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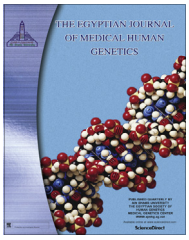

ORIGINAL ARTICLE

Andrographia paniculata a Miracle Herbs for cancer treatment: *In vivo* and *in vitro* studies against Aflatoxin B1 Toxicity

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KEYWORDS

Andrographia paniculata;
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Abstract *Background:* The history of natural products used in ancient times and in folk medicine these days, around the world, is the basis for the use of many therapeutic drugs in modern day medicine. *Andrographia paniculata* belongs to the family Acanthaceae or Kalmegh and is commonly known as 'king of bitters'. It is extensively used as home remedy for various diseases in Indian traditional system as well as in tribal system in India for multiple clinical applications.

Aim: In our present work, extracts of these ayurvedic plants were tested for their anticlastogenic, antimutagenic and anticarcinogenic properties against Aflatoxin B1 induced toxicity.

Materials and methods: We used the *in vitro* method i.e. human lymphocytes culture and *in vivo* method in bone marrow cells of albino mice, while 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 for *in vitro* studies, whereas total aberrant cells and the frequencies of aberrations were used for *in vivo* methods.

Abbreviations: CA, chromosomal aberrations; SCE, sister chromatid exchanges; RI, replication index; AFB1, Aflatoxin B1; AE, extracts of *Andrographia paniculata*.

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Results: *A. paniculata* extracts significantly reduced chromosomal aberrations from 35.0%, 62.0% and 69.0% level [at 24, 48, and 72 h due to Aflatoxin B1] to 21.72%, 44.0% and 52.0%, similarly sister chromatid exchanges were reduced from 14.60 per cell to 7.50 per cell at 48 h of treatments and replication index was enhanced *in vitro* for each concentration and duration of treatment.

Conclusion: In conclusion *A. paniculata* extracts significantly reduced the number of aberrant cells and frequencies of aberration per cell at each concentration and duration of exposure *in vivo*; similarly it reduced chromosomal aberrations and sister chromatid exchanges and replication index was enhanced *in vitro* that was statistically significant at <0.05 level.

1. Introduction

The history of natural product used in ancient times and in folk medicine around the world, is the basis for the use of many therapeutic drugs in modern day medicine. Traditionally, natural plant products have been the source in the search for new drugs by pharmaceutical companies [1]. Earlier we have worked on certain bioactivators like vitamins, carotenoids, flavonoids, ellagic acid and extracts of *Caesalpinia bonducella*, *Agaricus bisporus*, *Terminalia arjuna* and their antimutagenic and anticarcinogenic potentials were noticed, using *in vitro* and *in vivo* methods [2–6].

Andrographia paniculata belongs to the family Acanthaceae or Kalmegh and is commonly known as 'king of bitters'. It is widely distributed throughout tropical Asian countries often in isolated patches. Native populations of plants are spread throughout the south India and Sri Lanka which perhaps represent the center of origin and diversity of the species. It is extensively used in Ayurveda, Unani and Siddha medicines as home remedy for various diseases in India. The therapeutic value of kalmegh is due to its mechanism of action by enzyme induction. It is used to treat gastro intestinal tract, upper respiratory infections, fever, herpes, sore throat, hepatitis and a variety of other chronic and infectious diseases [7]. Therapeutically important active principal of kalmegh found in aerial part is Andrographolide ($C_{20}H_{30}O_5$, mp 230–239 °C). It is colorless, crystalline bitter in taste and known as diterpene lactone [8].

Sheeja et al. [9] reported that administration of methanolic extract of kalmegh produced complete inhibition of carrageenan induced inflammation compared with the control. Verma and Vinayak [10] studied the effect of the aqueous extract of *A. paniculata* on antioxidant defense system in lymphoma bearing AKR (an ecotropic N-tropic murine leukemia virus) mice in liver. Oral administration of the aqueous extract of plant in different doses caused a significant elevation of catalase, superoxide dismutase and glutathione-s-transferase activities. Sheeja et al. [9] explored the antioxidant and anti-inflammatory properties in methanolic extract of the plant and found it to inhibit the formation of oxygen derived free radicals such as superoxide (32%), hydroxyl radicals (80%), lipid peroxidation (80%) and nitricoxide (42.8%) *in vitro* system. *In vivo* studies using BALB/c mice models showed significant inhibition in phorbol-12-myristate-13-acetate (PMA) induced superoxide (32.4%) and nitric oxide (65.3%) formation. Tripathi and Kamat [11] examined aqueous extract for antioxidant activity using rat liver subcellular organelles as model systems and found that the extract shows potent antiradical agent against various pathophysiological oxidants. The isolated fractions effectively inhibited the toxic effect of snake venoms *in vitro* than *in vivo* [12].

