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Genotoxic property of poly herbal formulation of annona squomosa, zingiber officinalis and triticum aestivum plant extracts by *in vitro* chromosomal aberration test

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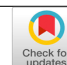
ABSTRACT



The present study was aimed to evaluate the genotoxic potential of polyherbal formulations by chromosomal aberration test. Cancer being the second most cause of death among all ailments even after the improvised medications needs alternative sources of treatment. Herbal sources were always among the one as prime treatment sources. Polyherbal formulations were taking the lead in medications because of their broad spectrum of activity and synergic effects. *Annona squamosa, Zingiber Officinalis, and Triticum Aestivum* formulation with 1:2:3 ratio has shown significant results in the definitive and confirmatory chromosome aberration assays. Indicated the test article PF3 did not induce a statistically significant increase in the percentage of cells with aberrations both in the presence and absence of metabolic activation. PF3 was found non-clostagenic at a maximum dose of 15 μg/ml in the CHO cell line. Inhibition of chromosomal aberration, DNA fragmentation, and maintenance of cellular integrity may prevent the genotoxic effect. Further optimization and *in vivo* authenticated studies need to be proven.

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INTRODUCTION

The Vedic knowledge of ayurvedic medicines was considered to be one of the oldest medical science and retained its significance even today. Herbal medicines are selective because of their non-resistive, broad-spectrum, non-accumulative, integral, and compatible with less side effects. Polyherbal formulations expose their broad-spectrum capacity and synergic effects in treatments. This synergic concept leads to the development of new polyherbal formulations. *Annona squamosa, Zingiber Officinalis, and Triticum Aestivum* were proven their efficacy as antimitoc, reduced chromosomal aberrations, and other genotoxic effects individually (Suresh *et al.*, 1970; Salah *et al.*, 2012; Jităreanu *et al.*, 2013). Chromosomal aberrations are due to genotoxic effects in relation to the initiation and development of cancer cells (Chen *et al.*, 2009; Scheutwinkel-Reich and Hude, 1984). The present study deals with the initial phase in determining the *in vitro* chromosomal aberration potential of polyherbal formulation.

MATERIALS AND METHODS

Based on the results of our previous studies, only Polyherbal Formulation-3 (PF3) was selected for *in vitro* Chromosomal Aberration Test.

Table 1: Relative cell growth in polyherbal PF3 in CHO cells (Range finding test)

Treatment Duration (Hrs) : 3 Hrs
Harvesting Time (Hrs) : 18 Hrs

Sl. No.	Test Samples	Trial Number	Test Concentration (μg/mL)	Number of cells (x 106) Per 60mm dish	Mean Number of cells (x 106) Per 60mm dish	Relative cell Growth (RCG) (%)	Cytotoxicity (%)
A	Without S9 activation						
	DMSO (0.1% in Media)	1	0	1.93	1.81	100.00	0.00
		2	0	1.68			
	Poly herbal PF3	1	05	1.76	1.70	93.91	6.09
		2	05	1.63			
		1	10	1.31	1.42	78.67	21.33
		2	10	1.53			
		1	20	0.50	0.43	23.82	76.18
		2	20	0.36			

- Relative Cell Growth = (No. Cells in Test Flask / No. Cells in Solvent Flask) X 100
- All values are expressed as Mean
- DMSO= Dimethyl sulfoxide

Table 2: Relative cell growth in polyherbal PF3 in CHO cells (Range finding test)

Treatment Duration (Hrs) : 3 Hrs
Harvesting Time (Hrs) : 18 Hrs

Sl. No.	Test Samples	Trial Number	Test Concentration (μg/mL)	Number of cells (x 106) Per 60mm dish	Mean Number of cells (x 106) Per 60mm dish		Cytotoxicity (%)
B	With S9 activation						
	DMSO (0.1% in Media)	1	0	1.48	1.50	100.00	0.00
		2	0	1.51			
	Poly herbal PF3	1	05	1.33	1.26	83.95	16.05
		2	05	1.18			
		1	10	1.13	1.12	74.58	25.42
		2	10	1.10			
		1	20	0.56	0.49	32.44	67.56
		2	20	0.41			

