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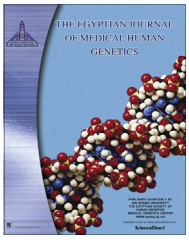

ORIGINAL ARTICLE

Terminalia arjuna, a herbal remedy against environmental carcinogenicity: An *in vitro* and *in vivo* study

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KEYWORDS

Ayurvedic medicine;
Carcinogen;
Chromosomal aberration;
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Replication index;
Terminalia arjuna

Abstract *Background:* Medicinal plants have been a major source of therapeutic agents from ancient times to cure diseases. The evaluation of rich heritage of traditional medicine is essential. The bark of *Terminalia arjuna* is rich in polyphenols (60–70%) including flavonoids and tannins.

Aim: The aim of the present investigation is to highlight the anticarcinogenic and antimutagenic potential of extracts of *T. arjuna*.

Subject and methods: In this experiment we have used human lymphocyte culture and bone marrow cells of albino mice as assay system. The parameters studied included chromosomal aberrations (CA), sister chromatid exchanges (SCEs) and cell growth kinetics (RI) both in the presence and in the absence of exogenous metabolic activation system for *in vitro* experiment, whereas total aberrant cells and the total frequencies of aberrations were taken for *in vivo* study.

Results: The role of *T. arjuna* extracts in reducing metaphase aberrations due to aflatoxin B_1 is quite significant, the reduction varying from 23.49%, 42.47%, and 59.65% down to 12.32%,

Abbreviations: CA, chromosomal aberration, SCE, sister chromatid exchange, RI, replication index, AFB1, aflatoxin B1, TA, *Terminalia arjuna* extracts, S_9 mix, liver microsomal metabolic activation system

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28.00%, and 36.88% respectively at the highest dose (TA_4) for the three different durations viz., 24, 48 and 72 h. Similarly the number of sister chromatid exchanges got reduced from a higher level of 15.00 ± 1.40 per cell to 7.70 ± 0.50 per cell with S_9 mix at 48 h of treatment. The replication index was enhanced from 1.33 to 1.55 *in vitro*. Similar trends were noticed in the *in vivo* experiments i.e., effective reductions in clastogeny ranging from 15.22% to 54.82% from the mutagen treated positive control and the total frequencies in aberrant cells got reduced from 429 due to AFB1 to 141 due to 5th concentration of *Terminalia* extracts at 32 h of exposure.

Conclusion: The ameliorating potential of *Terminalia* extracts was dose and time dependant.

1. Introduction

The evaluation of the rich heritage of traditional medicine is an important task for pharmacologist. In this regard the *Terminalia arjuna* is one such plant, which is distributed throughout the Indian Peninsula and is abundantly found in Sub-Himalayan tract. In the Indian system of medicine, the bark of the plant is used for curing ulcers, leucorrhoea, diabetes, tumor, asthma and inflammation etc. [1].

It was earlier observed that tannins and flavonoids are responsible for their anticancer properties [2]. There may be some chemical agents present in plants that may act as anticarcinogen or antimutagen by blocking or trapping ultimate carcinogen electrophile in a nucleophilic chemical reaction to form innocuous products. It was shown that the bark of *T. arjuna* is rich in polyphenols (60–70%) including flavonoids, tannins and triterpenoids [3]. These constituents are mainly responsible for anticancer activity. According to Sumitra et al. [4] the constituents of *T. arjuna* such as arjunolic acid and ascorbic acid arrest a decrease in antioxidant system, alpha tocopherol reduced glutathione as well as lipid peroxide and protect the heart from damage caused due to myocardial necrosis which is induced by isoproterenol. In a study by Devi et al. [5] the gastro-protective properties of *T. arjuna* in Diclofenac sodium induced gastric ulcer in rats due to its scavenging action of free radical and cyto-protective nature. The *T. arjuna* preparation at 50 mg/kg of body weight of dose, significantly decreases the higher level of the antioxidants produced by carbon tetrachloride induced oxidative stress and increases the reduced glutathione content. It also decreases the lipid peroxidation and its products [6]. A triterpenoid saponin, arjunin isolated from the *arjuna* plant ameliorates arsenic induced cyto-toxicity in isolated murine hepatocytes via normalizing the altered enzymatic and non-enzymatic levels [7]. In another study, the anti-inflammatory and immunomodulatory activities of the bark in mice and rats were also reported [8]. The aim of the present investigation is to highlight the anticarcinogenic and antimutagenic potential of *T. arjuna* extracts *in vitro* and *in vivo*.

