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RESEARCH ARTICLE

Genotoxicity Assessment of Hydroalcoholic Extract of Bark of *Terminalia arjuna* (Combretaceae)

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Abstract

The mutagenic potential of *Terminalia arjuna* hydroalcoholic bark extract was evaluated using Ames test with strains TA98, TA100, TA102, TA1535, and TA1537. The genotoxic potential was assessed by performing Chromosomal Aberration test with cell lines of CHO. In the assessment of mutagenic potential by the Ames test, hydroalcoholic extract of *Terminalia arjuna* induced a negative response and in Chromosomal Aberration test, it did not show any significant genotoxic activity in the CHO cell lines.

Keywords: Ames Test, Chromosomal Aberration Test, CHO Cell Lines, Hydroalcoholic Extract, *Terminalia arjuna*

1. Introduction

For thousands of years, local communities have used medicinal plants for their healing properties. Many people mistakenly assumed that plants that have medicinal properties are generally safe and free of undesirable side effects. However, very often some of these plants despite having therapeutic advantages can be potentially toxic, mutagenic, carcinogenic, or teratogenic[1]. Despite this inference, plants remain the main source of medicines for a large proportion of the world's population, particularly in the developing world. In spite of the advent of the pharmaceutical chemistry during the early twentieth century, which brought with it the ability to synthesize an enormous variety of medicinal drug molecules and allowed the treatment of previously incurable and/or life-threatening diseases.

Plants used in folk medicine have been researched intensively for their specific potential efficacy, safety, and toxicity; with special focus on the mutagenicity of plant extracts, herbal formulations, and specific phytocompounds. From scientific publications, it further appears that studies on the genotoxicity and/or antigenotoxicity of traditional medicinal plants are diverse and do not follow well-established protocols or are not conducted according to well-described and accepted guidelines.

Various applications of medicinal plants, such as used in cooking and to prepare infusions, decoctions, tinctures, sitz baths to treat diseases and used as a source of raw materials to develop herbal drugs are seen. Therefore, tests are needed to ensure not only efficacy and quality but also safety. However, many medicinal plants and herbal drugs are sold as dietary supplements, and in these cases do not require registration and supervision from regulatory agencies, which exposes individuals to products which have not been safely validated by preclinical and clinical trials, in contrast to other drugs.

As there is no harmonization of tests to evaluate the efficacy, quality, and safety of medicinal plants as is required for synthetic drugs across countries, regulatory agencies are improving the guidelines on the efficiency and safety of natural products. In this context, given the significant number of plants with mutagenic potential

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and the evidence from studies that demonstrate the commercialization of plants without regulation, it is important to study the genotoxic characteristics of medicinal plants. For this reason, we have conducted the genotoxic assessment for the *Terminalia arjuna* extract of our study.

Arjuna the common name of *Terminalia arjuna* is the plant used in our study which is indigenous to India. Based on the findings of ancient physicians, for centuries, the bark decoction of this tree has been used for congestive heart failure, anginal pain, hypertension, dyslipidaemia, blood diseases, fractures, ulcers, venereal, and viral disease. Calcium salts, tannins, polyphenols, flavonoids, triterpenoids, saponins, sterols, and minerals are found to be the major phytoconstituents of *T. arjuna.*

The genotoxic potential of *Terminalia arjuna* widely used in ethnomedicine and their ability to induce mutations and cytotoxicity has not been reported both *in vivo* and *in vitro* experiments. The antigenotoxic potential of this plant was discussed widely in literature but how tolerant it is to the genotoxicity test system was not revealed. Because of this backdrop, we tried to get details about the mutagenicity and clastogenicity of the hydroalcoholic bark extracts of *Terminalia arjuna*. We employed *Salmonella* reverse mutation assay followed by a chromosomal aberration assay in CHO cell lines to obtain more detailed knowledge of the genotoxic profile of this species.

2. Materials and Methods

2.1 Collection, Identification and Preparation of Plant Material

T. arjuna bark was freshly collected from the campus of Oushadhi, Thrissur, Kerala, India on April 2017. Bark was cut into small pieces. It was sun-dried for approximately one month and minced before pulverizing, coarsely powdered bark was passed through a sieve of no.10 to obtain uniform particle size, extracted using Soxhlet apparatus with water and ethanol mixture in the proportion of 1:1 as a solvent. The extractive value of its hydro-alcoholic extract was found to be 22.52%.