- Relative Cell Growth = (No. Cells in Test Flask / No. Cells in Solvent Flask) X 100
- All values are expressed as Mean
- DMSO= Dimethyl sulfoxide

Preparation of Plant Extracts

The three *Annona squamosa, Zingiber Officinalis and Triticum Aestivum* plant samples collected were chopped into small pieces, shade dried and grounded using hammer type milling machine at the Department of Pharmacology, East Point Pharmacy College, Bangalore. The powdered materials in 1:2: 3-gram equivalent ratio were transferred into and extracted in the Soxhlet extractor using methanol for 72 h. The extracts were filtered through a Whatman filter paper No. 42 (125 mm) and concentrated using a rotary evaporator with the water bath set at 40°C, then dried in a desiccator over anhydrous CuSO4. The powdered residue was transferred into vials and stored at 4°C in airtight vials before analysis.

Positive Controls

Mitomycin, Cyclophosphamide

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Table 3: Relative mitotic index in polyherbal PF3 in CHO cells (Range finding test)

Treatment Duration (Hrs): 3 Hrs Harvesting Time (Hrs) : 18 Hrs							
Sl. No.	Test Samples	Trial Number	Test Concentration (μg/mL)	Number of dividing cells /200 cells	Mean mitotic index	Relative Mitotic Index (RMI) (%)	Cytotoxicity (%)
A	Without S9 activation						
	DMSO (0.1% in	1	0	32	35	100.0	0.0
	Media)	2	0	38			
	Poly	1	05	28	29	82.86	17.14
	herbal	2	05	30			
	PF3	1	10	22	23	65.71	34.29
		2	10	24			
		1	20	18	16	44.29	55.71
		2	20	13			

- RMI = (Test Concentration MI / Solvent Control MI)X 100
- All values are expressed as Mean
- DMSO= Dimethyl sulfoxide

Table 4: Relative mitotic index in polyherbal PF3 in CHO cells (Range finding test)

Treatment Duration (Hrs): 3 Hrs Harvesting Time (Hrs): 18 Hrs							
Sl. No.	Test Samples	Trial Number	Test Concentration (μg/mL)	Number of dividing cells /200 cells	Mean mitotic index	Relative Mitotic Index (RMI) (%)	Cytotoxicity (%)
B	With S9 activation						
	DMSO (0.1% in	1	0	42	48	100.00	0.00
	Media)	2	0	53			
	Poly	1	05	36	35	72.63	27.37
	herbal	2	05	33			
	PF3	1	10	35	32	66.32	33.68
		2	10	28			
		1	20	24	20	42.11	57.89
		2	20	16			

- RMI = (Test Concentration MI / Solvent Control MI)X 100
- All values are expressed as Mean
- DMSO= Dimethyl sulfoxide

Table 5: Relative cell growth in polyherbal PF3 in CHO cells (Definitive chromosome aberration assay)

Treatment Duration (Hrs): 3 Hrs
Harvesting Time (Hrs): 18 Hrs

Sl. No	Test Samples	Trial Number	Test Concentration (μg/mL)	Number of cells (x 106) Per 60mm dish	Mean Number of cells (x 106) Per 60mm dish	Relative cell Growth (RCG) (%)	Cytotoxicity (%)
A	Without S9 activation						
	DMSO (0.1% in Media)	1	0	1.30	1.21	100.00	0.00
		2	0	1.12			
	Poly herbal PF3	1	5	1.09	1.15	95.04	4.96
		2	5	1.21			
		1	10	0.92	0.99	81.82	18.18
		2	10	1.06			
		1	15	0.81	0.80	65.70	34.30
		2	15	0.78			
	Mitomycin C	1	0.4	0.52	0.49	40.08	59.92
		2	0.4	0.45			
		1	0.8	0.31	0.30	24.38	75.62
		2	0.8	0.28			

- All values are expressed as Mean, DMSO= Dimethyl sulfoxide

Table 6: Relative cell growth in polyherbal PF3 in CHO cells (Definitive chromosome aberration assay)