Andrographolide inhibited LPS (lipopolysaccharide)-induced increase in tumor necrosis factor-alpha (TNF-α) and granulocyte–macrophage colony stimulating factor [13]. Neoandrographolide also inhibits PGE2 (Prostaglandin E2) synthesis and TNF-α in LPS-stimulated macrophages and its oral administration to mice significantly suppresses dimethyl benzene-induced ear edema and acetic acid-induced vascular permeability [14]. A refined extract of *A. paniculata*, also significantly reduces activities of lipid peroxide, while the activities of nitric oxide, cyclic guanosine monophosphate (cGMP) and superoxide dismutase are significantly enhanced in experimental atherosclerotic rabbits [15]. The aim of present investigation is to highlight the anticarcinogenic, antimutagenic, and anticlastogenic potential of extracts of *A. paniculata* in the *in vitro* and *in vivo* model.

2. Materials and methods

2.1. Materials

The whole plant was powdered into a mixture and the hydro-alcoholic extract was prepared by refluxing with the double distilled water (DDH_2O) and alcohol (3:1) in a round bottom flask for 48 h at 60 °C. The liquid extract was filtered, cooled and concentrated by evaporating its liquid contents in oven and collected. The powdered extract, termed *Andrographia* extract (AE), was re-dissolved in DDH_2O and the required doses for treatment were prepared and five concentrations of 50, 75, 100, 150 and 200 mg/kg body weight were selected for *in vivo* experiments and four concentrations of 50, 100, 200 and 250 mg/l of culture for *in vitro* experiments.

2.2. Aflatoxins B_1

Aflatoxins are produced by *Aspergillus flavus* and *A. parasiticus* at any time during growth and post harvest storage of a number of foodstuffs and the levels of contamination are enhanced under poor food harvesting and storage practices [6,16] that lead to aflatoxin B1 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 [17–19].

2.3. In vivo study

Albino mice were cultured in animal house following Codes and Commandments of Institutional Ethics Committee regarding caring, handling and use of animals in the Research and the work is carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). Albino mice 8–10 weeks old were exposed to different test chemicals by intra peritoneal 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 preparations, cells and chromosomal aberrations analyses are adopted from earlier published work [4,5]. The reduction factors due to test chemical treatments were calculated using the formula published earlier [5,6].

2.4. In vitro lymphocytes culture method

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 chromosomal aberrations, sister chromatid exchanges, cell cycle kinetics and statistical analysis were followed as per the methodology published earlier [4–6].

3. Results

3.1. In vivo effects

In these *in vivo* experiments we have selected five most optimum concentrations of aqueous extract of *A. paniculata* and their effect on chromosomal aberrations given at three different durations were calculated and presented in the tables.

As shown in Table 1 for 16 h of treatment duration, the percentage of aberrant cells were 14.8% due to treatment with Aflatoxin B1 (AFB1) which were reduced to 12.5%, 10.6%, 8.9%, 7.7% and 6.8%, respectively for five consecutive concentrations of *Andrographia* extract. Similarly reduction in the frequencies of clastogenic cells put the values at 15.54%, 28.37%, 39.86%, 47.97% and 54.05% against five different concentrations of *Andrographia* extract, respectively. The trend of effects was linear i.e., as concentration of *Andrographia* extract increases the effect also increases (Fig. 1). The gross effect on the total number of frequencies per thousand cells was 231, 176, 137, 117 and 94 aberrations due to five increasing concentrations of *Andrographia* extract along with aflatoxin B1 against 311 aberrations of AFB1 alone. The normal values were 21 for distilled water treatment (Table 2).