2.2 Ames Bacterial Reverse Mutation Test

The *Salmonella* mutagenicity assay suggested by Maron and Ames[7] was performed by pre-incubation method.

Bacteria: *Salmonella typhimurium* strains: TA98, TA100, TA102, TA1535, TA1537 obtained from DRDE, Gwalior, India.

Chemicals: Metabolic activation mixture: Aroclor-1254-induced rat-liver fraction (S9 mix) purchased (lyophilized) from Moltox Molecular Toxicology Inc. Bactoagar, nutrient broth nº 2 (Oxoid), mitomycin-C (MMC), Histidine, Sodium phosphate buffer, sodium azide, 9-Aminoacridine, 2-Nitrofluorene, Benzopyrene, Cyclophosphamide were obtained from Sigma-Aldrich.

2.2.1 Test Procedure

Genotyping of *Salmonella typhimurium* strains was performed before the initiation of the mutagenicity test to ensure genetic integrity[6]. Seven different concentrations of the extract of *T. Arjuna*; 5000, 2500, 1250, 625, 313, 156, and 78 µg/plate were evaluated with strains TA 98 and TA 100 for cytotoxicity both in the absence and presence of exogenous metabolic activation system in duplicates. Based on the cytotoxicity data, mutagenicity test was performed with all the 5 *S. typhimurium* strains (TA 98, TA 100, TA 102, TA 1535, and TA 1537), at doses 39, 78, 156, 313, and 625 µg/plate, in triplicates, in the absence and presence of exogenous metabolic activation system. Each concentration of the extract was added to the buffer (pH 7.4) and 0.1ml of bacterial culture and incubated at 37°C for 20-30 min. The influence of metabolic activation was evaluated by adding S9 mixture in the place of buffer. To this, 2ml of top agar was added and the mixture was poured onto a plate containing minimal agar medium. The plates were incubated at 37°C for 48 hrs and his+ revertant colonies were manually counted. The positive controls used in the absence of metabolic activation system for each strain were 2-Nitrofluorene (10 µg/plate) for TA98, sodium azide (1 µg/plate) for TA100 and TA1535, 9-Aminoacridine (50 µg/plate) for TA1537 and mitomycin C (0.5 µg/plate) and Benzo(a)pyrene (10 µg/plate) was the positive test control used for all strains, with metabolic activation and distilled water used as the negative control.

2.3 *In-vitro* Chromosomal Aberration (CA) Assay

In vitro Mammalian Chromosomal Aberration Test in CHO cell lines was conducted to assess chromosomal aberration potential of *Terminalia arjuna* extract[8]. A typical Chinese Hamster Ovary (CHO) cells were maintained in nutrient mixture (Ham's F-12), supplemented with fetal

bovine serum (5%). Cells (5×10^5 cells) were seeded on the day before experiment from a culture with 60-80% confluence into each T-75 cm^2 flask and incubated overnight at 37°C and 5% CO_2.

Stock solution (5 mg/ml) of test extract was prepared in sterile distilled water. Positive controls used were cyclophosphamide and mitomycin C with and without metabolic activation (10% S9), respectively. Seven concentrations of extract (i.e., 78, 156, 313, 625, 1250, 2500, and 5000 µg/ml) were taken for the initial test, and these test concentrations were added to cell culture medium containing fetal bovine serum. Cells with 10% S9 and without 10% S9 were exposed to extract for 3 hrs and 18 hrs, respectively.