Treatment Duration (Hrs): 3 Hrs
Harvesting Time (Hrs): 18 Hrs

Sl. No	Test Samples	Trial Number	Test Concentration (μg/mL)	Number of cells (x 106) Per 60mm dish	Mean Number of cells (x 106) Per 60mm dish	Relative cell Growth (RCG) (%)	Cytotoxicity (%)
B	With S9 activation						
	DMSO (0.1% in Media)	1	0	1.31	1.11	100.00	0.00
		2	0	0.91			
	Poly herbal PF3	1	5	0.78	0.95	85.59	14.41
		2	5	1.12			
		1	10	0.91	0.77	69.37	30.63
		2	10	0.63			
		1	15	0.58	0.59	53.15	46.85
		2	15	0.6			
	Cyclophos phamide	1	7.5	0.52	0.485	43.69	56.31
		2	7.5	0.45			
		1	15	0.48	0.43	38.74	61.26
		2	15	0.38			

- All values are expressed as Mean, DMSO= Dimethyl sulfoxide

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Table 7: Relative mitotic index in polyherbal PF3 in CHO cells (Definitive chromosome aberration assay)

Treatment Duration (Hrs): 3 Hrs Harvesting Time (Hrs): 18 Hrs							
Sl. No.	Test Samples	Trial Number	Test Concentration (μg/mL)	Number of dividing cells /200 cells	Mean mitotic index	Relative Mitotic Index (RMI) (%)	Cytotoxicity (%)
A	Without S9 activation						
	DMSO (0.1% in Media)	1	0	32	28.5	100.00	0.00
		2	0	25			
	Poly herbal PF3	1	5	22	20.5	71.93	28.07
		2	5	19			
		1	10	17	20	70.18	29.82
		2	10	23			
		1	15	18	16	56.14	43.86
		2	15	14			
	Mitomycin C	1	0.4	22	24	84.21	15.79
		2	0.4	26			
		1	0.8	16	15	52.63	47.37
		2	0.8	14			

- All values are expressed as Mean
- DMSO= Dimethyl sulfoxide

Table 8: Relative mitotic index in polyherbal PF3 in CHO cells (Definitive chromosome aberration assay)

Treatment Duration (Hrs): 3 Hrs Harvesting Time (Hrs): 18 Hrs							
Sl. No.	Test Samples	Trial Number	Test Concentration (μg/mL)	Number of dividing cells /200 cells	Mean mitotic index	Relative Mitotic Index (RMI) (%)	Cytotoxicity (%)
B	With S9 activation						
	DMSO (0.1% in Media)	1	0	38	31	100	0.00
		2	0	23			
	Poly herbal PF3	1	5	19	22	70.49	29.51
		2	5	24			
		1	10	17	18	59.02	40.98
		2	10	19			
		1	15	11	17	55.74	44.26
		2	15	23			
	Cyclophos phamide	1	7.5	11	9	27.87	72.13
		2	7.5	6			
		1	15	8	7	21.31	78.69
		2	15	5			

- All values areexpressed as Mean
- DMSO= Dimethylsulfoxide

Table 9: Chromosomal aberrations in polyherbal PF3 in CHO cells (Treatment: 3Hrs, Harvest: 18Hrs) (Definitive chromosome aberration assay)

Sl. No.	Test Samples	Trial Number	Test Concentration (μg/mL	Chromosomal aberrations /100 cells Type of Aberrations				% of cells	% of cells with Aberrations (Mean of trials)	Statistical Significance (p-value) Chi Square Test
				Aberratio not accounted	Chroma	Chromo	Other			
A	Without S9 activation									
	DMSO	1	0	2	1	0	0	1	1.5	-
	(0.1%)	2	0	0	1	1	0	2		
	Poly	1	5	1	2	0	0	2	1.5	1.0
	herbal	2	5	0	1	0	0	1		
	PF3	1	10	0	4	1	0	3	3.5	0.37
		2	10	2	5	1	0	4		
		1	15	0	11	3	0	6	5.0	0.17
		2	15	4	9	1	0	4		
	Mitom	1	0.4	1	15	5	1	21	19.5***	<0.001
	ycin C	2	0.4	0	18	0	0	18		
		1	0.8	3	28	10	1	25	30.5***	<0.001
		2	0.8	2	39	2	1	36		

- All values are expressed as Mean, #Others= Cells with Polyploidy and Endore duplication, DMSO=Dimethyl sulfoxide
- *p< 0.05, **p<0.01 and *** p<0.001, Chi Square Test to Negative control of the same treatment period. ## = Gap Aberrations

Cells

Chinese hamster ovary cell line from NCCS, Pune

Media

DMEM (Dulbecco's Modified Eagle Medium), Foetal Bovine Serum

Solvent control

0.1% DMSO was used as solvent control.

