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ALSTONIA SCHOLARIS EFFECTS ON PERIPHERAL HUMAN LYMPHOCYTE CULTURE AGAINST MMS INDUCED GENOTOXICITY

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ABSTRACT Use of medicinal plants in modern medicine for the prevention and treatment of cancer is an important aspect. For this reason, it is important to identify antitumor promoting agents present in medicinal plants commonly used by the human population, which can inhibit the progression of tumor. We used *in vitro* methods using chromosomal aberrations, sister chromatid exchange and replication index as markers, exposed by MMS as well as Alcoholic Extract of *Alstonia scholaris* in four increasing concentrations 150, 200, 250 and 300 µg/ml of culture) and of three different durations of 24, 48 and 72hrs in the presence as well absence of S_9-mix. Alcoholic extracts of *Alstonia scholaris* reduced *in vitro* experiments i.e., it reduces chromosomal aberrations 39.62, 32.83, and 38.48% ±S.E at 24, 48, and 72hrs of exposure respectively; but when experiments were carried out in the presence of liver S_9 fraction, these values were 45.31, 44.46, and 38.34% ± S.E respectively at <0.05 level, likewise it also reduces sister chromatid exchange from 7.70 to 4.20 ± S.E per cell and enhances replication index from 1.45 to 1.64 I. Alcoholic extracts of *Alstonia scholaris* significantly chromosomal aberrations and sister chromatid exchange and enhances replication index *in vitro* both of which were statistically significant at <0.05 level.

KEYWORDS : Antitumor, Ayurvedic medicine, *Alstonia scholaris*, Chromosomal aberration, Anticarcinogenic,

INTRODUCTION:

The use of medicinal plants in modern medicine for the prevention or treatment of cancer is an important aspect. For this reason, it is important to identify antitumor promoting agents present in medicinal plants commonly used by the human population, which can inhibit the initiation, progression and promotion of tumor. Recent development of medical treatment of human disease will be intimately connected to the natural products and greater emphasis has been given towards the researches on complementary and alternative medicine that deals with cancer management [1]. Many herbs have been evaluated in clinical studies and are currently being investigated phytochemically to understand their antitumour actions against various cancers. Certain biological response modifiers derived from herbs are known to inhibit growth of cancer by modulating the activity of specific hormones and enzymes. The traditional Indian system of medicines, Ayurveda, uses about 2,000 plant species, while the Chinese Pharmacopoeia lists over 5,700 traditional medicines, most of which are of plant origin [2].

The plant *Alstonia scholaris* is reported to have antimutagenic effect [3]. It is used in various Ayurvedic preparations like *Saptaparnasatvadi vati*, *Saptachadadi vati*, *Saptacchadadi kvatha* and *Saptaparna ghanasara* for uses of *Alstonia scholaris* mainly in whooping cough, malaria, jaundice, gastric complaint, headache, asthma, stomachache and fever. Ethanolic extract of *Alstonia scholaris* using various concentrations under *in vitro* tests were found to have significant (p 0.01) free radical scavenging and metal ion chelating properties [5].

The bark extract of *Alstonia scholaris* has immune-stimulating effects. The aqueous extracts at low dose induced the cellular immune response while at high dose inhibited the delayed type of hypersensitivity reaction [6]. Echitamine chloride, an indole alkaloid, extracted from the bark of *Alstonia scholaris* has promising anticancer effect against sarcoma [7]. The plant *Alstonia scholaris* is reported to possess *in vitro* nitric oxide scavenging activity in preliminary studies [8].

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. In addition to the cytotoxic effects, *Alstonia scholaris* has also been 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 [9].

MATERIALS AND METHODS

The whole plant of *Alstonia scholaris* 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 *Alstonia scholaris* was dissolved in DMSO to prepare different optimum concentrations for studies.

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 recommended by Moron and Ames [14] were followed. Swiss albino healthy rats (Wister strain, obtained from Animal house, Biotech, Varanasi, India), each weighing about 200g were given 0.1% (1mg/ml) of Phenobarbital in drinking water for one week for the induction of liver enzymatic activities. The removed livers were immediately placed in 0.15 M KCl chilled solution in culture tube. The whole procedure was carried out at 0-4°C using sterilized solutions and glassware. The livers were washed in chilled KCl several times so as to remove the traces of hemoglobin, which inhibits enzyme activity. The washed livers were transferred to a beaker containing three volumes of 0.15 M KCl (3 ml/g wet liver) and after mincing with sterile scissors, these were homogenized by a tissue homogenizer at 4°C. The homogenate was centrifuged in a refrigeration centrifuge for 10 min at 9000 rpm. The supernatant (S_9 fraction) was decanted and saved as 1ml aliquots in polypropylene storage vials and stored in liquid nitrogen till further use.

