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Anticancer Effect of *Alstonia Scholaris* Against the Methyl-Methanesulphonate-Induced Genotoxicity in Human Peripheral Lymphocytes in Vitro

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Abstract: ***Alstonia scholaris is used in various Ayurvedic preparations like Saptaparnasatvadi vati, Saptachadadi vati, Saptachhadadi vati, Saptacchadadi taila, Saptacchadadi kvatha and Saptaparna ghanasara or uses of Alstonia scholaris mainly in Whooping cough, Malaria, Jaundice, Gastric complaint, Headache, Asthma, Stomachache, Wound, Fever and also much effective in all types of cancer. The antigenotoxic potential of Alstonia scholaris extract (AS) was demonstrated on the methyl methanesulphonate (MMS) 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 Alstonia scholaris 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 AS shows dose – response relationship. The results suggest that AS was a potent anticarcinogen may contribute to the cancer prevention.***

Keywords: ***Alstonia scholaris extract, chromosomal aberration, Sister Chromatid Exchange,cell cycle kinetics, methyl methanesulphonate carcinogen.***

I. INTRODUCTION

Alstonia scholaris (also known as Devils tree) belonging to the family Apocynaceae, has been used since time immemorial in the folklore and traditional systems of medicine in India, to treat several diseases (Chandra *et al.*, 2003). Common names of *Alstonia scholaris* are chatium, white cheese wood, milky pine, black board tree, devil's tree, and satni (Chopra *et al* 1956).

The plant is grown in the lowland and mountain rainforests of India, the Asia-Pacific, Southern China and Queensland. The plant grows throughout the humid regions of India especially in West Bengal and west-coast forests of south India. The plant is used in Ayurvedic, Unani and Sidhha types of alternative medicinal systems (Dey, 2011; Joshi SG, 2000).The methanolic extract of this plant was found to exhibit pronounced antiplasmodial activity. The plant is reported to have antimutagenic effect (Arulmozhi *et al.*, 2010).

The plant is reported to have anti-mutagenic effect (Lim *et al*, 1990). The bark extract of *Alstonia scholaris* has immune-stimulating effect. The aqueous extract at low dose induced the cellular immune response while at high dose inhibited the delayed type of hypersensitivity reaction (Iwo *et al*, 2000). Echitamine chloride, an indole alkaloid, extracted from the bark of *Alstonia scholaris* has promising anticancer effect against sarcoma (Saraswathi *et al*, 1999; Saraswathi *et al*, 1998). The plant *Alstonia scholaris* is reported to possess *in vitro* nitric oxide scavenging activity in preliminary studies (Jagetia *et al*, 2004). The nitric oxide scavenging property of a compound is evidence for free radical and ROS scavenging properties (Lorenz *et al*, 2003). Several studies have demonstrated that plants produce potent antioxidants and represent important sources of natural antioxidants (Es-Safi *et al*, 2005; Harish *et al*, 2006).

Ethnomedicinal practices suggest it to be of use in treating cancer, and preclinical studies performed with cultured neoplastic cells and tumor-bearing animals having validated these observations. Additionally, the phytochemicals like echitamine, alstonine, pleiocarpamine, O-methylmacralstonine, macralstonine, and lupeol are also reported to possess antineoplastic effects. In addition to the cytotoxic effects, *Alstonia scholaris* is also observed to possess radiomodulatory, chemomodulatory, and chemopreventive effects and free-radical scavenging, antioxidant, anti-inflammatory, antimutagenic, and immunomodulatory activities, all of which are properties efficacious in the treatment and prevention of cancer (Baliga MS, 2010).

Chemopreventive agents can be targeted by intervention at the initiation, promotion, or progression stage of multistage carcinogenesis (Wattenberg, 1990; Kelloff *et al*, 1994; Morse and Stoner, 1993; Stoner and Mukhtar, 1995). The intervention of cancer at the promotion stage, however, seems to be the most appropriate and practical.

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The major reason for that relates to the fact that tumor promotion is reversible event at least in early stages and requires repeated and prolonged exposure of a promoting agent (Di Giovanni, 1992).

II. 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 methyl methanesulphonate (MMS) and the cultures were kept for 24, 48 and 72 hours. Noni juice 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).