When the treatment duration was increased to 24 h, the trends of effect were similar, with increasing values. The observed values were 11.7%, 10.4%, 8.8%, 7.7% and 7.1% due to five increasing concentrations of *Andrographia* extract, against 14.0% of AFB1 alone. The normal values were 1.9% and 2.2% for pure water and *Andrographia* extract alone, respectively. It also shows the dose–response relationship (Fig. 1). Effect of *Andrographia* extract on the frequency of aberrations per cell and total aberrations noticed were much significant statistically. The total aberrations per thousand cells were 196, 166, 134, 110 and 92 for *Andrographia* extract against the value of 242 with AFB1 alone.

At 32 h of treatment, we observed the percentage of aberrant cells as 15.3% for AFB1 alone, and 12.2%, 9.9%, 8.7%, 7.7% and 6.8%, respectively for five different concentrations of *Andrographia* extract given with AFB1, whereas the value for the normal control was 1.9%, and the same for *Andrographia* extract alone was 1.8%. In terms of the effects on the percent reduction in aberrant cells, the values were 20.26%, 35.29%, 43.13%, 49.67% and 55.56%, respectively. These values were statistically significant at <0.05 probability level due to *Andrographia* extract on the number and percentage of aberrant cells. It also showed almost dose dependent relationship, though the highest doses were much effective in comparison with preceding dose. More of chromosomal exchange types of aberrations were seen in contrast to the previous two durations of treatment (Fig. 1). The total frequencies per thousand cells were 324 for AFB1 only and 238, 157, 135, 114, and 87 aberrations, respectively for *Andrographia* extract along with AFB1.

Table 1 *In vivo* effects of *Andrographia paniculata* on the frequency of cells with chromosome aberrations induced by aflatoxin B1 (AFB1 x/kg.bw) at 16 h durations.

Treatment	AE (Y/kg.bw)	Cell with pulverized chromosome	Types of chromatic aberrations				Aberrant cell no. (%)	(%) Reduction
			Gaps	Breaks	Fragments	Exchanges		
DDH_2O	00	00	04	03	18	00	21 (2.1)	
AFB_1	00	12	35	53	80	04	148 (14.8)	
AE_5	AE_5	00	05	02	16	00	18 (1.8)	
AFB_1 + AE	AE_1	08	27	45	70	02	125 (12.5)	15.54*
	AE_2	05	20	38	62	01	106 (10.6)	28.37
	AE_3	03	22	32	54	00	89 (8.9)	39.86*
	AE_4	01	16	26	50	00	77 (7.7)	47.97*
	AE_5	00	05	21	47	00	68 (6.8)	54.05*

Note: AE_1-AE_5; concentrations of *Andrographia paniculata* extracts, DDH_2O; Double Distilled water, AFB1; Aflatoxin B_1 5 µg/ml /kg body weight at 16 h of treatment. Calculations were made excluding the gaps type of aberration.

* Significant at <0.05 probability. Y/kg.bw is the concentration of extracts of *Andrographia paniculata*.

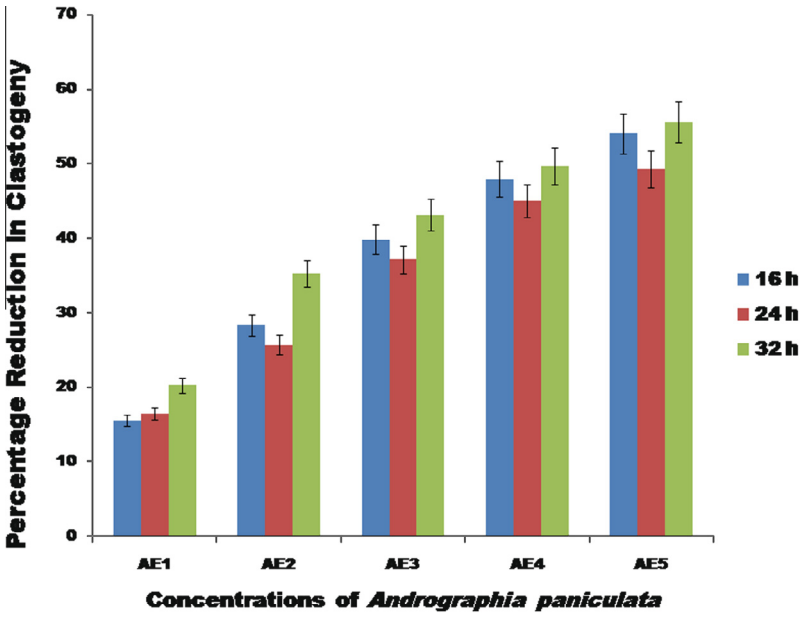

Figure 1 *In vivo* anticarcinogenic effect of *Andrographia paniculata* extracts at 16, 24, and 31 h of treatment durations against AFB1 genotoxicity in Albino mice bone marrow cell.