2. Materials and methods

2.1. Aflatoxins B_1

Aflatoxins are produced by *Aspergillus flavus* and *A. parasiticus* at any time during growth and post harvest storage of a

Table 1 *In vivo* effect of *Terminalia arjuna* extracts on the frequency of cells with chromosomal aberrations induced by Aflatoxin B1 (AFB1 x/kg.bw) at 16, 24 and 32 h durations.

Treatment	TA/kg.bw	Cell with pulverized chromosome	Types of chromatic aberrations				Aberrant cell no. (%)	Reduction (%)
			Gaps	Breaks	Fragments	Exchanges		
DDH_2O	00	00	05	04	27	00	31 (3.1)	
AFB_1	00	11	89	78	98	10	197 (19.7)	
TA	TA_5	00	03	06	28	00	34 (3.4	
AFB_1 + TA(16 h)	TA_1	08	60	66	86	07	167 (16.7)	15.22
	TA_2	07	56	57	80	06	150 (15.0)	23.85*
	TA_3	04	45	49	68	03	124 (12.4)	37.05*
	TA_4	03	41	49	60	03	115 (11.5)	41.62*
	TA_5	02	38	39	57	01	99 (9.9)	49.75*
AFB_1 + TA(24 h)	TA_1	07	60	63	81	06	157 (15.7)	20.30
	TA_2	05	56	54	76	05	140 (14.0)	28.93*
	TA_3	04	45	48	60	02	114 (11.4)	42.13*
	TA_4	03	41	46	54	02	105 (10.5)	46.70*
	TA_5	02	38	38	51	01	92 (9.2)	53.30*
AFB_1 + TA(32 h)	TA_1	07	60	60	83	05	155 (15.5)	21.32
	TA_2	06	56	57	78	04	145 (14.5)	26.40*
	TA_3	04	45	56	72	02	134 (13.4)	31.98*
	TA_4	01	41	48	60	01	110 (11.0)	44.16*
	TA_5	00	38	41	47	01	89 (8.9)	54.82*

Note: TA_1–TA_5; concentrations of *Terminalia arjuna* extracts, Aflatoxin B_1 5 μg/ml /kg body weight at at 16, 24 and 32 h of treatment. DH_2O; distilled water. Calculations were made excluding the gap type of aberration and *significant at <0.05 probability. TA/kg.bw is the concentration of *Terminalia arjuna* extracts.

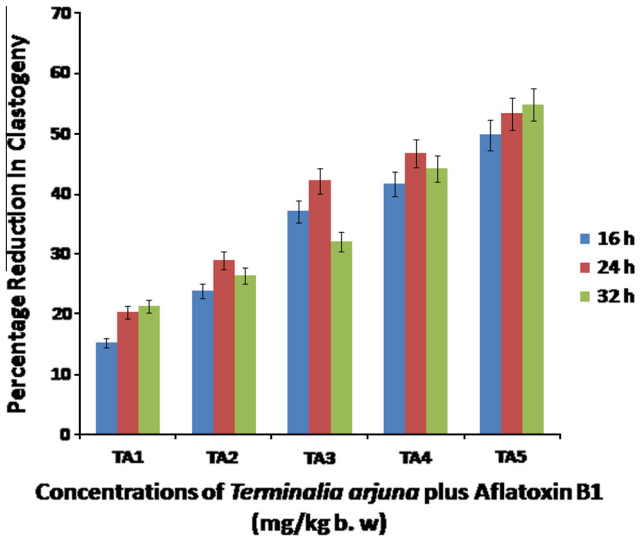

Figure 1 Showing *in vivo* anticarcinogenic effect of *Terminalia arjuna* at 16, 24, and 31 h of treatment durations against Aflatoxin B1 genotoxicity in Albino mice bone marrow cell (significant at $P < 0.05$ level).

number of foodstuffs and the levels of contamination are enhanced under poor food harvesting and storage practices [9,10] that lead to aflatoxin B_1 exposure to human. The major concern with respect to human health derives from the high potency of aflatoxins to produce cancer in laboratory animals and correlates with the evidence that AFB1 is a liver carcinogen in human populations [11–13].

