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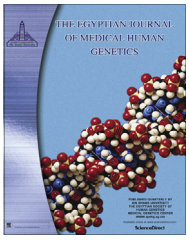

ORIGINAL ARTICLE

Cancer ameliorating potential of *Phyllanthus amarus*: *In vivo* and *in vitro* studies against Aflatoxin B1 toxicity

Md. Sultan Ahmad *, **Sultana Bano, Shafaat Anwar**

Department of Zoology, Shibli National (P.G) College, Azamgarh, U.P. 276001, India

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KEYWORDS

Antigenotoxicity;
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Green medicine

Abstract *Background:* Green medicine is safe and more dependable than the costly synthetic drugs. *Phyllanthus amarus* is one of them that have enormous medicinal potential.

Aim: In these experiments, we have evaluated the anticlastogenic, antigenotoxic and antimutagenic potential of *P. amarus* extract.

Subject and methods: In this experiment we have used *in vitro* human lymphocyte culture and *in vivo* bone marrow cells of Albino mice. The parameters used were chromosomal aberrations (CA), sister chromatid exchanges (SCEs) and cell growth kinetics (RI) both in the presence as well as in the absence of S_9-mix (for *in vitro* study) and total aberrant cells and the frequencies of aberrations per cell (for *in vivo* study). Alcoholic extracts of *P. amarus* was obtained by Soxhlet extractor.

Results: Extracts of *P. amarus* significantly reduces chromosomal aberrations from 34.49%, 68.47% and 78.65% at 24 h, 48 h, and 72 h produced by Aflatoxin B1 (AFB1) to 19.32%, 48.00% and 51.88% level. Similarly sister chromatid exchanges were reduced from 17.76 ± 1.50 (SE) to 09.76 ± 1.20 (SE) at 48 h of treatment, whereas replication index was enhanced *in vitro* for each concentration and duration of treatment. It also significantly reduced the number of aberrant cells and frequency of aberrations per cell *in vivo*.

Conclusion: Ameliorating potential of *P. amarus* was dose and duration dependant. These extracts significantly reduced the mutagenicity and genotoxicity that were produced due to AFB1 treatment both *in vitro* and *in vivo*.

1. Introduction

Bioactive components used as medicine are cheaper and safer than the costly synthetic drugs. Screening of compounds obtained from plants for their pharmacological activity has resulted in the isolation of innumerable therapeutic agents.

Abbreviations: CA, chromosomal aberration; SCE, sister chromatid exchange; RI, replication index; AFB1, Aflatoxin B1; S_9 mix, liver microsomal metabolic activation system

* Corresponding author. Mobile: +91 9335753122.
E-mail address: sultansnc@gmail.com (M.S. Ahmad).

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In these experiments, we have evaluated the anticlastogenic, antigenotoxic and antimutagenic i.e., anticancerous potential of *Phyllanthus* extract. *Phyllanthus amarus* is widely distributed in all tropical regions of the planet. It is a member of the Euphorbiaceae family, which contains 6500 species in 300 genera. In folk medicine, extracts of *P. amarus* have reportedly been used to treat jaundice, diabetes, swelling, skin ulcer, gastrointestinal disturbances and blocks DNA polymerase in the case of hepatitis B virus during reproduction [1]. The plant is bitter, astringent, diuretic, stomachic and antiseptic. Its antifungal, antiviral and anticancerous properties have also been demonstrated in experimental animals [2].

Several compounds including alkaloids, flavonoids, lignans, phenols and terpenes were isolated from this plant and some of them interact with most key enzymes. In traditional medicine, it is used for its hepatoprotective, antihypertensive, analgesic and anti-inflammatory properties [3].

Phyllanthenol, phyllanthenone and phyllantheol are the three triterpenes and two lignins i.e., nirphyllin and phyllnirurin were isolated from aerial parts of plant [4] The methanolic extract of *P. amarus* was found to inhibit lipid peroxidation, and scavenge hydroxyl and superoxide radicals in diabetic models and thus showed potent antidiabetic activity [5]. *P. amarus* treatment increased the activity of antioxidant enzymes, like superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPX) and glutathione reductase (GR) both in blood and tissue showing the antioxidant potential of the plant [6].

The methanolic extract of *P. amarus* leaves (50–800 mg/kg) caused a decrease in the levels of total cholesterol, urea, and total protein, prostatic, alkaline and acid phosphatases. These effects were dose and time-dependent. This shows that the leaves of *P. amarus* have hepato-protective, nephro-protective and cardio protective properties [7].