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, but the storage period never lasted longer than a week. About 1.574g of medium was dissolved in 100 ml of double distilled water by gentle shaking. Antibiotics, penicillin (100 IU/ml) and streptomycin (100 IU/ml) (Hoechst) were also added and pH was adjusted from 6.8-7.2 with N/10 $NaHCO_3$ and HCl. The medium was filtered and sterilized using Millipore filtration assembly by 0.45 µm

Millipore filters. The filtered medium was then stored in sterilized and tightly capped glass bottles.

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 37^0C in dark and colchicine was added 2 hrs prior to harvesting for arresting the cells at metaphase stage.

Harvesting of the cultures

After appropriate durations, the cultures were taken out from the incubator and their contents, after gentle shaking, were transferred to a centrifuge tube, the cells were spun down by centrifugation for 10 min, at 1200 rpm. Pellets were saved by discarding the supernatant. Hypotonic treatment (0.075 M KCl) was given for 10-12 minutes at 37^0C and the cells were recollected by centrifugation. The cell pellet was suspended in 5 ml freshly prepared chilled fixative (3:1; methanol: acetic acid), which was added drop by drop with a Pasteur pipette with continuous shaking to avoid formation of clots. In order to ensure the proper fixation, the cells were kept suspended in the fixative for a minimum period of one hour but preferably overnight. Two or three changes with fresh fixative were given before preparing the slides.

Slide preparation and staining

After giving final washing in the fixative the cells were re-suspended in 0.2 ml of fresh fixative. Two or three drops of cell preparation were dropped on clean, grease free, pre-chilled and wet microscopic slides and air-dried. One-day-old slides were stained in Giemsa (Sigma) for 15 minutes and rinsed in 95% alcohol and finally in absolute alcohol for proper differentiation, after air-drying these slides were dipped in xylene for 5 minutes before mounting in DPX.

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 300 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 [11].

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 [10].

Labeling of chromosomes with BrdU

The cells in the culture were exposed to nucleoside, BrdU (Sigma) after 24 hrs of culture initiation at the final concentration of 2 µg /ml. The culture vials were tightly capped and covered with aluminum foil to avoid light exposure and incubated at 37^0C for another 48 hrs in dark.

Slide preparation and staining

After 2hrs of colchicines treatment, the cultures were harvested and processed following the same procedure as desired for the chromosomal aberration analysis. For the differential staining of SCEs the methods of Latt *et al* [17] with slight modifications were followed. One-day-old slides were dipped in 0.5 µg/ml of 33258 Hoechst stain (Sigma) dissolved in double distilled water in horizontal coplin jar. The slides were then put in flat glass dish with the layer of cells facing upwards. These were covered by thick layer (2-3 cm) of phosphate buffer (pH 6.8) and exposed to UV lamp (15W, 254 µm, Philips) from a distance of 10-15 cm for 30-45 minutes. The slides were taken out from the buffer, washed twice in double distilled water and air-dried. These were then incubated in 2X SSC (0.3 M NaCl, 0.03M Sodium citrate, pH7.0) at 65^0C in water both for 90 minutes using vertical couplin jars. The slides were taken out and rinsed in distilled water. The air-dried slides were then stained in Giemsa for 20 minutes and rinsed in 90% alcohol followed by rinsing in absolute alcohol. The dried slides were dipped in xylene for 5 minutes and mounted in DPX.

Analysis of the cells

All slides were coded prior to scoring so as to avoid any ambiguity. Around 50 metaphases (25 metaphases/ donor) with differentially stained chromatid were scored for each test chemical treatment in absence of S_9 mix and 50 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 [12]. 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 [13] as given below. The deviation from the controls was determined by using Chi-square (x^2) test.

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

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 [14]. 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
MMS	5 µg/ml
Dimethyl Sulfoxide (DMSO)	5 µg/ml

(B) In vivo concentrations of phyto-chemicals

Phyto-products	1st Dose	2nd Dose	3rd Dose	4th Dose
Alcoholic extracts of *Alstonia scholaris in vitro*(µg/ml)	ALE_1 150	ALE_2 200	ALE_3 250	ALE_4 300

RESULTS

***In vitro* effects**

Treatment with MMS results in clastogenic abnormalities as observed in percent metaphase aberration, types of aberrations and aberration per cell viz., 39.75, 67.00, 69.50 percent and 0.40, 0.67 & 0.70 aberration per cell, whereas the control the normal and the DMSO plus Alstonia extract values are 04.00, 04.50 per cell at single standard dosage and for three various durations are 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 is lowered from 67.00% to 50.50, 43.35, 49.00 and 45.00 percent respectively by 1st to 4th concentrations of Alstonia extract. Similar trend was noticed, when the treatment durations was increased to 72 h. These values show linear increasing trend with dosages, but it does not depend on dose- durations. The maximum percentage reductions in the aberrations were 39.62 for 24 h which were 32.83 and 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 lowered 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.