After the initial test, a confirmatory test was done in a comparable manner, and the test concentrations employed were 313, 625, and 1250 µg/ml for 3 hr exposure, whereas, for 18 hr exposure they were 156, 313, and 625 µg/ml. Two hours prior harvesting, colchicine was added to arrest the metaphase, and hypotonic solution was added followed by fixation in 3:1 methanol and glacial acetic acid, and giemsa stain was used for staining. The Relative Population Doubling (RPD) of the treated cells was determined for evaluating cytotoxicity using the formula.

$$\text{Relative population doubling}\,(\%) = \frac{N \times 100}{n}$$

where:
N- Number of population doublings (treated culture)
n - Number of population doublings (control culture)

$$\text{Population doubling} = \frac{\left[\log\left(\text{post treatment cell number} \div \text{initial cell number}\right)\right]}{\log 2}$$

Two hundred well spread metaphases were scored per test and solvent control in duplicates, whereas one hundred metaphases were scored for the positive control.

3. Results

3.1 Ames Bacterial Reverse Mutation Test

Tester strain genotyping was performed and found intact.

Cytotoxic effect of the extract was assessed over a range of concentrations by pre-incubation method employing two tester strains TA98 and TA100. Seven concentrations were tested (Table 1). 78, 156, 313, 625, 1250, 2500, and 5000 µg/plate in the presence and absence of metabolic activation (i.e., 10% S9 mix). Concurrent solvent controls were maintained. Duplicates were used for all treatments and control. All plates were examined for the presence of any contamination throughout the incubation period.

No contamination was observed in any of the plates throughout the incubation period. At the end of the incubation period, the plates were observed for the signs of cytotoxicity by assessing the intactness of the background lawn. Extreme inhibition of the background lawn was observed in TA100 at doses of 2500 µg/plate and 5000 µg/plate in the treatment with and without exogenous metabolic activation. TA100 showed moderate inhibition of the background lawn at the dose of 1250 µg/plate in

Table 1. Genotype confirmations of tester strains

Test system	Strain				
	TA 98	TA 100	TA 102	TA 1535	TA 1537
Histidine dependence	-	-	-	-	-
UVrB mutation	-	-	-	-	-
Histidine and Biotin dependence	+	+	+	+	+
Rfa mutation	Z	Z	Z	Z	Z
Ampicillin resistance	+	+	+	-	-
Tetracycline resistance	-	-	+	-	-

'-' - No growth; '+' - Growth; 'Z' - Zone of inhibition

Table 2. Cytotoxicity test- Pre-incubation method

Tester strains	Plate No.	Number of revertant colonies at various concentrations (µg) per plate with 10% S9 Mix							Solvent control (Distilled water 0.1 ml/Plate)
		78	156	313	625	1250#	2500#	5000*	
TA98	1	64	62	69	56	38	28	*	68
	2	63	59	64	57	36	31	*	65
	AHR	**63.50**	**60.50**	**66.50**	**56.50**	**37.00**	**29.50**		**66.50**
	SD	**0.71**	**2.12**	**3.54**	**0.70**	**1.41**	**2.12**		**2.12**
TA100	1	126	113	121	72	58	*	*	148
	2	111	127	104	68	55	*	*	139
	AHR	**118.5**	**120**	**112.5**	**70**	**56.5**			**143.5**
	SD	**10.61**	**9.90**	**12.02**	**2.83**	**2.12**			**6.36**
		Number of revertant colonies at various concentrations (µg) per plate without S9 Mix							
Tester strains	**Plate No.**	**78**	**156**	**313**	**625**	**1250#**	**2500***	**5000***	**Solvent control (Distilled water 0.1 ml/Plate)**
TA98	1	61	58	60	54	36	*	*	62
	2	57	61	59	57	38	*	*	64
	AHR	**59.0**	**59.5**	**59.5**	**55.5**	37.0			**63**
	SD	**2.83**	**2.12**	**0.71**	**2.12**	1.41			**1.41**
TA100	1	128	119	84	81	52	*	*	131
	2	106	84	89	88	59	*	*	129
	AHR	**117.0**	**102.0**	**87.0**	**85.0**	**55.5**			**130**
	SD	**15.56**	**24.75**	**3.54**	**4.95**	**4.94**			**1.41**

AHR = Average Histidine Revertants, *- Extreme Lawn Inhibition, # - Moderate Lawn Inhibition and @ - Mild Lawn Inhibition