2. Materials and methods

2.1. Materials

The whole plant was powdered in a mixture-grinder and the hydro-alcoholic extract was prepared by refluxing with the double distilled water (DDW) and alcohol (3:1) in a round bottom flask for 36 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 *Phyllanthus* extract (PA), was re-dissolved in DDW prior to the administration in mice or used in the culture. The required doses for treatment were prepared by dissolving the extract in DDW and we have selected 200, 300, 400, 450 and 500 mg/kg body weight for *in vivo* experiments whereas 150, 200, 250 and 300 mg/l for *in vitro* experiments.

2.2. In vivo method

Albino mice 8–10 weeks old (25–35 g in weight) were exposed to the above mentioned doses of *Phyllanthus* extract by intra peritoneal (IP) injection. The animals were sacrificed at sequential intervals of 16 h, 24 h, and 32 h of stipulated treatment time. The samples were taken separately after each treatment as mentioned above. The central sampling interval was 24 h, since cell cycle kinetics could be influenced by the test substances. The earlier and late sampling intervals were adequately spaced within the range of 6–48 h. The additional dose levels were tested in subsequent experiments; these samples were taken at the predetermined duration as earlier published work [8,9]. 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.

2.2.1. Preparation of slides

Immediately after sacrifice, the bone marrow contents were obtained by standard procedure and exposed to hypotonic solution and cells were fixed in Carnoy's fixative. Chromosome preparations were made from bone marrow cells following standard procedure as described. The stained slides were examined and metaphase cells were scored for chromosomal aberrations. Prior to sacrifice, mice were further treated with colchicine, a spindle inhibitor to arrest the cells in C-metaphase. The slides were stained in 10% aqueous Giemsa solution and 100 bone marrow metaphase cells from each animal were scored under code as earlier published work [8,9]. The types of chromosomal aberrations considered were chromatid and chromosome gaps, breaks, and fragments, exchanges and pulverization (severely damaged cells). The reduction factors due to test chemical treatments were calculated using the formula:

$$\%\,\text{Reduction} = \frac{(\text{Aberrant cells in control} - \text{aberrant cells in MMS and test chemicals})}{(\text{Aberrant cells in control} - \text{aberrant cells in negative control})}$$

2.3. In vitro lymphocyte culture method

Human peripheral blood lymphocytes are extremely sensitive indicators of the *in vitro* assay system. The chromosomal changes (numerical and structural) were utilized for investigation of the genotoxic as well as antigenotoxic potentiality of test chemicals. The parameters studied included chromosomal aberrations (CA), sister chromatid exchanges (SCEs) and cell growth kinetics (RI) both in the presence and in the absence of exogenous metabolic activation system [8,9].

2.3.1. Preparation of S_9 liver/microsome fraction

The S_9 liver/microsome fraction formed the exogenous metabolic activation system utilized for the present investigation and was prepared following the standard procedures as recommended by Maron and Ames [10].

2.3.2. Chromosomal aberrations

2.3.2.1. Preparation of culture media. Tissue culture medium RPMI-1640 (flow Laboratories) with L-glutamine and Hepes buffer without $NaHCO_3$ was prepared in advance and stored at 4 °C, but the storage period never lasted longer than a week. About 1.574 g of medium was dissolved in 100 ml of double distilled water by gentle shaking. Antibiotics, penicillin (100 IU/ml) and streptomycin (100 IU/ml) (Hoechst) were also added and pH was adjusted at 6.8–7.2 with N/10 $NaHCO_3$ and HCl. The medium was filtered and sterilized using millipore filtration assembly by 0.45 μm millipore size. The filtered medium was then stored in sterilized tightly capped glass bottles.

2.3.2.2. Collection of blood samples and culture setting. Peripheral blood from the healthy donors was taken fresh every time through veinal puncture under aseptic conditions (disposable needle and disposable syringes, Unitech) and Heparin (500 IU/ml; Micro Lab) was used as anticoagulant. The tightly capped glass vials were gently mixed and stored at 4 °C for half an hour to separate blood cells from plasma.

Lymphocyte culture was carried out by adding 0.8 ml of plasma containing white blood cells (WBC) in 4.5 ml of culture medium supplemented with 0.1 ml phytohaemagglutinin–P (PHA–P, Micro lab) and 15% fetal calf serum (Gibco). The culture vials were then tightly capped to avoid CO_2 loss and after gently mixing, culture tubes were incubated at 37 °C in the dark and colchicines were added 2 h prior to harvesting for arresting the cells at the metaphase stage [8,9].