Table 2 *In vivo* effects of *Andrographia paniculata* extract on the frequency of cells with chromosomal aberrations induced by aflatoxin B1 (AFB1 x/kg.bw) at 16 h durations.

Treatment	AE/kg.bw	Cell with aberration							Total number of aberration
		0	1	2	3	4	5	6–9	
DDH_2O	00	979	17	04	00	00	00	00	25
AFB_1	00	852	81	25	14	12	11	05	311
AE	AE_5	982	16	02	00	00	00	00	20
AFB_1 + AE	AE_1	875	79	18	10	09	07	02	231*
	AE_2	894	73	14	07	06	06	00	176*
	AE_3	911	62	16	05	02	04	00	137*
	AE_4	923	55	13	03	03	03	00	117*
	AE_5	932	50	14	01	02	01	00	94*

Note: AE_1-AE_5; concentrations of *Andrographia paniculata* extracts, DDH_2O; Double Distilled water, AFB1; Aflatoxin B_1 5 μg/ml /kg body weight at 16 h of treatment. Calculations were made excluding the gaps type of aberration.
* Significant at <0.05 probability. Y/kg.bw is the concentration of *Andrographia paniculata* extracts. The animals were sacrificed 16 h after AFB1 treatment 1000 cells from 10 animals were analyzed for each point.

3.2. *In vitro effects*

Here the culture treated with Aflatoxin B1 (AFB1) resulted in clastogenic abnormalities as observed in percent metaphase aberrations, types of aberrations and aberrations per cell (35.07%, 72.57% and 83.75% or 0.35, 0.73 and 0.84 aberrations per cell) at single standard dose and three durations viz., 24, 48 and 72 h, respectively whereas, with control, the normal and DMSO plus *Andrographia* extract, these values were (0.05, 0.05) per cell. *Andrographia* extract bring down aberrations from 35.0% to 29.0%, 26.62%, 24.0% and 21.72% with four consecutive doses of the extract at 24 h of duration, whereas at 48 h, it gets lowered from 62.0% to 55.92%, 49.0%, 47.00%, and 44.0% by administration of 1st to 4th concentrations of *Andrographia* extract, respectively. The same trends were noticed, when the treatment durations were increased to 72 h. These values showed linear increasing trends with doses, but it does not depend on durations. The maximum percentage reductions in the aberrations were 37.94% for 24 h and 29.03% and 24.63%, respectively for 48 h and 72 h that were caused by highest doses of *Andrographia* extract (Table 3 Fig. 2).

Similarly when the experiment was setup along with metabolic activation system (+S_9 mix) the effect of AFB1 got increased. The effect of *Andrographia* extract also showed similar trend; they lower the clastogenic activity of AFB1. These values showed linearly increased with doses (Fig. 3). The maximum effective percentage reductions were 42.60%, 26.08%, and 23.14% for 24, 48 and 72 h, respectively.

When sister chromatid exchanges were counted (Table 4, Fig. 4) the reduction was evident both in the absence as well as in the presence of metabolic activation; thereby lowering

Table 3 *In vitro* analysis of chromosomal aberration after treatment with aflatoxin B1 (AFB1) along with *Andrographia paniculata* extract, in the absence of S_9 mix.