Metabolic activation

Rat Liver Homogenate (S9 Mixture)

Test Procedure (OECD, 1997; Ishidate and Sofuni, 1980)

Solubility and pH Determination

Polyherbal PF3 extract of 10 mg was taken in a volumetric flask, and DMSO was added to the flask in 0.1 mL increments until the Polyherbal PF3 extract forms a uniform suspension. The 10 mg/mL stock in DMSO was serially diluted in DMEM medium to obtain a working stock of 0.1mg/mL and checked for any precipitation. The working stock was checked for any pH variations in the media using pH meter. There was no variation in pH was observed in the media.

Preparation of Test Cultures

Test cultures were prepared and harvested by growing stock cultures in T-25 cm2 tissue culture flasks with antibiotic-free medium and showing approximately 60-95% confluence. The culture medium from the T-25 cm2 flasks was discarded, and the cells were washed with Ca++ and Mg++ free phosphate buffer saline (PBS). The cells were dissociated then by incubating along swith 0.05% Trypsin at 37 ± 2ºC. The cells were re-suspended in complete culture medium containing 10% Fetal Bovine Serum (FBS), 2 μM L-glutamine, 50 units/mL of penicillin, and 50 μg/mL of streptomycin. An aliquot of pooled cell suspensions were diluted to the appropriate concentration before counting with a cell counter. Depending upon the cell counts, 1x105 cells/mL of separate cell suspension was prepared in complete medium. Five (5.0) mL of this suspension was seeded in each T-25 cm2 tissue culture flask to give 5x 10^5 cells per flask. The separated test cultures were used for Range Finding studies and for Chromosomal Aberration Tests. Prior to 24 h of the treatment, the test cultures were incubated at 37°C, 5%

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Table 10: Chromosomal aberrations in polyherbal PF3 in CHO cells (Treatment: 3Hrs, Harvest: 18Hrs) (Definitive chromosome aberration assay)

Sl. No.	Test Samples	Trial Number	Test Concentration (μg/mL)	Chromosomal aberrations /100 cells Type of Aberrations				% of cells with Aberrations	% of cells with Aberrations (Mean of trials)	Statistical Significance (p-value) Chi Square Test
				Aberratic not accountec	Chrom	Chromos	Others#			
B	With S9 activation									
	DMSO (0.1%)	1	0	0	2	0	0	2	1.5	-
		2	0	1	1	0	0	1		
	Poly herbal PF3	1	5	0	2	0	0	1	1.0	0.75
		2	5	4	1	1	0	1		
		1	10	5	0	0	0	0	1.5	1.0
		2	10	2	3	1	0	3		
		1	15	1	8	1	0	7	6.0	0.1
		2	15	4	10	5	0	5		
	Cyclophos phamide	1	7.5	2	38	8	1	35	28.0***	<0.001
		2	7.5	1	23	6	2	21		
		1	15	4	52	11	2	51	42.0***	<0.001
		2	15	0	35	8	1	33		

- All values are expressed as Mean, #Others= Cells with Polyploidy and Endore duplication, DMSO=Dimethyl sulfoxide
- *p< 0.05, **p<0.01 and *** p<0.001, Chi Square Test to Negative control of the same treatment period. ## = Gap Aberrations

CO_2 in culture flasks.

Preparation of Test Article/Positive Control Solutions

The test article solutions were prepared just prior to the treatment. The required quantity of test article was weighed and dissolved in an appropriate volume of DMSO to get the stock solution of 1mg/100μL concentration. The stock solution was diluted further using a cell culture medium to prepare the working stock solutions of required concentrations for the Range Finding Test, the Definitive, and the Confirmatory Assays.