2.3.2.3. Harvesting of the cultures and slide preparation. After appropriate durations, the cells were spun down by centrifugation for 10 min, at 1200 rpm, and pellets saved by discarding the supernatant. Hypotonic treatment (0.075 M KCl) was carried for 10–12 min at 37 °C and the cells were recollected by centrifugation. The cell pellet was suspended in 5 ml of freshly prepared chilled fixative (3:1 of methanol and acetic acid), which was added drop by drop with a pipette while continuously shaking the tube to avoid formation of clots. In order to ensure the proper fixation, the cells were kept suspended in the fixative for a minimum period of 1 h though preferably overnight. Three changes with fresh fixative were given before preparing the slides. Two or three drops of cell preparation were dropped over clean, grease free, pre-chilled and wet microscopic slides and air-dried. One-day-old slides were stained in Giemsa (Sigma) for 15 min and rinsed in 95% alcohol and finally in the absolute alcohol for proper differentiation. After proper air-drying these slides were dipped in Xylene for 5 min before mounting in dibutyl phthalate xylene (DPX).

2.3.2.4. Analysis of the cells. A total of 300 well spread metaphases were analyzed for each concentration of the test chemicals and for each time duration to analyze various chromosome and chromatid type aberrations using the method of Evans [11].

2.3.3. Sister chromatid exchange analysis

Sister chromatid exchange is a sensitive rapid and objective method of observing reciprocal exchange between sister chromatids. This method depends upon the phenomenon of 5-bromo-2-deoxyuridine (BrdU) incorporation into DNA in place of thymidine. After two rounds of cell division, the chromatids were labeled with BrdU and consequently differentially stained with Hoechst. The BrdU incorporation quenches the fluorescence of 33258 Hoechst. Therefore, the light energy is absorbed but not emitted by such dyes, which results in the reduced staining of chromatid with Giemsa [12].

2.3.3.1. Labeling of chromosomes with BrdU. The sister chromatid exchange analysis was carried out following the standard procedure of Latt et al [13]. One-day-old slides were dipped in 0.5 μg/ml of 33,258 Hoechst stain (Sigma) dissolved in double distilled water in a horizontal coplin jar. The slides were then put in flat glass dish with the layer of cells facing upwards. These were covered with thick layer (2–3 cm) of phosphate buffer (pH 6.8) and exposed to UV lamp (15 W, 254 μm, Philips) from a distance of 10–15 cm for 30–45 min. The slides were taken out from the buffer, washed twice in double distilled water and air-dried. These were then incubated in 2X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) at 65 °C in water bath for 90 min using vertical coplin jars. The slides were taken out and rinsed in distilled water. The air-dried slides were then stained in Giemsa for 20 min and rinsed in 90% alcohol followed by rinsing in absolute alcohol. The dried slides were dipped in Xylene for 5 min and mounted in DPX.

2.3.3.2. Analysis of the cells. Around 50 metaphases (25 metaphases/donor) with differentially stained chromatids were scored for each dose of treatment in the absence of S_9 mix. Similarly 50 metaphases were also scored in the presence of S_9 mix. Student's '*t*' test was applied for calculating the significance of difference between the treated and the controls.

2.3.4. Cell cycle kinetics analyses

The cells undergoing first (M_1), second (M_2) and third (M_3) divisions were detected by studying the BrdU labeled and differentially stained chromosomes, following the method as earlier published [8,9]. The cells with both the chromatids being darkly stained were scored as M_1 cells and those with one dark and one lightly stained chromatid were taken as M_2 cells, whereas those having a mixture of both the differentially stained and uniformly stained chromatids were scored as M_3 metaphase. Around 200 well spread metaphases were scored for each concentration and each treatment of duration from each donor in the absence as well as in the presence of S_9 mix. The replication index (RI) was calculated according to the formula of Tice et al [14] as given below. The deviation from the controls was determined by using Chi-square (χ^2) test.

$$\text{R.I} = \frac{(M_1X1) + (M_2X2) + (M_3X3)}{100} \quad (1)$$

2.4. Statistical Analyses

Chi-square (χ^2) test for homogeneity test of variance was used to analyze the cell growth kinetics exchange with the normal control. The level of significance was tested from standard statistical tables of Fisher and Yates [15].

Student two-tailed '*t*' test was used for calculating the statistical significance in SCE and chromosomal aberrations by comparing the effect induced by different doses of extracts with the respective control. The statistical significance was calculated from Fisher and Yates table at $(n_1 + n_2 - 2)$ degree of freedom (df) at 0.05% level of significance.

3. Results

3.1. *In vivo* effects

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

Table 1 Effect of *Phyllanthus amarus* on the frequency of cells with chromosomal aberrations induced by Aflatoxin B1 (AFB1 x/kg bw) at 16 h duration.