Table 3. Main study- Pre-incubation method with 10% S9 mix

Tester Strains	Plate No.	Test concentrations (µg/plate)					Solvent control (Distilled water 0.1 ml/Plate)	@Positive Control
		39	78	156	313	625		
TA98	1	62	61	59	55	59	64	121
	2	66	53	61	57	51	68	128
	3	58	59	63	51	53	62	136
	AHR	**62**	**57.67**	**61**	**54.33**	**54.33**	**64.67**	**128.33**
	S.D.	**4**	**4.16**	**2**	**3.06**	**4.16**	**3.06**	**7.51**
TA100	1	112	103	113	93	91	136	238
	2	109	116	101	99	94	128	235
	3	119	107	108	95	99	139	246
	AHR	**113**	**109**	**107**	**96**	**95**	**134**	**240**
	S.D.	**5.13**	**6.66**	**6.03**	**3.06**	**4.04**	**5.69**	**5.69**
TA102	1	351	349	372	382	364	381	634
	2	356	358	361	376	359	378	682
	3	348	344	379	368	347	389	619
	AHR	**351.67**	**350.33**	**370.67**	**375.33**	**356.67**	**382.67**	**645**
	S.D.	**4.04**	**7.09**	**9.07**	**7.02**	**8.74**	**5.69**	**32.91**

Table 3 to be continued...

Tester Strains	Plate No.	Test concentrations (µg/plate)					Solvent control (Distilled water 0.1 ml/Plate)	@Positive Control
		39	78	156	313	625		
TA1535	1	37	32	34	46	35	48	135
	2	41	43	45	48	42	47	148
	3	47	40	46	46	42	48	151
	AHR	**41.67**	**38.33**	**41.67**	**46.67**	**39.67**	**47.67**	**144.67**
	S.D.	**5.033**	**5.69**	**6.66**	**1.15**	**4.04**	**0.58**	**8.50**
TA1537	1	15	17	19	15	16	24	94
	2	12	14	15	18	15	23	85
	3	14	15	17	16	14	19	104
	AHR	**13.67**	**15.33**	**17**	**16.33**	**15**	**22**	**94.33**
	S.D.	**1.53**	**1.53**	**2**	**1.53**	**1**	**2.65**	**9.50**

@ - Benzo(a)pyrene (10 µg/plate) for all five strains (TA100, TA102, TA1535, TA1537 and TA98)
AHR = Average Histidine Revertants, S.D. = Standsard Deviation

Table 4. Main study - Pre-incubation method without S9 mix

Tester strains	Plate No.	Test concentrations (µg/plate)					Solvent control (Distilled water 0.1 ml/Plate)	@Positive Control
		39	78	156	313	625		
TA98	1	55	58	56	54	56	57	104
	2	53	58	51	51	53	54	109
	3	56	55	54	59	55	59	112
	AHR	**54.67**	**57.00**	**53.67**	**56.33**	**54.67**	**56.67**	**108.33**
	S.D.	**1.53**	**1.73**	**2.52**	**2.52**	**1.53**	**2.52**	**4.04**
TA100	1	96	81	82	78	83	128	211
	2	88	75	81	83	77	117	215
	3	82	78	86	79	81	132	206
	AHR	**89**	**78**	**83**	**80**	**80**	**126**	**211**
	S.D.	**7.02**	**3.00**	**2.65**	**2.65**	**3.06**	**7.77**	**4.51**
TA102	**1**	305	319	324	301	294	329	648
	2	312	321	335	329	307	338	636
	3	310	314	319	309	297	321	671
	AHR	**309**	**318**	**326**	**313**	**299.33**	**329.33**	**651.67**
	S.D.	**3.61**	**3.61**	**8.19**	**14.42**	**6.81**	**8.50**	**17.79**
TA1535	**1**	42	37	49	36	33	46	124
	2	39	40	41	33	35	43	113
	3	41	35	46	39	38	45	109
	AHR	**41**	**37**	**45**	**36**	**35**	**45**	**115**
	S.D.	**1.53**	**2.52**	**4.04**	**3.00**	**2.52**	**1.53**	**7.77**

(continued)

Table 4 to be continued...

