65	27	8	1.43	Significant
			Control			
Normal	150	34	56	8	1.7	Significant
DMSO	150	35	54	11	1.76	Significant
DMSO + OIE_3	150	36	56	13	1.87	Significant

In the in vitro experiment, it was to shown the effects of extracts on anti-cancer activity. “Tumor Necrosis Factor” (TNF- α) was recognized as the serum mediator of innate immunity having the ability of inducing hemorrhagic necrosis in tumor and it has the capacity to directly kill tumor cells *in vitro* [13]. Immuno-stimulatory cytokines like TNF-a, IL-1β, MIP-1α act as effective messenger molecules in an immune response. A well-balanced secretion of these cytokines under normal conditions ensures proper functioning of the immune system. An agent which stimulates the secretion of immune-stimulatory cytokines resulting in dendritic cell activation and subsequently enables efficient functioning of T-cells is said to possess strong immune-stimulatory activity. In a study, it was found that a substantial increase was observed in secretion of cytokines and TNF-α from RAW 264.7 and THP-1 cells after 24 hours of treatment with extracts, indicating the immuno-stimulatory potential of *Oroxylum indicum* [14]. This could be attributed to the constituent, a polyphenolic compound chrysin which block TNF-a triggered ICAM-1 expression by inhibiting ERK, JNK and P38 in epithelial cells [15].

Major constituents of *O. indicum* are baicalein, baicalein are 6-glucuronides, baicalein-7-glucuronides, oroxylin-A, scutellarein, chrysin and irridoids [16]. The antiproliferative potential of *Oroxylum indicum* was investigated on human breast tumor cell lines and found to be active against MCF7 and MDA-MB-231 breast cancer cell lines [3].

Table 6 Effects of cell kinetic after treatment with Aflatoxin B_1 along with *Oroxylum indicum* extract (OIE)in vitro in the Absence -S_9 mix

Treatment	Cell scored	(%) cell in			Replication Index	2 × 3 chi square test
		M_1	M_2	M_3		
Aflatoxin B_1	150	64	24	5	1.27	Significant
AFT B_1 + OIE_1	150	60	34	6	1.46	Significant
AFT B_1 + OIE_2	150	72	26	3	1.33	Significant
AFT B_1 + OIE_3	150	72	24	5	1.35	Significant
AFT B_1 + OIE_4	150	57	32	5	1.36	Significant
			Control			
Normal	150	34	50	8	1.58	Significant
DMSO	150	36	55	9	1.73	Significant
DMSO + OIE_3	150	30	67	13	2.03	Significant

Oroxylum indicum was also examined on the sea urchin eggs and found to inhibit development of cell cycle by inhibiting the first cleavage (IC50= 13.5 µg/ml) [5]. The ethylacetate extract of *Oroxylum indicum* was also found to be the potent NF- B inhibitor with IC_{50} values 47.45 g/ml. Furthermore, ethylacetate extract of *Oroxylum indicum* was also found to inhibit PGE2 as well as in vitro lipid-peroxidation [6]. Antiproliferative and antimitotic activity of stem bark of *Oroxylum indicum* was studied and found that hydroalcoholic extract at concentrations of 4, 5 and 6 mg/ml showed significant inhibitory effect against the dividing cells of *Allium* roots and thus decreased root growth and mitotic index, as we have seen in this study. Also, antiproliferative studies revealed that hydroalcoholic extract of *Oroxylum indicum* at concentrations of 4, 5 and 6 mg/ml led to apparent decline in number of dividing cells and inhibition of cell viability of *Saccharomyces cerevisiae,* and thus exhibits interruption in cell proliferation, when compared to control [17]. Dhru *et al*. [18] studied the inhibitory effect of n-butanol fraction of root bark of *Oroxylum indicum* on breast cancer cells at different concentrations and found that it showed the highest activity against *Artemia salina nauplii* and significant cytotoxic activity i.e., 70.41% inhibition as evident through MTT assay on the MCF7 breast carcinoma cell line.

Zazali *et al*. [19] reported an antiproliferative activity of methanolic leaf extract of *Oroxylum indicum* against HeLa cell line (human cervical cancer cell lines). Leaf extract helped to arrest the cellcycle at G1/S phase via p53-mediated pathway which contributes to its antiproliferative activity. The methanolic extract of *Oroxylum indicum* leaves was found to be more active as compared to cisplatin,an anticancer drug which is used as a positive control, by preventing the proliferation of HeLa cells atlower dose viz. IC_{50} 3.87 µg/ml [19). Chakma *et al*. [20] described the cytotoxic activity of different fractions of *Oroxylum indicum*. Petroleum ether fraction of crude methanol extractof leaf was found to be most cytotoxic [20).

To ascertain whether the cytotoxicity against HeLa cells was mediated through apoptosis, several studies including morphological, biochemical, and sub-G0/G1 population studies were carried out on treated HeLa cells. In morphological study, most of the dead cells showed characteristic features of apoptosis such as cytoplasmic membrane blebbing, nuclear fragmentation and apoptotic bodies on treatment with extracts. Furthermore, based on flow cytometry analysis of HeLa cells after treatment with extracts, it was found that the sub-G0/G1 population, a biochemical marker of apoptosis [21) with hypo-diploid DNA increased considerably in all extracts. This accumulation directly relates to the decrease of the cell population in the other phases of the cell cycle, indicating cell death through interference on cell cycle programme. There are 1,100 publications reporting anticancer activities of polyphenols in the peer-reviewed journals [22).

CONCLUSION

Oroxylum indicum, has been well recognized as an important anticancerous plant due to presence of several chemotherapeutic molecules in it. It acts as an impending foundation of various cancer preventing and treating components, and holds a significant position among other anticancer medicinal plants. There is a great need to study the correlation of various metabolites with various types of cancer to discover the fundamental mechanisms underlying the compound activity. Considering its wide distribution and various medicinal properties, it needs to be further explored to accelerate the development of promising new molecules at a very minimal cost due to its wide availability. Looking into the potential of this plant for treatment of cancer, studies on identification of active principles and scientific valuation of the plants are required.

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