Treatment	PA/kg bw	Cell with pulverized chromosome	Types of chromatic aberrations				Aberrant cell No. (%)	(%) Reduction
			Gaps	Breaks	Fragments	Exchanges		
DH_2O	00	00	02	07	20	0	27 (2.7)	
AFB1	00	15	71	81	99	11	206 (20.6)	
PA	PA_5	00	03	06	19	00	25 (2.5)	
AFB1 + PA	PA_1	08	85	74	86	09	177 (17.7)	14.07
	PA_2	04	54	63	77	07	151 (15.1)	26.70*
	PA_3	02	58	53	64	04	123 (12.3)	40.29*
	PA_4	01	61	45	56	02	104 (10.4)	49.51*
	PA_5	00	51	38	47	01	86 (8.6)	58.25*

Note: PA; concentrations of extracts of *Phyllanthus amarus,* Aflatoxin B_1 5 μg/ml/kg body weight at 16 h of treatment. Calculations were made excluding the gaps type of aberration and *significant at <0.05 probability. PA/kg bw is the concentration of extracts of *Phyllanthus amarus*.

As shown in Table 1 for 16 h of treatment duration, the percentage of aberrant cells was 20.6% due to treatment with Aflatoxin B1 (AFB1) which was reduced to 17.7%, 15.1%, 12.3%, 10.4% and 8.6% respectively for five consecutive concentrations of *Phyllanthus* extract. The fragment types of aberrations were most prominent followed by breaks and gaps. Similarly reduction in the frequencies of clastogenic cells put the values at 14.07%, 26.70%, 40.29%, 49.51% and 58.25% against five different concentrations of *Phyllanthus* extract respectively. The trend of effects was linear i.e., as concentration of *Phyllanthus* extract increases the effect also increases (Fig. 1). The gross effect on the total frequencies of aberrations per thousand cells was 347, 269, 215, 172 and 147 of five different increasing concentrations of *Phyllanthus* extract against 430 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 effects were similar, with increasing values. The observed values were 17.3%, 15.0%, 13.4%, 11.5% and 9.9% due to five increasing concentrations of *Phyllanthus* extract, against 19.7% of AFB1 alone. The normal values were 2.1% and 2.6% for pure water and *Phyllanthus* extract alone respectively. It also shows dose-response relationship (Table 3). Effect of *Phyllanthus* extract on the frequency of aberrations per cell and total decreased in aberrations noticed were statistically much significant. The total aberrations per thousand cells were 345, 267, 222, 176 and 143 for *Phyllanthus* extract against the value of 400 with AFB1 alone (Table 4).

At 32 h of treatment, we observed 19.8% aberrant cells for AFB1 alone, and were reduced to 17.1%, 15.5%, 13.3%, 11.9% and 10.4% respectively for five increasing concentrations of *Phyllanthus* extract given with AFB1, whereas the values for normal control was 2.7%, and the same for *Phyllanthus* extract alone were 2.3%. In terms of the effects on the percent reduction in aberrant cells, the values were 13.63%, 21.71%, 32.82%, 39.90% and 47.47% respectively. These values were statistically significant at <0.05 probability level due to *Phyllanthus* extract on the number and percentage of aberrant cells. It also showed

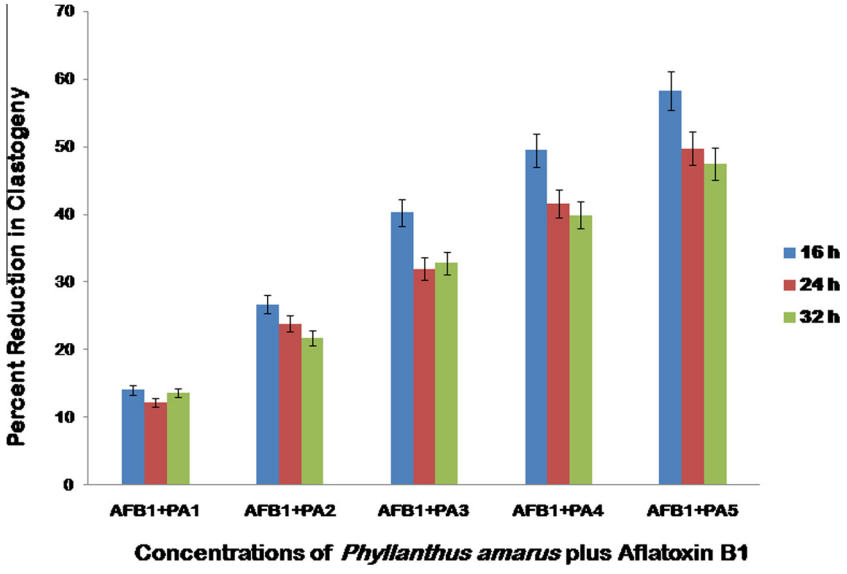

Figure 1 Showing *in vivo* anticarcinogenic effect of *Phyllanthus* extract at 16 h, 24 h, and 32 h of treatment durations against AFB1 induced genotoxicity in Albino mice bone marrow cell.