Tester strains	Plate No.	Test concentrations (µg/plate)					Solvent control (Distilled water 0.1 ml/Plate)	@Positive Control
		39	78	156	313	625		
TA1537	1	14	19	17	15	16	21	71
	2	17	16	13	14	14	18	59
	3	15	18	15	13	18	21	65
	AHR	**15.33**	**17.67**	**15**	**14.00**	**16.00**	**20.00**	**65**
	S.D.	**1.53**	**1.53**	**2**	**1.00**	**2.00**	**1.73**	**6**

@ - Positive control: Mitomycin C (0.5 µg/plate) -TA102, Sodium azide (1 µg/plate) - TA100 and TA1535, 2 - Nitrofluorene (10 µg/plate) - TA98, 9 - Aminoacridine (50 µg/plate) - TA1537, AHR = Average Histidine Revertants and S.D. = Standard Deviation

Table 5. Cell concentration data of hydroalcoholic extract of *Terminalia arjuna* - Initial experiment (with and without S9 mix (10%) - 3/18 hrs exposure)

Concentration µg/ml	ICN (106)			PTCN (106)			PTCN ÷ICN (106)		
	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)
Solvent control (50 µl/ml culture)	1.00	1.00	1.00	3.80	3.60	3.00	3.80	3.60	3.00
78	1.00	1.00	1.00	3.50	3.30	2.70	3.50	3.30	2.70
156	1.00	1.00	1.00	3.40	3.30	2.60	3.40	3.30	2.60
313	1.00	1.00	1.00	3.30	3.30	2.60	3.30	3.20	2.60
625	1.00	1.00	1.00	3.30	3.20	2.60	3.30	3.20	2.60
1250	1.00	1.00	1.00	3.30	3.20	#	3.30	3.20	-
2500	1.00	1.00	1.00	#	#	#	-	-	-
5000	1.00	1.00	1.00	#	#	#	-	-	-
Concentration µg/ml	**Log (PTCN÷ICN)**			**Log [(PTCN÷ICN)] ÷Log2**			**%RPD**		
	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)
Solvent control (50 µl/ml culture)	0.57	0.49	0.46	1.92	1.63	1.53	NA	NA	NA
78	0.54	0.51	0.43	1.80	1.72	1.43	92.10	91.66	90.00
156	0.53	0.51	0.41	1.76	1.72	1.37	89.47	91.66	86.66
313	0.51	0.50	0.41	1.72	1.67	1.37	86.84	91.66	86.66
625	0.51	0.50	0.41	1.72	1.67	1.37	86.84	88.88	86.66
1250	0.51	0.50	-	1.72	1.67	-	86.84	88.88	-
2500	-	-	-	-	-	-	-	-	-
5000	-	-	-	-	-	-	-	-	-

*: Mild detachment of the cells; #: Complete detachment of cells; PTCN: Post treatment cell number; ICN: Initial cell number; -: No live cells; NA - Not applicable; SC: Solvent control (sterile distilled water, 50 µl/ml culture).

Table 6. Cell concentration data of hydroalcoholic extract of *Terminalia arjuna* - Confirmatory experiment (with and without 10% S9 mix - 3/18 hrs exposure)

Concentration µg/ml	ICN (106)			PTCN (106)			PTCN ÷ICN (106)		
	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)
Solvent control (50 µl/ml culture)	1	1	1	3.7	3.3	2.9	3.7	3.3	2.9
	1	1	1	3.8	3.2	3	3.8	3.2	3
156	NA	NA	1	NA	NA	2.8	NA	NA	2.8
			1			2.9			2.9
313	1	1	1	3.4	3.2	2.9	3.4	3.2	2.9
	1	1	1	3.3	3.2	2.8	3.3	3.2	2.8
625	1	1	1	3.3	3.1	2.8	3.3	3.1	2.8
	1	1	1	3.2	3	2.9	3.2	3	2.9
1250	1	1	1	3.3	3	NA	3.3	3	NA
	1	1	1	3.2	3.1		3.2	3.1	
#CYP-10 µg/ml	1	1	1	2.3	2.2	1.8	2.3	2.2	1.8
	1	1	1	2	2.4	2.3	2	2.4	2.3
Concentration µg/ml	**Log (PTCN÷ICN)**			**Log [(PTCN÷ICN)] ÷Log2**			**%RPD**		
	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)
Solvent control (50 µl/ml culture)	0.56	0.51	0.46	1.88	1.72	1.53	NA	NA	NA
	0.47	0.5	0.43	1.92	1.67	1.58			
156	NA	NA	0.44	NA	NA	1.48	NA	NA	96.79
			0.46			1.53			
313	0.53	0.5	0.46	1.76	1.67	1.53	91.57	98.23	96.79
	0.51	0.5	0.44	1.72	1.67	1.48			
625	0.51	0.49	0.44	1.72	1.63	1.48	89.47	94.7	96.79
	0.5	0.47	0.46	1.67	1.58	1.53			
1250	0.51	0.47	0.46	1.72	1.58	1.53	89.47	94.7	NA
	0.5	0.49	0.44	1.67	1.63	1.48			
#CYP-10 µg/ml	0.36	0.34	0.25	1.14	1.07	0.85	56.31	67.05	67.94
	0.30	0.30	0.36	1.00	1.20	1.26			