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

III. RESULT AND DISCUSSION

A. In Vitro Result

In this experiments shows treatment with MMS 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 Alstonia extract 04.00, 04.50 per cell at single standard dosage and three various durations viz, 24, 48 and 72 h. Alstonia extract bring down aberrations from 39.75 % to 32.50 ,28.75, 26.25 and 24.00 percent with four consecutive dosages of Alstonia 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 Alstonia 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 MMS increased. Similarly the effects of Alstonia extract also lower the clastogenic activity of MMS. 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.

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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 Alstonia extract on replication index (Table 5,6) show on elevated level when compared from the MMS 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 Alstonia 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 A. scholaris showed antitumor and radiation sensitising activity against a mouse transplantable tumor and is cytotoxic to human tumour cell lines (Baliga *et al*, 2004). The ethanolic extract of *Alstonia scholaris* was found to decrease the malondialdehyde level and prevented lipid peroxidation (Arulmozhi *et al*, 2007). Reports suggested presence of nitric oxide scavenging activity in *Alstonia scholaris* (Jagetia *et al*, 2004). In addition to flavonoids and phenolic compounds, some of the alkaloids, saponins and triterpenoids are reported to possess antioxidant activity (Sujay *et al*, 2006). The presence of flavonoids, alkaloids and triterpenoids in alcoholic extract of *Alstonia scholaris* has been reported (Khan *et al*, 2003) and the results of preliminary phytochemical investigation in the present study also further substantiates this. Hence, the observed *in vitro* antioxidant activity may be because of these phytoconstituents, which needs further investigation.

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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	
MMS	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
MMS+AS_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
MMS +AS_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
MMS + AS_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
MMS + AS_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+AS_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 Methylmethanesulphonate along with *Alstonia scholaris* 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	
MMS	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
MMS+AS_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
MMS +AS_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
MMS + AS_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
MMS + AS_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+AS_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 Methylmethanesulphonate along with *Alstonia scholaris* extract *in vitro*, in the absence of $-S_9$ mix.

Treatment	Duration (h)	METAPHASE SC	Total	Range	SCE /Cell ± SE
MMS	48	50	385	1 — 11	7.70 ± 1.50
MMS + AS_1	48	50	330	1 — 11	6.60 ± 1.50
MMS + AS_2	48	50	275	1 — 10	5.50 ± 1.50
MMS + AS_3	48	50	245	1 — 10	4.90 ± 1.50
MMS + AS_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+AS_2	48	50	90	0 — 4	1.80 ± 1.00

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Table 4. Analysis of Sister chromatid exchange after treatment with Methylmethanesulphonate along with *Alstonia scholaris* extract *in vitro*, in the presence of $+S_9$ mix.

Treatment	Duration (h)	METAPHASE SCC	Total	Range	SCE /Cell ± SE
MMS	48	50	360	3 — 12	7.20 ±1.50
MMS + AS_1	48	50	310	1 — 11	6.20 ±1.50
MMS + AS_2	48	50	270	2 — 11	5.40± 1.50
MMS + AS_3	48	50	250	1 — 10	5.00 ± 1.50
MMS + AS_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+AS_2	48	50	97	0 — 5	1.94 ± 1.00