Table 2 Effect of *Phyllanthus amarus* extract on the frequency of cells with chromosomal aberrations induced by Aflatoxin B1 (AFB1 x/kg bw) at 16 h duration.

Treatment	PA/kg bw	Cell with aberration							Total number of aberration
		0	1	2	3	4	5	6–9	
DH_2O	00	973	23	04	00	00	00	00	31
AFB1	00	794	121	29	19	16	12	09	430
PA	PA_5	975	22	03	00	00	00	00	28
AFB1 + PA	PA_1	823	113	21	15	12	09	07	347
	PA_2	849	106	15	11	08	06	05	269*
	PA_3	877	81	17	09	10	05	01	215*
	PA_4	896	74	11	07	08	03	01	172*
	PA_5	914	58	09	08	08	03	00	147*

Note: PA; concentrations of extracts of *Phyllanthus amarus,* Aflatoxin B_1 5 µg/ml/kg body weight at 16 h of treatment. Calculations were made excluding the gaps type of aberration and *significant at < 0.05 probability. PA/kg bw is the concentration of *Phyllanthus amarus* extracts. The animals were sacrificed 16 h after AFB1 treatment. 1000 cells from 10 animals were analyzed for each point.

Table 3 Effect of *Phyllanthus amarus* on the frequency of cells with chromosomal aberrations induced by Aflatoxin B1 (AFB1 x/kg bw) at 24 h duration.

Treatment	PA/kg bw	Cell with pulverized chromosome	Types of chromatic aberrations				Aberrant cell No. (%)	(%) Reduction
			Gaps	Breaks	Fragments	Exchanges		
DDH_2O	00	00	05	04	17	00	21 (2.1)	
AFB1	00	11	89	75	101	10	197 (19.7)	
PA	PA_5	00	03	06	20	00	26 (2.6)	
AFB1 + PA	PA_1	09	60	66	90	08	173 (17.3)	12.18
	PA_2	07	56	57	80	06	150 (15.0)	23.85*
	PA_3	04	45	53	72	05	134 (13.4)	31.98*
	PA_4	03	41	49	60	03	115 (11.5)	41.62*
	PA_5	02	38	39	57	01	99 (9.9)	49.75*

Note: PA; concentrations of extracts of *Phyllanthus amarus,* Aflatoxin B_1 5 µg/ml/kg body weight at 24 h of treatment. Calculations were made excluding the gap type of aberration and *significant at < 0.05 probability. PA/kg bw is the concentration of extracts of *Phyllanthus amarus.*

Table 4 Effect of *Phyllanthus amarus* extract on the frequency of cells with chromosomal aberrations induced by Aflatoxin B1 (AFB1 x/kg bw) at 24 h duration.

Treatment	PA/kg bw	Cell with aberrations							Total number of aberration
		0	1	2	3	4	5	6–9	
DH_2O	00	979	18	03	00	00	00	00	24
AFB1	00	803	119	29	17	13	09	10	400
PA	PA_5	973	24	03	00	00	00	00	30
AFB1 + PA	PA_1	827	106	24	15	14	07	07	345
	PA_2	850	99	20	11	11	06	03	267*
	PA_3	866	91	20	09	09	04	01	222*
	PA_4	885	81	18	07	07	02	00	176*
	PA_5	901	71	15	06	05	02	00	149*

Note: PA; concentrations of extracts of *Phyllanthus amarus,* Aflatoxin B_1 5 µg/ml/kg body weight at 24 h of treatment. Calculations were made excluding the gaps type of aberration and *significant at < 0.05 probability. PA/kg bw is the concentration of *Phyllanthus amarus* extracts. The animals were sacrificed 24 h after AFB1 treatment. 1000 cells from 10 animals were analyzed for each point.

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 (Table 5 and Fig. 1). The total frequencies of aberrations per thousand cells were 416 for AFB1 only and 325, 282, 228, 203, 167 respectively for *Phyllanthus* extract along with AFB1. These values were significantly

Table 5 Effect of *Phyllanthus amarus* on the frequency of cells with chromosomal aberrations induced by Aflatoxin B1 (AFB1 x/kg bw) at 32 h duration.