Positive control - Cyclophosphamide (CYP); PTCN - Post treatment cell number; ICN - Initial cell number; NA - Not applicable

the treatment with and without exogenous metabolic activation.

TA98 showed extreme inhibition of the background lawn at the dose of 2500 and 5000 µg/plate in the treatment without exogenous metabolic activation and at 5000 µg/plate in metabolic activation. Moderate inhibition of the background lawn was observed at the doses of 1250 µg/plate and 2500 µg/plate in the treatment with exogenous metabolic activation and 1250 µg/plate without exogenous metabolic activation (Table 2).

Mutagenicity evaluation was performed based on the cytotoxicity data, employing five tester strains (TA100, TA102, TA1535, TA98, and TA1537) at the following concentrations with and without metabolic activation,

39, 78, 156, 313, and 625 µg/plate. No contamination was observed in any of the plates throughout the incubation period. At the end of the incubation period, the plates were observed for intactness of the background lawn, and colonies were counted. The Average Histidine Revertant (AHR) colonies per plate of all hydroalcoholic extracts of bark of *Terminalia arjuna* treated cultures fell within the concurrent solvent control values. There was no evident increase (2-3-fold) in the AHR colonies in any of the extract-treated cultures. The concurrent strain-specific positive controls exhibited a two to multi-fold increase in the number of histidine positive colonies (His^+) in the presence and absence of the S9 mix (Tables 3 and 4).

3.2 *In-vitro* Chromosomal Aberration Assay

In the initial test, complete detachment of cells was observed at 2500 and 5000 µg/ml for 3 hrs exposure,

Table 7. Chromosomal aberration data of hydroalcoholic extract of bark of *Terminalia arjuna* - Confirmatory experiment (with and without 10% S9 mix - 3/18 hrs exposure)

Concentration (µg/ml)	Structural aberrations																						
	Chromatid																						
	(+S9/3hrs)							(-S9/3hrs)							(-S9/18hrs)								
	tg	tb	tf	del	ex			tg	tb	tf	del	ex			tg	tb	tf tr	del qr	ex		
					tr	qr	cr					tr	qr	cr					tr	qr	cr
SC 50 µl/5 mL culture)	1	2	1	0	2	0	0	1	2	2	1	1	0	0	1	2	2	1	1	0	0
156	NA							NA							2	2	1	2	2	2	0
313	1	2	1	1	2	0	0	1	2	2	0	2	1	0	1	2	2	2	2	1	0
625	2	2	2	2	2	0	0	1	2	2	2	1	3	0	1	3	1	2	3	2	1
1250	1	2	2	2	3	2	0	2	2	2	2	2	2	1	NA						
CYP - 10 µg/ml	6	10	4	4	18	6	0	NA							NA						
MMC - 0.8 & 0.4µg/ml	NA							6	14	2	3	16	3	2	4	16	4	8	12	7	2
	Chromosome																						
Concentration (µg/ml)	(+S9/3hrs)							(-S9/3hrs)							(-S9/18hrs)								
	ig	sb	af	del	ex			ig	sb	af	del	ex			ig	sb	af	del	ex		
					r/acr	d	tc	plc					r/acr	d	tc	plc					r/acr	d	tc	plc
SC 50 µl/5 mL culture)	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
156	NA							NA							0	0	0	1	2	1	0	0
313	0	1	1	2	1	1	0	0	2	0	0	1	1	2	0	0	1	0	0	1	3	2	0	0
625	2	0	0	1	2	1	0	0	0	1	0	2	1	1	0	0	2	1	0	1	1	1	0	0
1250	1	0	0	2	2	1	0	0	1	2	0	1	1	1	0	0	NA							
CYP - 10 µg/ml	4	0	0	4	2	0	0	0	NA								NA							
MMC - 0.8 & 0.4µg/ml	NA							1	1	0	2	2	1	0	0	2	0	0	2	2	1	0	0