The positive controls stock solution was prepared by dissolving the required quantity inappropriate volume of DMSO (0.1mg/mL). Positive controls stock solution was further subsequently diluted using cell culture medium to prepare required concentrations respectively for Range Finding, Definitive, and for Confirmatory studies.

Range Finding Test

The toxicity of the test sample was determined by Range Finding Study. Approximately 24 h old Test cultures were used as seed culture for Range Finding Studies. At each concentration, duplicate cultures were used for the activation of both metabolic systems. Range Finding study was performed at the concentration from 5 to 20μg/mL. Treated cells were assessed by Relative Cell Growth (RCG) and Relative Mitotic Index (RMI) to determine the cytotoxicity of the test sample. The medium of test culture was taken out from the flask in both metabolic activated and non-activated systems, and to each flask, fresh 5.0 mL of complete medium or 5.0 mL of serum-free medium, and S-9 activation mixture were added respectively. Test culture was exposed 3 h with test samples. The cells were removed from the culture medium and rinsed with DPBS followed by reefing with 5.0 mL of complete medium. Cells were incubated further 15 hours along with 0.1 μg/mL Colcemid for the final 2 hours.

Post-incubation cells were harvested by transferring the medium into centrifuge tubes and labeled. PBS was used to wash the monolayer of cells.

Table 11: Relative cell growth in polyherbal PF3 in CHO cells (Confirmatory chromosome aberration assay)

Treatment Duration (Hrs): 18 Hrs
Harvesting Time (Hrs): 18 Hrs

Sl.No.	Test Samples	Trial Number	Test Concentration (μg/mL)	Number of cells (x 106) Per 60mm dish	Mean Number of cells (x 106) Per 60mm dish	Relative cell Growth (RCG) (%)	Cytotoxicity (%)
A	Without S9 activation						
	DMSO (0.1% in	1	0	1.30	1.21	100	0.00
	Media)	2	0	1.12			
	Poly	1	5	1.09	1.15	95.04	4.96
	herbal	2	5	1.21			
	PF3	1	10	0.92	0.99	81.82	18.18
		2	10	1.06			
		1	15	0.81	0.80	65.70	34.30
		2	15	0.78			
	Mitomycin-C	1	0.4	0.31	0.30	24.38	75.62
		2	0.4	0.28			

- All values are expressed as Mean
- DMSO= Dimethyl sulfoxide

Table 12: Relative mitotic index in polyherbal PF3 in CHO cells (Confirmatory chromosome aberration assay)

Treatment Duration (Hrs): 18 Hrs
Harvesting Time (Hrs): 18 Hrs

Sl. No	Test Samples	Trial Number	Test Concentration (μg/mL)	Number of dividing cells /200 cells	Mean mitotic index	Relative Mitotic Index (RMI) (%)	Cytotoxicity (%)
A	Without S9 activation						
	DMSO (0.1% in	1	0	32	28.5	100.00	0.00
	Media)	2	0	25			
	Poly	1	5	22	20.5	71.93	28.07
	herbal	2	5	19			
	PF3	1	10	17	20	70.18	29.82
		2	10	23			
		1	15	18	16	56.14	43.86
		2	15	14			
	Mitomycin-	1	0.4	9	10	35.09	64.91
	C	2	0.4	11			

- All values are expressed as Mean
- DMSO= Dimethyl sulfoxide

Table 13: Chromosomal aberrations in polyherbal PF3 in CHO cells (treatment: 18hrs, harvest: 18hrs) (Confirmatory chromosome aberration assay)

Sl. No.	Test Samples	Test Samples	Test Concentration (μg/ml	Chromosomal aberrations /100 cells Type of Aberrations				% of cells with Aberrations	% of cells with Aberrations (Mean of trials)	Statistical Significance (p-value) Chi Square Test
				Aberra not accoun	Chrom	chromo	Others			
A	Without S9 activation									
	DMSO (0.1%)	1	0	1	1	1	0	2	1.5	-
		2	0	2	0	1	0	1		
	Poly herbal PF3	1	5	3	2	0	0	2	2.0	0.78
		2	5	1	0	2	0	2		
		1	10	3	2	2	0	4	3.5	0.37
		2	10	1	3	1	0	3		
		1	15	5	16	5	0	8	6.5	0.077
		2	15	3	8	3	0	5		
	Mitomycin C	1	0.4	6	33	16	3	30	35.5***	<0.001
		2	0.4	3	49	5	0	41		