Treatment	PA/kg bw	Cell with Pulverized chromosome	Types of chromatic aberrations				Aberrant cell No. (%)	(%) Reduction
			Gaps	Breaks	Fragments	Exchanges		
DH_2O	00	00	03	04	23	00	27 (2.7)	
AFB1	00	14	68	87	85	12	198 (19.8)	
PA	PA_5	00	01	03	20	00	23 (2.3)	
AFB1 + PA	PA_1	11	65	74	77	09	171 (17.1)	13.63
	PA_2	08	62	69	71	07	155 (15.5)	21.71*
	PA_3	05	54	57	70	01	133 (13.3)	32.82*
	PA_4	02	49	52	64	01	119 (11.9)	39.90*
	PA_5	01	38	42	61	00	104 (10.4)	47.47*

Note: PA; concentrations of extracts of *Phyllanthus amarus,* Aflatoxin B_1 5 μg/ml/kg body weight at 32 h of treatment. Calculations were made excluding the gaps type of aberration and *significant at <0.05 probability. PA/kg bw is the concentration of extracts of *Phyllanthus amarus.*

Table 6 Effect of *Phyllanthus amarus* extract on the frequency of cells with chromosomal aberrations induced by Aflatoxin B1 (AFB1 x/kg bw) at 32 h duration.

Treatment	PA/kg bw	Cell with aberration							Total number of aberration
		0	1	2	3	4	5	6–9	
DH_2O	00	973	21	06	00	00	00	00	33
AFB1	00	802	115	29	19	14	11	10	416
PA	PA_5	977	20	03	00	00	00	00	26
AFB1 + PA	PA_1	829	113	19	14	10	08	07	325
	PA_2	845	106	17	13	07	06	06	282*
	PA_3	867	92	16	11	07	04	03	228*
	PA_4	881	82	14	11	05	05	02	203*
	PA_5	896	74	12	10	04	03	01	167*

Note: PA; concentrations of extracts of *Phyllanthus amarus,* Aflatoxin B_1 5 μg/ml/kg body weight at 32 h of treatment. Calculations were made excluding the gap type of aberration and *significant at <0.05 probability. PA/kg bw is the concentration of *Phyllanthus amarus* extracts. The animals were sacrificed 32 h after AFB1 treatment. 1000 cells from 10 animals were analyzed for each point.

reduced, showed the effects of *Phyllanthus* extract on the total aberrations as well as aberrations per cell as shown in Table 6.

3.2. In vitro effects

Here the culture treated with Aflatoxin B1 (AFB1) resulted in clastogenic abnormalities as observed in percent metaphase aberration, types of aberrations and aberration per cell (34.49%, 68.47% and 78.65% or 0.35, 0.68 and 0.79 aberrations per cell) at single standard dose and three durations viz., 24 h, 48 h and 72 h respectively whereas, with control, the normal and DMSO plus *Phyllanthus* extract, these values were 0.03, 0.04 per cell. *Phyllanthus* extract brings down aberrations from 34.49% to 28.87%, 24.47%, 21.0% and 19.32% with four consecutive doses of the extract at 24 h of duration, whereas at 48 h, it gets lowered from 68.47% to 61.53%, 57.26%, 52.95%, and 48.0% by administration of 1st to 4th concentrations of *Phyllanthus* 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 43.98% for 24 h and 29.90% and 34.03% respectively for 48 h and 72 h that were caused by the highest doses of *Phyllanthus* extract (Table 7 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 *Phyllanthus* extract also showed a similar trend; they lower the clastogenic activity of AFB1. These values were linearly increased with doses (Table 8, Fig. 3). The maximum effective percentage reductions were 40.40%, 32.00%, and 35.30% for 24 h, 48 h and 72 h respectively. When sister chromatid exchanges were counted (Table 9 and 10) the reduction was evident both in the absence as well as in the presence of metabolic activation; there being a lowering of the mean range and the total SCEs and SCE per cell from 15.92 to 08.38 and from 17.76 to 09.76. For the analysis of SCE, only 48 h cultures were used and 50 metaphases were scanned. The effects of *Phyllanthus* extract on replication index (Table 11 and 12, Fig. 4) showed an elevated level when compared with the AFB1 treatment alone i.e., from 1.35 to 1.56, though still being lower than the normal level of 1.78. The effect, after treatment with metabolic activation system, shows the level from 1.23 to 1.54, i.e., again being much effective in comparison with that without metabolic activation system.

Table 7 Analysis of chromosomal aberration after treatment with Aflatoxin B1 (AFB1) along with *Phyllanthus amarus* extract *in vitro,* in the absence of $-S_9$ mix.