Table 8. Chromosomal aberration data of hydroalcoholic extract of bark of *Terminalia arjuna* - Confirmatory experiment (with and without 10% S9 mix - 3/18 hrs exposure)

Concentration (µg/ml)	Numerical aberrations																	
	(+S9/3hrs)						(-S9/3hrs)						(-S9/18hrs)					
SC 50 µl/5 mL culture)	p+/pc	gt	en	po	an	others	p+/pc	gt	en	po	an	others	p+pc	gt	en	po	an	others
	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0
156	NA						NA						0	0	2	1	0	0
313	0	0	1	0	0	0	0	1	2	0	0	0	0	0	1	2	0	0
625	0	0	1	0	0	0	0	0	1	2	0	0	0	0	2	1	0	0
1250	0	0	1	1	0	0	0	0	0	1	2	0	NA					
CYP - 10 µg/ml	0	0	6	0	0	0	NA						NA					
MMC - 0.8& 0.4 µg/ml	NA						0	5	0	0	0	0	0	2	0	4	0	0

tg = chromatid gap, tb = chromatid break, tf = fragment, del = deletion, ex = exchange, tr = triradial, qr = quadriradial, cr = complex rearrangement, ig = isochromatid gap, sb = chromosome break, af = acentric fragment, dmin = double minutes, r = centric ring, acr = acentric ring, d = dicentric, tc = tricentric, plc = polycentric, p+ = pulverized chromosomes, pc = pulverized cell, gt = cell with greater than 10 aberrations/multiple aberations, po = polyploidy, en = endoreduplication, an = aneuploidy. Chromatid structural aberrations (tb, tf, del, and ex combined) of CYP- 10 µg/ml significantly different from solvent control at 5% probability level (Mann Whitney - U test).

Table 9. Confirmatory experiment summary on *in vitro* Chromosomal aberrations in CHO cell lines - 3 hrs exposure to hydroalcoholic bark extract of *Terminalia arjuna* (with and without S9 mix) and 18 hrs continuous exposure (without S9 mix)

Concentration (µg/ml)	3h exposure				Continuous exposure	
	With S9 (10%) mix		Without S9 mix		Without S9 mix	
	% Structural aberrations (exclusive of gaps)	% Numerical aberrations	% Structural aberrations (exclusive of gaps)	% Numerical aberrations	% Structural aberrations (exclusive of gaps)	% Numerical aberrations
SC	4.5	0.5	3.5	1	4.5	1
156	NA	NA	NA	NA	7.5	1.5
313	6.5	0.5	7	1.5	8.5	1.5
625	8	0.5	8	1.5	9.5	1.5
1250	9	1	9.5	1.5	NA	NA
Cyclophosphamide (10 µg/ml)	58	6	NA	NA	NA	NA
MMC - 0.4 (µg/ml)	NA	NA	NA	NA	60	6
MMC - 0.8 (µg/ml)	NA	NA	52	5	NA	NA

NA - Not applicable; Total Number of metaphases counted = 200 (for the solvent control and the extract concentrations); Total Number of metaphases counted = 100 (for the positive controls)

and the % RPD was within the cytotoxic limit for extract concentrations, 1250 to 78 µg/ml. Mild detachment of cells was observed at 1250 µg/ml, whereas complete detachment for 2500-5000 µg/ml. And for concentrations, 625 to 78 µg/ml the % RPD was within the cytotoxic limits for 18 hrs exposure (Table 5).