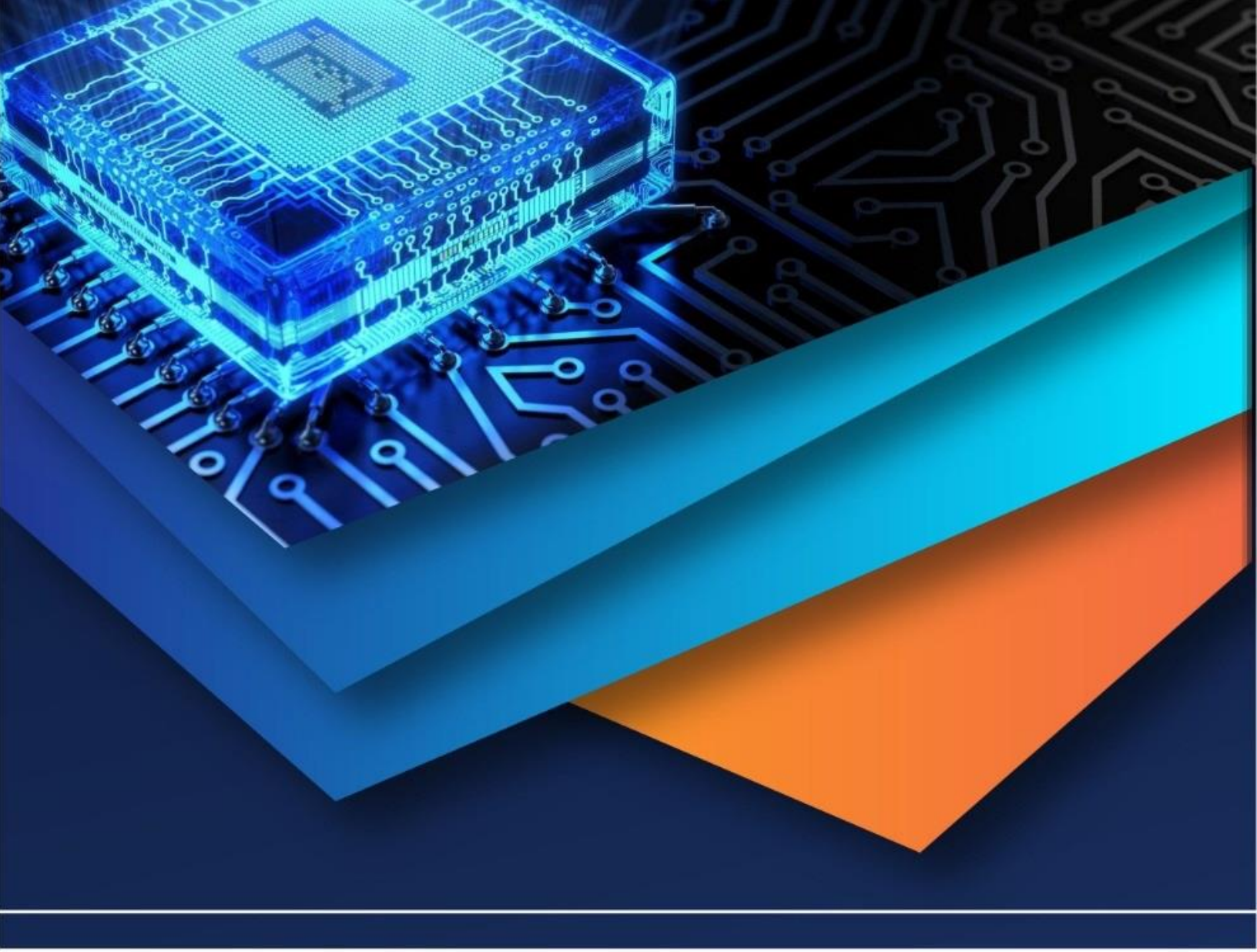
Table 5. Analysis of cell cycle kinetics after treatment with Methylmethanesulphonate along with *Alstonia scholaris* extract *in vitro*, in the absence of $-S_9$mix.

Treatment	Cell scored	(%) cell in			Replication Index	2×3 chi square Test
		M_1	M_2	M_3		
MMS	200	61	34	05	1.44	
MMS + AS_1	200	58	36	06	1.48	
MMS + AS_2	200	54	35	11	1.57	Significant
MMS + AS_3	200	50	38	12	1.62	Significant
MMS + AS_4	200	49	40	13	1.68	Significant
CONTROL						
Normal	200	44	41	15	1.71	
DMSO	200	41	45	14	1.73	
DMSO +AS_2	200	38	46	16	1.78	

Table 6. Analysis of cell cycle kinetics after treatment with Methylmethanesulphonate along with *Alstonia scholaris* extract, *in vitro*, in the presence of $+S_9$mix.

Treatment	Cell scored	(%) cell in			Replication Index	2×3 chi square Test
		M_1	M_2	M_3		
MMS	200	61	35	04	1.43	
MMS + AS_1	200	60	34	06	1.46	
MMS + AS_2	200	57	32	11	1.54	Significant
MMS + AS_3	200	53	34	13	1.60	Significant
MMS + AS_4	200	49	36	15	1.66	Significant
CONTROL						
Normal	200	47	37	16	1.69	
DMSO	200	46	38	16	1.70	
DMSO +AS_2	200	43	40	17	1.74	

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