Treatments	Durations (h)	Metaphase scored	Percent aberration metaphase		Types of aberration (%)			Aberratio/cell ± SE
			Including gap	Excluding gap	Chromatid	Chromosome	Total	
AFB_1								
	24	200	24.00	21.50	24.00	11.00	35.00	0.35 ± 0.04
	48	200	38.00	34.00	42.00	20.00	62.00	0.62 ± 0.08
	72	200	41.00	36.00	45.00	24.00	69.00	0.69 ± 0.09
$AFB_1 + AE_1$	24	200	22.98	20.50	22.00	7.00	29.00	0.29 ± 0.04
	48	200	34.00	31.00	38.00	17.92	55.92	0.56 ± 0.06
	72	200	39.00	34.57	42.00	22.67	64.67	0.65 ± 0.09
$AFB_1 + AE_2$	24	200	21.77	18.54	19.67	6.95	26.62	0.27 ± 0.03
	48	200	32.00	29.00	34.00	15.00	49.00	0.49 ± 0.06
	72	200	37.88	32.90	39.00	20.00	59.00	0.59 ± 0.08
$AFB_1 + AE_3$	24	200	19.00	16.00	18.00	6.00	24.00	0.24 ± 0.04
	48	200	30.54	27.84	32.50	14.60	47.10	0.47 ± 0.05
	72	200	34.00	30.00	37.00	18.62	55.62	0.56 ± 0.09
$AFB_1 + AE_4$	24	200	17.92	15.00	16.72	5.00	21.72	0.22 ± 0.03
	48	200	29.85	26.00	31.00	13.00	44.00	0.44 ± 0.06
	72	200	32.00	29.55	35.00	17.00	52.00	0.52 ± 0.06
Control								
DDH_2O	72	200	2.75	2.00	1.98	0.96	2.94	0.03 ± 0.01
AE_2	72	200	3.85	1.56	2.88	1.00	3.88	0.04 ± 0.01

Note: AE_1–AE_4; concentrations of *Andrographia paniculata* extracts, DDH_2O; Double Distilled water, AFB1; Aflatoxin B1 5 µg/ml/culture, gaps type of aberration is not included, SE; Standard error, Calculations were significant at <0.05 probability level.

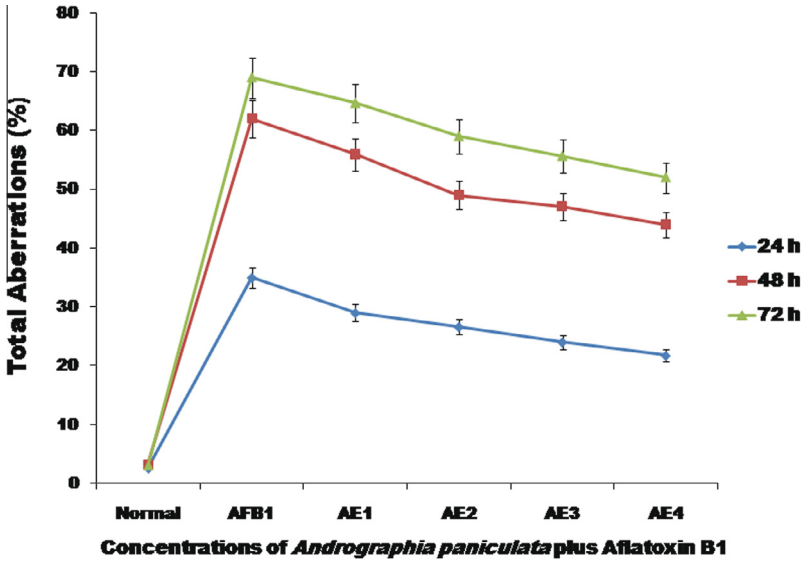

Figure 2 Comparative *in vitro* anticlastogenic effect of *Andrographia paniculata* extracts in the absence of S_9 mixture at 24, 48 and 72 h of treatment duration.

of the mean range and the total SCEs and SCE per cell from 13.40 to 06.80 and in the presence of S_9 from 14.60 to 07.50. For the analysis of SCE, only 48 h of cultures were used and 50 metaphases were scanned.

The effects of *Andrographia* extract on replication index (Table 5) showed an elevated level when compared with the AFB1 treatment alone i.e., from 1.26 to 1.52, though still being lower than the normal level of 1.76. The effect, after treatment with metabolic activation system, shows the level from 1.35 to 1.51, i.e., again being much effective in comparison to that without metabolic activation system.

4. Discussion

Cancer results when cells do not respond to signals that are intended to limit growth. If a cancer cell can be made to mature