For the confirmatory test, 1250 µg/ml and 625 µg/ml of the extract were selected as the highest concentrations based on the cytotoxicity results for 3- and 18-hrs exposure, respectively. The pH was approximately 7 and no visible contamination was observed post treatment.

DNA damage may be caused when potential genotoxicity agents interact with DNA. One of the manifestations of damage to the genome is chromosomal aberrations in proliferating cells. However, there was no significant increase in the frequency of breaks, gaps, fragments, exchanges, and endoreduplication in the CHO cell culture treated with hydroalcoholic extract of bark of *Terminalia arjuna* as demonstrated by the results of the present study when compared with the control. There was a statistical increase in the percentage of structurally damaged cells in the MMC (positive control) treatment when compared to the solvent control which indicates the cells responsiveness in this test system.

Cell concentration data about the treatment of hydroalcoholic extract of bark of *Terminalia arjuna* is presented in (Table 6). The % RPDs of treated cultures with S9 mix (10%) for 3 hrs at a concentration of 313, 625, and 1250 µg/ml were 92, 89, and 89. Whereas the % RPDs without S9 mix were 98, 95, and 95. For 18 hrs continuous exposure without S9, % RPDs at a concentration of 156, 313, and 625 µg/ml were 97. The % RPDs calculated are within the acceptable cytotoxic limits.

The aberration data of hydroalcoholic extract of bark of *Terminalia arjuna* is presented in Table 7. There was no marked change in chromosomal aberration in cultures treated with extract at any concentration as compared to solvent control. Whereas, significant increase in aberration in the chromatid region was observed in the positive control which validates the test system and experimental procedure (Table 8).

Summary of the percent structural aberrations of chromosomes indicates hydroalcoholic extract of bark of *Terminalia arjuna* did not induce any structural chromosomes aberrations in CHO cells when compared to positive control and the values obtained were within the acceptable limits (Table 9).

4. Discussion

Ames test is an extensively used testing method which is employed to assess the mutagenicity of compounds. It detects gene mutations such as point mutations, frameshift mutations, and transversion events.

It is reported that when tested for antimutagenic effect of *Terminalia arjuna against* 2-aminofluorene and sodium azide, fractions isolated from *Terminalia arjuna* inhibited the mutagenicity[9]. Antimutagenic effect of *Terminalia arjuna* may be due to direct protection of DNA from electrophilic mutagens or by the adduct formation which can prevent genotoxic damage[10]. From the results, we did not find increase in the histidine revertant with or without metabolic activation system in any of the tester strains. Hence, it can be concluded that *Terminalia arjuna* hydroalcoholic extract is non-mutagenic.

Hydroalcoholic extracts of bark of *Terminalia arjuna* did not induce % structural aberrations (exclusive of gaps) and % numerical aberrations (exclusive of gaps) at any of the concentrations used either in the presence and absence of metabolic activation system for 3 hrs and continuous exposure period. Hence, hydroalcoholic extract of bark of *Terminalia arjuna* may be interpreted as non-mutagenic at the concentrations tested and the experimental conditions employed. Literature search revealed that ethyl ether extract of bark of *Terminalia arjuna* when tested for comet' assay and micronuclei test was very effective in reducing the DNA damage induced by 4-Nitroquinoline 1-oxide[9]. Treatment of mice with aqueous extract of *Terminalia arjuna* significantly reduced the aberrations induced by Aflatoxin. Whereas, treatment with aqueous extract of bark of *Terminalia arjuna* did not induce any aberrations. It indicates that *Terminalia arjuna* may have potential antigenotoxic compounds[10]. These results are supportive of our findings and hydroalcoholic extract of *Terminalia arjuna* would be non-mutagenic and clastogenic.

5. Conclusion

Based on the results of the study, it can be concluded that hydroalcoholic extracts of bark of *Terminalia arjuna* is non-mutagenic in the Ames test. And, it does not possess mutagenicity and clastogenicity as confirmed by the Chromosomal Aberration test at the different concentrations tested and the experimental conditions employed.

6. References

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