2.2. Terminalia arjuna

The name *Terminalia* is derived from latin 'Teminalis' due to terminal crowding of the leaves in many species of the genus *Terminalia* [14]. It belongs to the family Combretaceae. It is a large deciduous tree with buttressed roots, and reaches up to 60–70 feet. Stem is covered with white–gray bark which changes its color to pink according to season and age of the bark, and flakes off in large flat pieces from the trunk [1].

2.3. Extract preparation

The sun dried bark of *T. arjuna* was brought to the laboratory and powdered and extracted in water (1:8, after passing through 80 mesh size) by boiling (4 h). The extracts were subsequently filtered through muslin cloth and the filtrate was spray dried. For preparation of water solution of *T. arjuna* extracts, dried powder (500 mg), was dissolved in distilled water and then centrifuged (500*g*, 15 min). The supernatants were transferred to micro-centrifuge tubes and stored (at −20 °C). The amount of total soluble solids in supernatant was measured using gravimetric analysis which served as the basis to formulate *T. arjuna* concentrations.

We have selected four optimum doses of *T. arjuna* suspension viz., 75, 100, 150 and 200 μg/ml for lymphocyte culture *in vitro* and five doses viz., 50, 100, 150, 250 and 350 mg/kg body weight for *in vivo* experiments.

2.4. In vivo study

Albino mice 8–10 weeks old (25–35 gm in weight) were exposed to mutagen and different concentrations of Terminalia

Table 2 *In vivo* effect of *Terminalia arjuna* extracts on the frequency of cells with chromosomal aberrations induced by Aflatoxin B1 (AFB1 x/kg.bw) at 16, 24 and 32 h durations.

Treatment	TA/kg.bw	Cell with aberration							Total number of aberration
		0	1	2	3	4	5	6–9	
DH_2O	00	969	27	04	00	00	00	00	35
AFB_1	00	803	103	32	25	16	12	09	429
TA	TA_5	966	28	06	00	00	00	00	40
AFB_1 + TA(16 h)	TA_1	833	105	25	19	12	09	07	357
	TA_2	850	97	19	15	08	06	05	279*
	TA_3	876	83	17	12	06	05	03	239*
	TA_4	885	83	13	09	06	03	01	183
	TA_5	901	75	09	07	05	03	00	149*
	TA_1	843	89	22	18	13	08	07	329
	TA_2	860	87	17	16	09	06	05	272*
AFB_1 + TA(24 h)	TA_3	886	75	14	12	06	04	03	205*
	TA_4	895	78	10	09	04	03	01	163*
	TA_5	908	68	9	07	05	03	00	142*
	TA_1	845	90	23	17	11	08	06	316
	TA_2	855	92	19	15	08	06	05	274*
AFB_1 + TA(32 h)	TA_3	866	91	17	12	06	05	03	233
	TA_4	890	79	12	09	06	03	01	177
	TA_5	911	64	09	08	05	03	00	141*

Note: TA_1–TA_5; concentrations of *Terminalia arjuna* extracts; AFB1, Aflatoxin B_1 5 μg/ml /kg body weight at16, 24 and 32 h of treatment. DH_2O; distilled water. Calculations were made excluding the gap type of aberration and *significant at <0.05 probability. TA/kg.bw is the concentration of *Terminalia arjuna* extracts. The animals were sacrificed at 16, 24 and 32 h after AFB1 treatment of 1000 cells from 10 animals was analyzed for each point.