Treatments	Durations (h)	Metaphase scored	Percent aberration metaphase		Types of aberration (%)			Aberration/ Cell ±SE
			Including gap	Excluding gap	Chromatid	Chromosome	Total	
AFB1	24	200	26.12	23.00	26.15	8.34	34.49	0.34 ± 0.03
	48	200	44.00	40.17	47.47	21.00	68.47	0.68 ± 0.05
	72	200	47.27	42.00	52.12	26.53	78.65	0.79 ± 0.08
AFB1 + PA_1	24	200	22.22	19.51	21.00	7.87	28.87	0.29 ± 0.04
	48	200	40.00	36.41	43.53	18.00	61.53	0.62 ± 0.06
	72	200	43.18	38.21	48.18	21.50	69.68	0.70 ± 0.09
AFB1 + PA_2	24	200	21.32	18.32	17.00	7.47	24.47	0.24 ± 0.03
	48	200	38.73	34.00	41.13	16.13	57.26	0.57 ± 0.06
	72	200	41.00	36.67	44.39	17.25	61.64	0.62 ± 0.08
AFB1 + PA_3	24	200	20.00	16.00	16.00	5.00	21.00	0.21 ± 0.04
	48	200	37.65	32.38	37.68	15.27	52.95	0.53 ± 0.05
	72	200	39.00	34.77	40.98	15.75	56.73	0.57 ± 0.09
AFB1 + PA_4	24	200	19.12	15.12	15.22	4.10	19.32	0.19 ± 0.03
	48	200	37.00	31.89	35.00	13.00	48.00	0.48 ± 0.06
	72	200	38.19	33.61	35.88	16.00	51.88	0.52 ± 0.06
Control								
Normal	72	200	2.78	2.33	1.98	0.77	2.75	0.03 ± 0.01
DMSO + PA_2	72	200	3.88	1.56	2.58	1.00	3.58	0.04 ± 0.01

Note: PA; concentrations of *Phyllanthus amarus* 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.

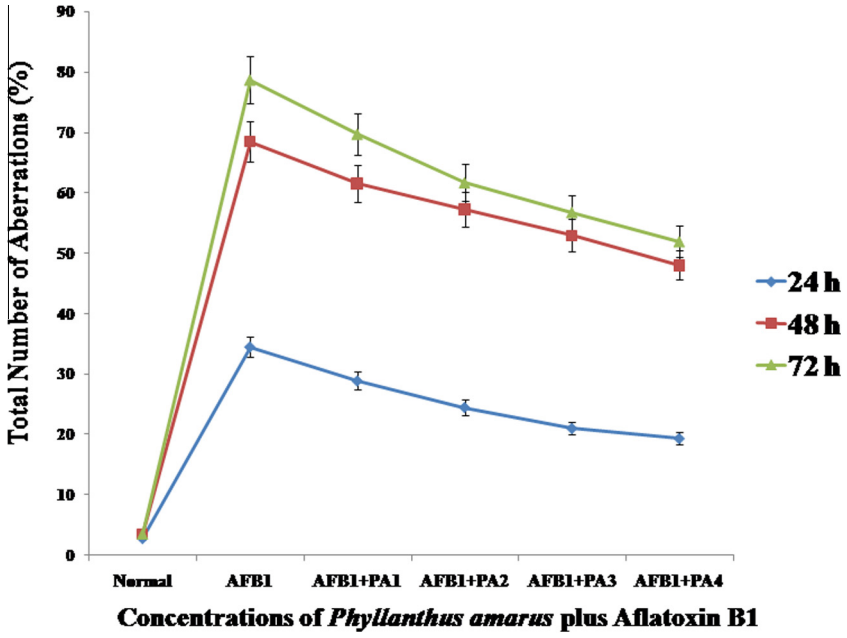

Figure 2 Comparative *in vitro* anticlastogenic effect of *Phyllanthus* extract in absence of S_9 mixture at 24 h, 48 h and 72 h of treatment duration.

4. Discussion

Aqueous extract of *P. amarus* exhibited potent anticarcinogenic activity against 20-methylcholanthrene induced sarcoma and increased the survival of tumor harboring mice. The extract administration was also found to prolong the life span of Dalton's Lymphoma Ascites and Ehrlich Ascites Carcinoma bearing mice and reduced the volume of

Table 8 Analysis of chromosomal aberration after treatment with Aflatoxin B1 (AFB1) along with *Phyllanthus amarus* extract *in vitro,* in the presence of $+S_9$ mix.