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 07.70 to 04.30 and from 7.20 to 04.20. For conducting SCE assay, only 48 h of cultures were done and 50 metaphases were scored for counting the number of exchanges.

The effects of Alstonia extract on replication index [Table 5, 6] show an elevated level when compared with the MMS treatment i.e., rising from 1.45 to 1.58, though still lower than the normal level of 1.69. The effect, after treatment with metabolic activation system shows to elevated from 1.45 to 1.64 i.e., being much effective than one without metabolic activation system. Therefore, we observed that Alstonia extract has potent anti-clastogenic activities in these experiments.

DISCUSSION

Free radical chain reactions are stopped by the action of antioxidants. In our experiment the protective effects of Alcoholic extracts of *Alstonia scholaris* may be due to this reason i.e quenching the free radicals that were generated due to mutagen and carcinogen. The anticancer effect of various doses of an alkaloid fraction of *Alstonia scholaris* (ASERS), was studied in vitro in cultured human neoplastic cell lines (HeLa, HepG2, HL60, KB and MCF-7) and in Ehrlich ascite carcinoma bearing mice [15]. Treatment of HeLa cells with 25 g/Ml ASERS resulted in a time dependent increase in the antineoplastic activity and the greatest activity was observed when the cells were exposed to ASERS for 24 h [15].

The possible chemopreventive and anti-oxidative properties of this medicinal plant on two-stage process of skin carcinogenesis induced by a single application of 7,12-dimethyabenz (a) anthrecene (100lg/100 ll acetone), whereas two weeks later, these are promoted by repeated application of croton oil (1% increase in incidence, tumor yield, tumor burden and cumulative number of papillomoas). These changes are found to be higher in the carcinogen treated control (without ASE treatment) as compared to experimental animals (ASE treated). Furthermore, a significant increase in reduced glutathione, superoxide dismutase and catalase level but decrease in lipid peroxidation was measured in ASE administered experimental groups than the carcinogen treated controls [16].

Tumour multiplicity incidence was significantly reduced (91.93% with extract versus 100% in benzo(a)pyrene-treated mice) by 4 mg/mL dose that was added to the drinking water during the post-initiation period, starting at 48 h after the last dose of benzo(a)pyrene (post-treatment) these continued for 8 weeks. These findings were corroborated by the observation that micronuclei frequency reached the lowest point at 4 mg/mL of the extract. The extract was able to inhibit benzo(a)pyrene-induced mutagenic changes as the frequency of splenocytes bearing one micronuclei and also cells which bear multiple micronuclei were reduced by the administration of extract [17]. In our *in vitro* experiments, we observe the enhancement of replication indices that support the above finding.

The anticancer properties of this medicinal plant was evaluated and the tumor incidence, tumor yield, tumor burden and cumulative number of papillomas were found to be higher in the carcinogen treated control compared to animals treated with *Alstonia scholaris* extract. Furthermore, a significant increase in reduced glutathione, superoxide dismutase and catalase level but decrease in lipid peroxidation was observed in ASE administered experimental groups than the carcinogen with control treated. This study demonstrated the chemopreventive potential of *Alstonia scholaris* bark extract in DMBA- induced skin tumor genesis in Albino mice [18]. The aqueous extract at 50mg/kg b.w. induced the cellular immune response while at 100mg/kg b.w. inhibited the delayed type of hypersensitivity reaction [19].

An 85% of ethanolic bark extract of *Alstonia scholaris* showed antitumor and radiation sensitising activity against a mouse transplantable tumor and is cytotoxic to human tumour cell lines [20]. The ethanolic extract of *Alstonia scholaris* was also found to decrease the malondialdehyde level that prevented lipid peroxidation [21]. Other reports also suggested presence of nitric oxide scavenging activity in case of *Alstonia scholaris* [9].

Besides flavonoids and phenolic compounds, some of the alkaloids, saponins and triterpenoids are also reported to possess antioxidant activity [22]. The results of the present phytochemical investigation further add to these conclusions.

CONCLUSION

Alcoholic extracts of *Alstonia scholaris* reduces chromosomal aberrations 39.62, 32.83, and 38.48% ±S.E at 24, 48, and 72hrs of exposure respectively; but when experiments were carried out in the presence of liver S_9 fraction, these values were 45.31, 44.46, and 38.34% ± S.E respectively at <0.05 level, likewise it also reduces sister chromatid exchange from 7.70 to 4.20 ± S.E per cell and enhances replication index from 1.45 to 1.64.

Alcoholic extracts of *Alstonia scholaris* significantly reduces the number of aberrant cells and frequency of aberration per cell at each concentration and duration of exposure *in vivo;* similarly it reduces chromosomal aberrations and sister chromatid exchange and enhances replication index *in vitro* both of which were statistically significant at <0.05 level.