Table 3 *In vitro* analysis of chromosomal aberration after treatment with Aflatoxin B_1 (AFB1) along with *Terminalia arjuna* extracts, 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	
AFB_1	24	200	26.12	23.00	17.15	6.34	23.49	0.23 ± 0.03
	48	200	44.00	40.17	29.47	13.00	42.47	0.42 ± 0.05
	72	200	47.27	42.00	38.12	21.53	59.65	0.60 ± 0.08
$AFB_1 + TA_1$	24	200	22.22	19.51	14.00	5.87	19.87	0.20 ± 0.04
	48	200	40.00	36.41	26.53	11.00	37.53	0.38 ± 0.06
	72	200	43.18	38.21	35.18	18.50	53.68	0.54 ± 0.09
$AFB_1 + TA_2$	24	200	21.32	18.32	12.00	5.47	17.47	0.17 ± 0.03
	48	200	38.73	34.00	21.13	10.13	31.26	0.31 ± 0.06
	72	200	41.00	36.67	34.39	15.25	49.64	0.50 ± 0.08
$AFB_1 + TA_3$	24	200	20.00	16.00	10.00	4.00	14.00	0.14 ± 0.04
	48	200	37.65	32.38	19.68	8.27	27.95	0.28 ± 0.05
	72	200	39.00	34.77	27.98	12.75	40.73	0.41 ± 0.09
$AFB_1 + TA_4$	24	200	19.12	15.12	08.22	4.10	12.32	0.12 ± 0.03
	48	200	37.00	31.89	20.00	08.00	28.00	0.28 ± 0.06
	72	200	38.19	33.61	25.88	11.00	36.88	0.37 ± 0.06
Control								
Normal	72	200	2.78	2.33	1.98	0.77	2.75	0.03 ± 0.01
DMSO + TA_2	72	200	3.88	1.56	2.58	1.00	3.58	0.04 ± 0.01

Note: TA_1–TA_4; concentrations of *Terminalia arjuna* extracts, AFB1 x/kg.bw; Aflatoxin B1 5 µg/ml/culture, gap type of aberration is not included, SE; Standard error, DMSO; dimethyl sulphoxide. Calculations were significant at <0.05 probability level.

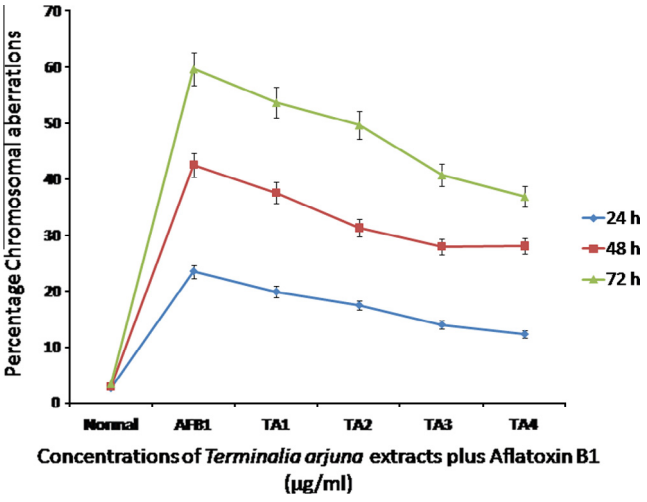

Figure 2 Comparative *in vitro* anticlastogenic effect of *Terminalia arjuna* in the absence of S_9 mixture at 24, 48 and 72 h of treatment durations (significant at $P < 0.05$ level).

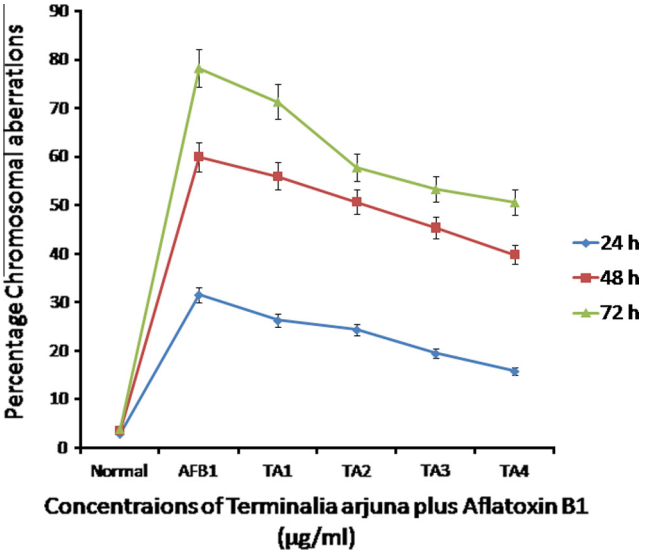

Figure 3 *In vitro* anticlastogenic effect of *Terminalia arjuna* in the presence of S_9 mixture at 24, 48 and 72 h of treatment durations (significant at $P < 0.05$ level).

extract preparation by appropriate routes (intra peritoneal i.e., I.P injection) and were sacrificed at sequential intervals of 16, 24, and 32 h of stipulated treatment time. Animals were treated with each test substance as mentioned above. Further processes of slide preparation, the cells and chromosomal aberration analyses are adopted from an earlier published work [15].