- All values are expressed as Mean, #Others= Cells with Polyploidy and Endore duplication, DMSO=Dimethyl sulfoxide
- *p< 0.05(significance), ** p<0.01(significance) and *** p<0.001 statistically highly significant, Chi Square Test to Negative control of the same treatment period. ## = Gap Aberrations

Trypsin 0.05% was used for dissociation and resuspended in the medium collected. Different concentrations of cell suspension was read at an electronic cell counter. For each concentration, the cell number were calculated per flask. Excel 2013 spreadsheet was used to calculate the Relative Cell Growth (RCG) as of the following,

$$RCG = \frac{No.\ Cells\ in\ Test\ Flask \times 100}{No.\ Cells\ in\ Solvent\ Flask}$$

The Relative Mitotic Index (RMI) was determined by the remaining processed cell suspension, as described below.

The cells were allowed to swell in 0.075 M hypotonic KCI solution and fixed in 3:1 methanol: glacial acetic acid fixative. The cells were then centrifuged at 800 rpm, collected were stored at 1-5 °C. The cells were again centrifuged recollected and suspended again in fresh fixative and placed on microslides. The slides were allowed to air dry and stained in Giemsa stain (5 %). The slides were mounted with #1coverglasses using Cytoseal. Mitotic Index (MI) were scored for the coded slides. For each duplicate concentration (500 from each), overall, 1000 cells were scored and recorded the number of dividing cells. The MI was calculated for each concentration with Excel 2013 spreadsheet as of the following,

$$MI = \frac{No.\ of\ Dividing\ Cells\ from\ 1000\ Cells}{10}$$

$$RMI = \frac{Test\ Concentration\ MI\ \times 100}{Solvent\ Control\ MI}$$

Based on the reduction of RCG and RMI, the cytotoxicity of cells was determined. The highest test concentration in RCG and RMI, causing approximately 50% reduction, was selected for the Chromosomal Aberration Test. In addition to this, three to six lower concentrations were also included in the Test. If cytotoxicity was not observed at the maximum concentration, Chromosomal Aberration Test was performed at four decreasing concentrations from the maximum concentration tested or the concentrations formed with precipitates.

Definitive Chromosome Aberration Assay

The Definitive Chromosomal Aberration Test was conducted based on the Range Finding Study results. The assay was performed with a single harvest at 1.5 X, the time of the normal cell cycle. The test culture medium was prepared, as described earlier. The duplicate 24 h old seeded cultures with

$1x10^5$ cells/mL were treated in metabolic activated and non-activated systems at each concentration level. Based on the Range Finding Study results, the cell culture were treated for both with and without metabolic activation at the concentrations, 5, 10, and 15 μg/mL. Cyclophosphamide (CP) (at 7.5 and 15 μg/mL) and Mitomycin-C (MMC) (at 0.4 and 0.8 μg/mL) were used as the positive controls both in activated and non-activated systems respectively. For both metabolic activated and non-activated systems and to each flask, fresh 5.0 mL of complete medium or 5.0 mL of serum-free medium and S-9 activation mixture were added respectively. Test culture was exposed 3 h with test samples. The cells were removed from the culture medium and rinsed with DPBS followed by reefing with 5.0 mL of complete medium. Cells were incubated further 15 hours along with 0.1 μg/mL Colcemid for the final 2 hours. Post incubation of 18 hours cells were harvested (1.5 x normal cell cycle).

200 μl of cell suspension was used for the determination of Cytotoxicity, determined with the reduction in the RCG and RMI. The slides were also used in the determination of the RMI of chromosomal aberrations. Chromosomal aberrations were scored based on the results of RCG / RMI of treated cells with 5, 10, and 15μg/mL of concentrations in both with and without activation. The untreated, corresponding solvent control and both concentrations of the positive controls (Mitomycin-C at 0.4 and 0.8μg/mL; and Cyclophosphamide at 7.5 and 15 μg/mL) were also scored. Two hundred metaphases were scored from each concentration and the controls.