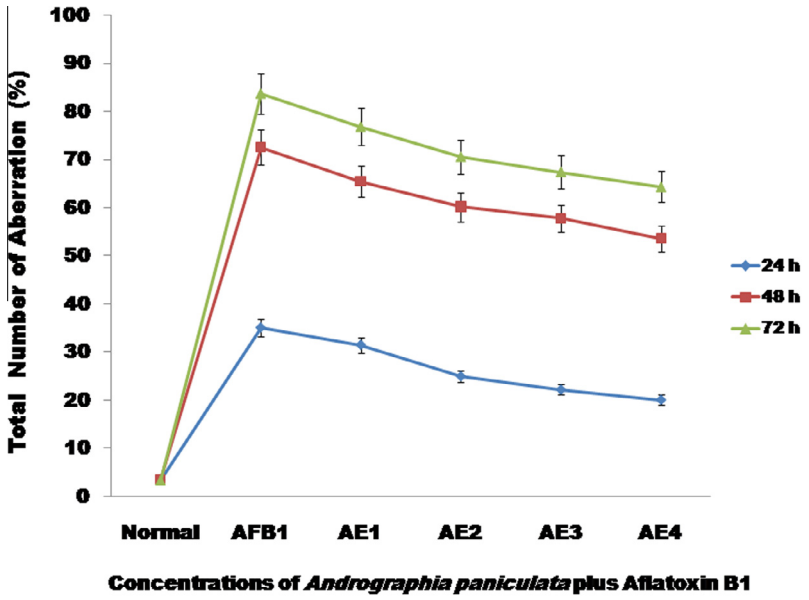

Figure 3 *In vitro* anticlastogenic effect of *Andrographia paniculata* extracts in the presence of S_9 mixture at 24, 48 and 72 h of treatment duration.

Table 4 *In vitro* analysis of sister chromatid exchanges (SCE) after treatment with aflatoxin B1 along with *Andrographia paniculata* extract, in the presence of S_9 mix.

Treatment	Duration (h)	Metaphase scored	Total	Range	SCE/cell ± SE
Aflatoxin B_1	48	50	730	3–12	14.60 ± 1.00
AF B_1 + AE_1	48	50	580	3–12	11.60 ± 1.00
AF B_1 + AE_2	48	50	490	2–12	09.80 ± 0.70
AF B_1 + AE_3	48	50	410	1–11	08.20 ± 0.50
AF B_1 AE_4	48	50	375	1–11	07.50 ± 0.50
Control					
DDH_2O	48	50	240	0–6	04.80 ± 0.40
AE_3	48	50	238	0–6	04.76 ± 0.40

Note: AE_1–AE_4; concentrations of *Andrographia paniculata* extracts, DDH_2O; Double Distilled water, AFB1; Aflatoxin B1 5 μg/ml/culture, SCE; sister chromatid exchange, SE; Standard error, Calculations were significant at <0.05 probability level.

(or differentiate), it will not have the ability to grow out of control. *A. paniculata* was chosen because it contained substances (terpenes) that were known to cause differentiation of cancer cells. The results of the previous study were demonstrated that *A. paniculata* had potential cell differentiation-inducing activity on leukemia cells [20].

The extracts have shown a broad-range anti-proliferative activity on a variety of cancer cell lines including breast cancer, colon cancer, hepatoma, cervical cancer, leukemia and prostate cancer [21,22]. Among various breast cancer cell lines, the MCF-7 cell lines were found to be most sensitive. As the colorectal and colon cancer cells are observed to be more sensitive toward andrographolide treatment.

A. paniculata possessed anticancer activity and the inhibition of the proliferation of colon cancer cells and augmentation of the proliferation of human peripheral blood lymphocytes at low concentration were revealed by the dichloromethane crude extract of the medicinal plant [23]. The anticancer activity was observed due to the presence of andrographolide which demonstrated their direct anticancer activity at G0/G1 phase of the cell cycle through induction of cell cycle inhibitory protein p^{27}. The expression of cyclin dependent kinase 4 (CDK4) was decreased and the production of tumor necrosis factor -α was increased that was contributed to cytotoxicity of lymphocytes against cancer cells [24]. The mechanism involved in the inhibition of tumor growth was, to stimulate the cytotoxic T lymphocyte production through increased secretion of interlukin-2 and interferon–γ [25]. Another study in the *in vivo* demonstrated that the 70% ethanol extract of *A. paniculata* and andrographlide increased the life spans of mice injected with thymoma cells [26].