The reduction factors due to test chemical treatments were calculated using the formula published earlier [16]. The work was carried out following the guideline of institutional ethics committee and in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for humans and animals.

2.5. *In vitro* lymphocyte culture method

The chromosomal changes (numerical and structural) were utilized for the 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 and in the absence of exogenous metabolic activation system. The *in vitro* culture methods, preparation of S_9 (microsomal fraction), media preparation and analyses of

Table 4 *In vitro* analysis of sister chromatid exchanges (SCE) after treatment with Aflatoxin B_1 along with *Terminalia arjuna* extract, in the absence as well as presence of S_9 mix.

Treatment	Duration (h)	Metabolic activation	Metaphase scanned	Total SCE	Range	SCE/cell ± SE
Aflatoxin B_1	48	$-S_9$	50	610	2–12	12.20 ± 1.00
		$+S_9$		750		15.00 ± 1.40
AFB_1 + TA_1	48	$-S_9$	50	555	2 – 10	11.10 ± 1.00
		$+S_9$		640		12.80 ± 1.00
AFB_1 + TA_2	48	$-S_9$	50	490	1–08	9.80 ± 0.74
		$+S_9$		505		10.10 ± 1.00
AFB_1 + TA_3	48	$-S_9$	50	370	0 – 7	7.40 ± 0.50
		$+S_9$		455		9.10 ± 0.70
AFB_1 + TA_4	48	$-S_9$	50	318	0 – 6	6.36 ± 0.44
		$+S_9$		385		7.70 ± 0.50
Normal	48	$+S_9$	50	75	0 – 6	1.50 ± 0.20
DMSO	48	$+S_9$	50	66	0 – 7	1.32 ± 0.20
DMSO + TA_2	48	$+S_9$	50	72	0–6	1.44 ± 0.20

Note: TA_1–TA_4; concentrations of *Terminalia arjuna*, AFB1 x/kg.bw; Aflatoxin B1 5 μg/ml/culture, SE; Standard error, DMSO; dimethyl sulphoxide. Calculations were significant at <0.05 probability level.

chromosomal aberrations, sister chromatid exchanges, cell cycle kinetics and statistical analysis were followed as per the methodology published earlier [15,16].

3. Results

3.1. *In vivo* effect of *Terminalia arjuna* extracts

After 16 h of treatments the percentages of aberrant cells obtained were 16.7%, 15.0%, 12.4%, 11.5% and 9.9% respectively at the five increasing concentrations of *T. arjuna* extracts as compared to 19.7% of aberrant cells induced by Aflatoxin B_1 alone. Fragment types of aberrations were most prominent followed by breaks and gaps. In terms of percentage reduction in the frequencies of aberrant cells, the values were 15.22%, 23.85%, 37.055%, 41.62% and 49.75% due to five different concentrations of *T. arjuna* extracts given with AFB_1 as compared with positive control respectively (Table 1 Fig. 1).

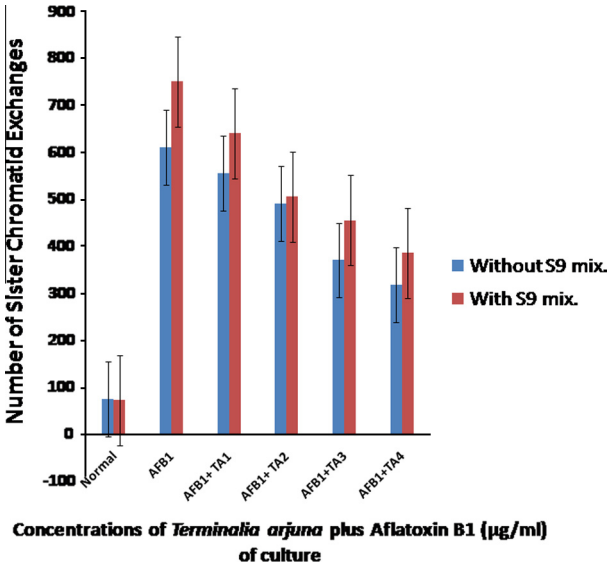

Figure 4 Antigenotoxic effect of *Terminalia arjuna* on sister chromatid exchanges in the absence as well as in the presence of metabolic activation system. Total 50 metaphase plates were scored at 48 h of treatment durations (significant at $P < 0.05$ level).