Conflict of interest

The authors declare that there is no conflict of interest.

TABLE: 1 Analysis of Chromosomal aberrations after treatment with Methyl methane sulphonate along with alcoholic extracts of *Alstonia scholaris 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	
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+AL_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 +AL_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 +AL_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 +AL_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+AL_2	72	200	4.50	1.70	3.00	1.50	4.50	0.05 ± 0.01

Note: SE; Standard error, AL or ALE; concentrations of alcoholic extracts of *Alstonia scholaris,* MMS; Methyl methane sulphonate 5 µg/ml culture. Calculations were made excluding the Gaps type of an aberration and at <0.05 probability.

TABLE: 2 Analysis of Chromosomal aberrations after treatment with Methyl methane sulphonate along with alcoholic extracts of *Alstonia scholaris 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+AL_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+AL_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+AL_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+AL_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

Note: SE; Standard error, AL or ALE; concentrations of alcoholic extracts of *Alstonia scholaris,* MMS; Methyl methane sulphonate 5 µg/ml culture. Calculations were made excluding the Gaps type of an aberration and at <0.05 probabilit

TABLE: 3 Analysis of sister chromatid exchange after treatment with Methyl methane sulphonate along with alcoholic extracts of *Alstonia scholaris in vitro*, in the absence of -S_9 mix.

Treatment	Duration (h)	Metaphase Scored	Total	Range	SCE /Cell ± SE
MMS	48	50	385	1 — 11	7.70 ± 1.50
MMS + AL_1	48	50	330	1 — 11	6.60 ± 1.50
MMS + AL_2	48	50	275	1 — 10	5.50 ± 1.50
MMS + AL_3	48	50	245	1 — 10	4.90 ± 1.50
MMS + AL_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 +AL_2	48	50	90	0 — 4	1.80 ± 1.00

Note: SCE; Sister chromatid exchange, SE; Standard error, AL or ALE; concentrations of alcoholic extracts of *Alstonia scholaris,* MMS; Methyl methane sulphonate 5 µg/ml culture. Calculations were made at <0.05 probability.

TABLE: 4 Analysis of Sister chromatid exchange after treatment with Methyl methane sulphonate along with alcoholic extracts of *Alstonia scholaris* in vitro, in the presence of +S_9 mix.

Treatment	Duration (h)	Metaphase scored	Total	Range	SCE /Cell ± SE
MMS	48	50	360	3 — 12	7.20 ±1.50
MMS + AL_1	48	50	310	1 — 11	6.20 ±1.50
MMS + AL_2	48	50	270	2 — 11	5.40± 1.50
MMS + AL_3	48	50	250	1 — 10	5.00 ± 1.50
MMS + AL_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+AL_2	48	50	97	0 — 5	1.94 ± 1.00

Note: SCE; Sister chromatid exchange, SE; Standard error, AL or ALE; concentrations of alcoholic extracts of *Alstonia scholaris,* MMS; Methyl methane sulphonate 5 µg/ml culture. Calculations were made at <0.05 probability.

TABLE: 5 Analysis of cell cycle kinetics after treatment with Methyl methane sulphonate along with alcoholic extracts of *Alstonia scholaris* 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	60	35	05	1.45	Significant
MMS + AL_1	200	58	36	06	1.48	„ „
MMS + AL_2	200	58	37	05	1.47	„ „
MMS + AL_3	200	55	33	12	1.57	„ „
MMS + AL_4	200	53	36	11	1.58	„ „
CONTROL						
Normal	200	55	30	15	1.60	„ „
DMSO	200	51	35	14	1.63	„ „
DMSO + AL_2	200	52	31	17	1.65	„ „

Note: 2×3 chi square test were conducted, AL; concentrations of alcoholic extracts of *Alstonia scholaris,* MMS; Methyl methane sulphonate 5 µg/ml culture. Calculations were made at <0.05 probability.

TABLE: 6 Analysis of cell cycle kinetics after treatment with Methyl methane sulphonate along with alcoholic extracts of *Alstonia scholaris* 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		
MMS	200	59	37	04	1.45	Significant
MMS + AL_1	200	58	34	08	1.50	„
MMS + AL_2	200	54	35	11	1.57	„
MMS + AL_3	200	52	35	13	1.61	„
MMS + AL_4	200	50	36	14	1.64	„
CONTROL						
Normal	200	47	37	16	1.69	„
DMSO	200	46	38	16	1.70	„
DMSO +AL_2	200	45	38	17	1.72	„

Note: 2×3 chi square test were conducted, AL; concentrations of alcoholic extracts of *Alstonia scholaris,* MMS; Methyl methane sulphonate 5 µg/ml culture. Calculations were made at <0.05 probability.

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