Andrographolide induces apoptosis in human cancer cells via the activation of caspase 8, pro-apoptotic Bcl-2 family members Bax conformational change, release of cytochrome C from mitochondria and activation of caspase cascade [27] and also through the activation of tumor suppressor p53 by ROS-dependent c-Jun NH_2-terminal kinase (JNK) activation,

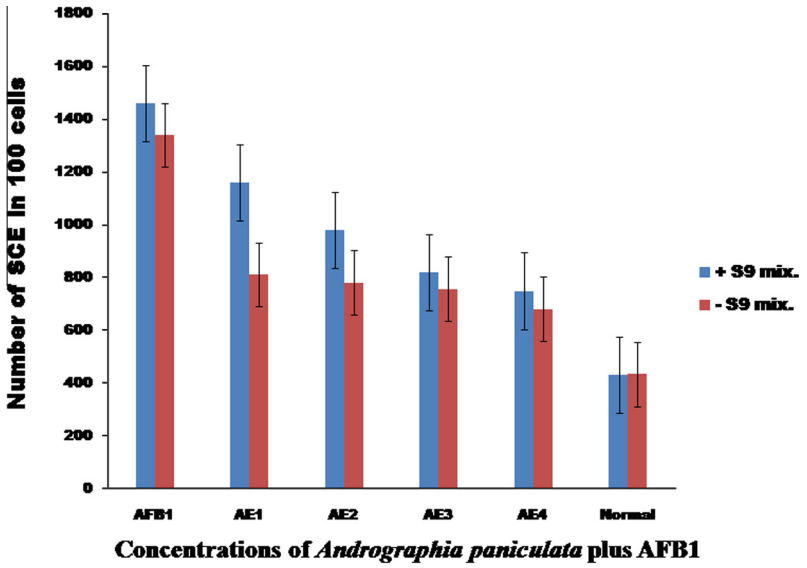

Figure 4 Antigenotoxic effect of *Andrographia paniculata* extracts on sister chromatid exchanges in the absence as well as in the presence of metabolic activation system.

Table 5 *In vitro* analysis of cell cycle kinetics after treatment with aflatoxin B1 along with *Andrographia paniculata* extract, in the presence of S_9 mix.

Treatment	Cell scored	Percentage (%) cell in			Replication index	2×3 Chi square test
		M_1	M_2	M_3		
Aflatoxin B_1	200	75	24	01	1.26	
$AFB_1 + AE_1$	200	70	26	04	1.34	
$AFB_1 + AE_2$	200	66	28	06	1.40	Significant
$AFB_1 + AE_3$	200	62	31	07	1.45	Significant
$AFB_1 + AE_4$	200	58	32	10	1.52	Significant
Control						
DDH_2O	200	37	52	11	1.74	
AE_3	200	38	50	12	1.74	

Note: 2×3 Chi square (χ^2) test was conducted, AE_1–AE_4; concentrations of *Andrographia paniculata* extracts, DDH_2O; Double Distilled water, AFB1; Aflatoxin B_1 5 μg/ml/culture. Calculations were made at < 0.05 probability level.

thereby increasing p53 phosphorylation and protein stabilization [28,29]. Andrographolide may suppress an oncogene v-Src-induced transformation and down-regulate v-src protein expression via the attenuation of ERK1/2 signaling pathway [30].

Andrographolide inhibits the adhesion of cancer cells to the activated endothelium by blocking E-selectin expression [31]. Andrographolide may also inhibit angiogenesis for tumor metastasis via down-regulating matrix metalloproteinases-7 (MMP-7) expression, possibly by inactivating activator protein-1 (AP-1) through suppressing PI3K/Akt signaling pathway [32,33]. Chao et al. [34] demonstrated that andrographolide at nontoxic to subtoxic concentrations (0.3–3 μM) suppressed the invasion ability of CT26 cells in Matrigel-based invasion assays.

Therapeutic efficacy of a drug is reflected by its bioavailability, when taken in the form of an extract, andrographolide is readily absorbed in blood and maximum plasma concentrations were reached after 1.5–2 h of oral administration [35]. It is suggested that P-glycoprotein participates in the intestinal absorption of andrographolide [36]. The available information on the metabolism of the compound reflects that metabolic fate of andrographolide in humans after oral administration might involve a sulfonate reaction at C-12 [37].

5. Conclusion

A. paniculata extracts significantly reduced the number of aberrant cells and frequencies of aberration per cell at each concentration and duration of exposure *in vivo*; similarly it reduced chromosomal aberrations and sister chromatid exchanges and replication index was enhanced *in vitro* that was statistically significant at < 0.05 level.

Conflict of interest

The authors declare that there is no conflict of interest regarding any financial and personal relationships with other people or organization.

Acknowledgment

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