The gross effects on the total frequencies of aberrations per thousand cells were 357, 279, 239, 183 and 149 due to five different doses of *Terminalia* extracts along with AFB_1, against 429 aberrations of Aflatoxin B_1 when given alone. The normal control value was 40 for only *Terminalia* extract treatment without AFB_1 added. Most of the cells have one or two aberrations per cell. When the treatment durations were increased to 24 h, the effects followed similar trends, but with increasing values. The observed values were 15.7%, 14.0%, 11.4%, 10.5% and 9.2% respectively at five concentrations of *Terminalia* extracts given with AFB_1 against only 19.7% aberrant cells for Aflatoxin B_1 alone, whereas normal value was 3.4 for *Terminalia* extracts as shown in Fig. 1.

The effect of *Terminalia* extracts in reducing the frequency of aberrations per cell and the total number of aberrations were statistically significant at <0.05 level. The total aberrations per thousand cells were 329, 272, 205, 163 and 142 for *Terminalia* extracts given with AFB_1 against 429 with Aflatoxin B_1 alone. Further, the animals were exposed to 32 h; the increase in aberrant cells observed was 15.5%, 14.5%, 13.4%, 11.0% and 8.9% for five increasing doses of *Terminalia* extracts against the normal control value which was 3.1%. The effects on reduction in aberrant cells were 21.32%, 26.40%, 31.98%, 44.16% and 54.82% respectively, which were statistically significant. It shows almost a dose-dependent relationship as shown in Tables 1 and 2 and Fig. 1.

3.2. *In vitro* effect of *Terminalia arjuna* extracts

The role of *T. arjuna* extracts in reducing metaphase aberrations due to Aflatoxin B_1 is quite significant, varying from untreated positive control values (23.49%, 42.47%, and 59.65%) to treated ones (12.32%, 28.00%, and 36.88%) at the highest dose (E_4) for the three different durations viz., 24, 48 and 72 h. There was no change in basal clastogeny of the cell (2.75% and 3.58%) by *T. arjuna* extracts. The effects showed

Table 5 *In vitro* analysis of cell cycle kinetics after treatment with Aflatoxin B_1 along with *Terminalia arjuna* extract, in the presence of S_9 mix.

Treatment	Cell scored	Metabolic activation	Percent aberration metaphase			Replication index	2*3 chi square test
			M1	M2	M3		
Aflatoxin B_1	200	$-S_9$	64	33	03	1.37	
		$+S_9$	69	29	02	1.33	
$AFB_1 + TA_1$	200	$-S_9$	60	35	05	1.45	Significant
		$+S_9$	65	32	03	1.38	
$AFB_1 + TA_2$	200	$-S_9$	56	37	07	1.51	Significant
		$+S_9$	62	33	05	1.41	
$AFB_1 + TA_3$	200	$-S_9$	50	41	09	1.58	Significant
		$+S_9$	59	35	06	1.47	
$AFB_1 + TA_4$	200	$-S_9$	45	45	10	1.65	Significant
		$+S_9$	53	39	08	1.55	
Normal	200	$-S_9$	39	47	14	1.75	
DMSO	200	$+S_9$	39	46	15	1.76	
DMSO + TA_2	200	$+S_9$	38	47	15	1.77	

Note: 2×3 Chi square (χ^2) test was conducted, TA_1–TA_4; concentrations of *Terminalia arjuna*, AFB1 x/kg.bw; Aflatoxin B_1 5 μg/ml/culture, DMSO; dimethyl sulphoxide. Calculations were made at < 0.05 probability level.

a linear dose–response relationship. The effective maximum reductions in the clastogeny were 47.55%, 34.07% and 38.17% percent at three different durations respectively as shown in Table 3 Fig. 2. Similarly in the presence of S_9 metabolic activation system, the same values were reduced to 49.93%, 33.67% and 35.34% (Fig. 3).