Confirmatory Chromosome Aberration Assay

The Confirmatory Chromosomal Aberration Study was conducted only by without activation, because of the negative results of Definitive Assay and Mitomycin-C 0.4 μg/mL was positive. The cells were exposed to 5, 10, and 15 μg/mL of test item dilutions and 0.4 μg/mL of Mitomycin-C dilution up to 18 hours. The harvest time was of 18 h after treatment initiation (1.5 x normal cell cycle). The positive control, MMC, was used at the concentration of 0.4 μg/mL. The solvent and positive, MMC at 0.4 μg/mL controls were also scored. From each concentration and controls, a minimum of two hundred metaphases were scored. By determining the RCG and RMI, the toxicity was measured along with the percentage of polyploid and end reduplicated cells at each concentration.

Method-II: Preincubation

Evaluation Criteria

1. Dose concentration-related the significant increase in the percentage of cells with chromosomal aberrations compared to negative control indicates that the test substance has the potential to induce chromosomal aberrations.
2. An increase in the number of polyploidy cells compared to negative control indicates the test substance has the potential to inhibit the mitotic process and to induce numerical chromosome aberrations.
3. An increase in the number of cells with end reduplicated chromosomes, compared to the negative control, indicates the test substance has the potential to inhibit cell cycle progression.
4. Positive controls, both with or without metabolic activation, must show an increase in the percentage of cells with aberrations and cells with polyploidy and endoreduplication when compared to a negative control.
5. Negative control must not show either an increase in the percentage of cells with aberrations, polyploidy, or endoreduplication.
6. The test substance for which the results do not meet the above criteria is considered as non-mutagenic.

Statistical Analysis

Total chromosomal aberrations were compared with solvent controls, and statistical analysis was performed using the Chi-square test using Microsoft Excel Program, 2013 (Microsoft Corporation, Redmond, WA, USA). A value of $P < 0.05$ was considered to be statistically significant.

1. * $p< 0.05$ (significance),
2. ** $p<0.01$ (significance),
3. *** $p<0.001$ (statistically highly significant)

RESULTS AND DISCUSSION

Precipitation test

Working stock solution (0.1mg/mL) of 1.0 mL was added to 4.0 mL of complete medium, resulting in a final test item concentration of 20μg/mL and formed a clear solution. No precipitation and pH value changes were observed after dilutions.

Range finding test

The cytotoxicity was evaluated on the basis of the reduction in the RCG and RMI. The concentrations

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of 5, 10, and 20 μg/mL were used in both without and with metabolic activation. The test item, at 20 μg/mL concentration, showed a reduction in RCG and RMI by 76.18% and 55.71%, respectively, in the absence of metabolic activation (-S9); and by 67.56% and 57.89% respectively in the presence of metabolic activation (+S9). Since 20 μg/mL concentration causing >50% reduction in RCG and RMI, the next lower concentration of 15 μg/mL was selected as the highest test concentration for the Chromosome Aberration Assay (Tables 1 and 2 and Tables 3 and 4). No precipitation and pH value changes were observed in the test.

Definitive Chromosome Aberration Assay

In the absence of metabolic activation, Polyherbal PF3 exposure for 3 hours at the concentrations of 5, 10, and 15 μg/mL showed 4.96%, 18.18%, and 34.30% of relative cell growth, and 28.07%, 29.82% and 43.86% of relative mitotic index, respectively. 0.1% DMSO (Negative Control) showed 100% of relative cell growth and relative mitotic index while the positive control Mitomycin-C at 0.4 and 0.8 μg/mL showed 59.92% and 75.62% of relative cell growth, and 15.79% and 47.37% of relative mitotic index, respectively, under culture conditions.

In the presence of metabolic activation, Polyherbal PF3 exposure for 3 hours at the concentrations of 5, 10, and 15 μg/mL showed 14.41%, 30.63%, and 46.85% of relative cell growth, and 29.51%, 40.98% and 44.26% of relative mitotic index, respectively. 0.1% DMSO (Negative Control) showed 100% of relative cell growth and relative mitotic index while the positive control Cyclophosphamide at 7.5 and 15 μg/mL showed 56.61% and 61.26% of relative cell growth, and 72.13% and 78.69% of relative mitotic index, respectively, under culture conditions.