The effect on sister chromatid exchange counts was similarly reduced; however the experiments were conducted only for 48 h for all treatments including control. The 50 metaphases were scored for each treatment along with S_9 treatment as shown in Table 4 Fig. 4. The aflatoxin B_1 produced 12.20 per cell and 15.0 per cell SCE in the absence as well as in the presence of S_9 mix respectively. These values were reduced to 06.36 SCE per cell and 07.70 SCE per cell due to the highest concentration of *Terminalia* extracts as compared with aflatoxin B1 values.

The replication index calculated showed a significant elevation of R.I in comparison to aflatoxin B_1treatment. Here, the dose–effect relationship was linear as shown in Table 5.

4. Discussion

Due to global environmental pollution and modern life style, there has been an increase in the rate of mutations leading to cancer. The ways to neutralize the effect of such mutagenic and carcinogenic agents is to identify the substances that can antagonize their effects. Plants are the promising sources of antimutagens found in them as secondary metabolites [17]. In earlier studies, we have shown the antimutagenic and anticarcinogenic potential of vitamins, carotenoids and extracts of *Caesalpinia bonducella* and *Agaricus bisporus* [15,16,18,19]. These antimutagenic agents may help in strengthening the cell defense mechanism against environmental carcinogens. It has been suggested earlier that halving the rate of mutations would delay the onset of most cancers and might be adequate in the lifetime of many individuals [20]. *T. arjuna* is a well known medicinal plant, particularly its bark is extensively used in ayurvedic medicines. Keeping in mind the medicinal importance of *T. arjuna*, the present study aimed at observing the anticarcinogenic and antimutagenic potential of this medicinal plant with the potential to combat a number of mutagens and carcinogens.

Tannins and flavones in the leaves, barks and stems of *T. arjuna* were reported to be responsible mainly for anticancer activity [21]. Antimutagenic assay of ethyl ether extracts of *arjuna* bark carried out by using the 'comet' assay and micronuclei test revealed that extracts are effective in reducing the DNA damage induced by 4NQO [22]. Increased levels of plasma and the liver glycolytic enzymes and decreased level of glucose- 6-phosphatase were reverted to normal by depleting the energy metabolism and inhibiting the cancer growth accounting for its anticancer potential [23]. Luteolin, a flavone isolated from the butanol fraction of *T. arjuna* was found to be effective in inhibiting a series of solid tumors (Renal A-549, ovary SK-OV-3, Brain SF-295, etc.). It also acted as an antitumor promoter and had antimutagenic properties [24]. It was also reported the efficacy of *T. arjuna* in inhibiting the proliferation of the human hepatoma cell lines (HepG2) as well as a potent inhibitor of CYP isoform that prevents the conversion of cyclophosphamide or aflatoxin B_1 to its genotoxic metabolite [25,26]. Its antimutagenic effect may be due to the direct protection of DNA from electrophilic mutagens or their metabolites or by formation of adducts that may result in the prevention of genotoxic damage [27]. The effect of bark extract of *T. arjuna* was studied on the alteration of adriamycin (ADR)-induced micronuclei formation in cultured human peripheral blood lymphocytes. These results demonstrate that extract of *T. arjuna* protects DNA against ADR-induced damage [28] that was parallel to our finding *in vitro* using replication index parameter.

5. Conclusion

The role of *T. arjuna* extracts in reducing metaphase aberrations due to aflatoxin B_1 is quite significant, the reduction varying from 23.49%, 42.47%, and 59.65% down to 12.32%, 28.00%, and 36.88% respectively at the highest dose (E_4) for the three different durations viz., 24, 48 and 72 h. Similarly the number of sister chromatid exchanges got reduced from higher level of 15.00 ± 1.40 per cell to 7.70 ± 0.50 per

cell with S_9 mix at 48 h of treatment. The replication index was enhanced from 1.33 to 1.55 *in vitro*. Similar trends were noticed in the *in vivo* experiments i.e., effective reductions in clastogeny ranging from 15.22% to 54.82% from the mutagen treated positive control and the total frequencies in aberrant cells got reduced from 429 due to AFB1 to 141 due to 5th concentration of *Terminalia* extracts at 32 h of exposure. The ameliorating potential of *Terminalia* extracts was dose and time dependant.

Conflict of interest

All authors declare that there is no conflict of interest as regards financial and personal relationships with other people or organizations that inappropriately influence the work.

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