In absence of metabolic activation, Polyherbal PF3, at the tested concentrations of 5, 10 and 15 μg/mL, exposed for 3 hours and harvested for 18 hours showed 1.5%, 3.5% and 5.0% of cells with aberrations which was not significantly different from the negative control which showed 1.5% of cells with aberrations. The positive control (Mitomycin-C) at the tested concentrations of 0.4 and 0.8 μg/mL showed a significant increase in the % of cells with aberration (19.0 and 30.5%, respectively).

In presence of metabolic activation, Polyherbal PF3, at the tested concentrations of 5, 10 and 15 μg/mL, exposed for 3 hours and harvested for 18 hours showed 1.0%, 1.5% and 6.0% of cells with aberrations which was not significantly different from the negative control which showed 1.5% of cells with aberrations. The positive control (Cyclophosphamide) at the tested concentrations of 7.5 and 15 μg/mL showed a significant increase in the % of cells with aberration (28.0 and 42.0%, respectively) (Tables 5 and 6 & Tables 7 and 8 & Tables 9 and 10). No precipitation and pH value changes were observed in this test.

Confirmative Chromosome Aberration Assay

In the absence of metabolic activation, Polyherbal PF3 exposure for 18 hours at the concentrations of 5, 10, and 15 μg/mL showed 4.96%, 18.18%, and 34.30% of relative cell growth, and 28.07%, 29.82% and 43.86% of relative mitotic index, respectively. 0.1% DMSO (Negative Control) showed 100% of relative cell growth and relative mitotic index while the positive control Mitomycin-C at 0.4 μg/mL showed 75.62% of relative cell growth, and 64.91% of relative mitotic index, respectively, under culture conditions.

Polyherbal PF3, at the tested concentrations of 5, 10, and 15 μg/mL, exposure for 18 hours showed 2.0%, 3.5%, and 6.5% of cells with aberrations, which was not significantly different from the negative control which showed 1.5% of cells with aberrations. The positive control (Mitomycin-C) at the tested concentrations of 0.4 μg/mL in the absence of metabolic activation showed a significant increase in the % of cells with aberration (35.5%) (Tables 11 and 12 and Table 13). No precipitation and pH value changes were observed in this test.

Physico-chemical alterations lead to the causes of genotoxicity, in turn to cancer. The prevention of cellular metabolic and genetic integrity is the main target in designing new cancer treatments. The present study was carried out to evaluate the genotoxic potential by the chromosomal aberration method. The results from the definitive and confirmatory chromosome aberration assays indicate that the test article Polyherbal Formulation-3 (PF3) did not induce a statistically significant increase in the percentage of cells with aberrations both in presence and absence of metabolic activation when compared to the negative control. Therefore, under the conditions of this test, according to the criteria set for evaluating the test results, PF3 was found nonclostagenic at a maximum dose of 15 μg/ml in the chromosome aberration assay using CHO cell line, both in presence and absence of metabolic activation.

The studies carried out in *Annona squamosa, Zingiber Officinalis, and Triticum Aestivum* individually attributes to the study carried out. Polyherbal formulations were also studied for their improved action as antidiabetic, antihyperlipidemic, cellulite, and genotoxicity evaluations (Srivastava *et al.*, 2012; Parasuraman *et al.*, 2010; Yimam *et al.*, 2017; Lee

et al., 2018; Salah *et al.*, 2012). Natural anti-cancer compound Zerumbone of ginger were tested on CHO cell lines for their genotoxic potentials (Al-Zubairi, 2012), inhibited the colon and lung carcinogenesis in mice (Kim *et al.*, 2009), inhibited cellular growth and moderate factors of angiogenic ovarian cancer cells (Rhode *et al.*, 2007). Phytotoxic and mutagenic effects were observed in *Triticum* with similar methods of evaluation (Jităreanu *et al.*, 2013) represents the present work.

CONCLUSION

The Presence of rich flavonol compounds and other phytoconstituents may inhibited the mutagenic changes, DNA fragmentation and chromosomal aberration and their by prevented the genotoxicity. Further optimization and *in vivo* authenticated studies needed in this regard.

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