Treatments	Durations (h)	Metaphase scored	Percent aberration metaphase		Types of Aberration (%)			Aberration/ Cell ± SE
			Including gap	Excluding gap	Chromatid	Chromosome	Total	
AFB1	24	200	27.28	23.84	27.55	8.98	36.53	0.37 ± 0.04
	48	200	45.00	40.67	49.89	21.87	71.76	0.72 ± 0.08
	72	200	50.55	43.25	54.50	28.35	82.85	0.83 ± 0.09
AFB1 + PA_1	24	200	25.00	22.00	23.50	8.72	32.22	0.32 ± 0.04
	48	200	40.00	36.50	45.00	20.00	65.00	0.65 ± 0.06
	72	200	46.66	42.00	49.30	27.00	76.30	0.76 ± 0.09
AFB1 + PA_2	24	200	24.00	20.88	20.00	7.30	27.30	0.27 ± 0.03
	48	200	36.00	33.00	39.34	18.35	57.69	0.58 ± 0.06
	72	200	43.99	39.00	46.32	23.45	69.77	0.70 ± 0.08
AFB1 + PA_3	24	200	22.32	19.32	19.54	5.38	24.92	0.25 ± 0.04
	48	200	36.12	32.22	34.25	18.10	52.35	0.52 ± 0.05
	72	200	41.63	36.50	42.32	21.00	63.32	0.63 ± 0.09
AFB1 + PA_4	24	200	21.00	19.00	17.00	4.77	21.77	0.22 ± 0.03
	48	200	34.00	29.79	33.00	15.80	48.80	0.49 ± 0.06
	72	200	37.65	32.00	36.00	17.60	53.60	0.54 ± 0.06
Control								
Normal	72	200	2.70	2.00	1.98	0.90	2.88	0.03 ± 0.01
DMSO + Phy_2	72	200	3.55	1.50	2.88	1.00	3.88	0.04 ± 0.01

Note: PA; concentrations of *Phyllanthus amarus* 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.

Table 9 Analysis of sister chromatid exchanges (SCE) after treatment with Aflatoxin B1 along with *Phyllanthus amarus* extract *in vitro,* in the absence of S_9 mix.

Treatment	Duration (h)	Metaphase scored	Total	Range	SCE/Cell ± SE
Aflatoxin B1	48	50	796	3–12	15.92 ± 1.50
AFB1 + PA_1	48	50	702	3–12	14.04 ± 1.50
AFB1 + PA_2	48	50	610	2–12	12.20 ± 1.50
AFB1 + PA_3	48	50	526	1–11	10.52 ± 1.50
AFB1 + PA_4	48	50	419	1–11	08.34 ± 1.50
Control					
Normal	48	50	214	0–6	04.28 ± 1.00
DMSO	48	50	219	0–6	04.38 ± 1.00
DMSO + PA_3	48	50	215	0–6	04.30 ± 1.00

Note: PA; concentrations of *Phyllanthus amarus* extracts, AFB1 x/kg bw; Aflatoxin B1 5 µg/ml/culture, SE; standard error, DMSO; dimethyl sulphoxide. Calculations were significant at <0.05 probability level.

transplanted solid tumors. Antitumor and anticancer activity of *P. amarus* may be evident by the inhibition of metabolic activation of carcinogen as well as the inhibition of cell cycle regulators [2]. In our experiments *P. amarus* extracts counteract the genotoxicity produced due to AFB1, a potent carcinogen significantly *in vitro* and *in vivo* models.

It has been reported earlier that *P. amarus* prevents the induction of tumor in rats by inhibition of N-methyl N-nitrosoguanidine (MNNG) induced gastric carcinogenesis [16]. Revathia et al, [17], observed increased lipid peroxidation and decreased levels of antioxidant status in azaserine-induced rat pancreatic carcinoma. Tumor cells generally display high levels of lipid peroxidation, which in turn can stimulate cell division and promote tumor growth primarily by setting up an oxidant–antioxidant imbalance that favors neoplastic transformation [18]. *P. amarus* administration to azaserine treated rats restored lipid peroxidation levels to near control levels. *P. amarus* exhibits anti-proliferative activity against various types of cancer and thus suggesting that it may be able to regulate cell proliferation [19].

The chemoprotective effect of methanolic extract of the *P. amarus* plant was studied against cyclophosphamide (CTX) induced toxicity in mice. Administration of CTX produced significant myelo-suppression as seen from the

Table 10 Analysis of sister chromatid exchanges (SCE) after treatment with Aflatoxin B1 along with *Phyllanthus amarus* extract *in vitro,* in the presence of S_9 mix.

Treatment	Duration (h)	Metaphase scored	Total	Range	SCE /Cell ± SE
Aflatoxin B1	48	50	888	3–12	17.76 ± 1.50
AFB1 + PA_1	48	50	715	3–12	14.30 ± 1.50
AFB1 + PA_2	48	50	652	2–12	13.04 ± 1.50
AFB1 + PA_3	48	50	590	1–11	11.80 ± 1.50
AFB1 + PA_4	48	50	488	1–11	09.76 ± 1.50
Control					
Normal	48	50	213	0–6	04.26 ± 0.40
DMSO	48	50	217	0–6	04.34 ± 0.40
DMSO + PA_3	48	50	215	0–6	04.30 ± 0.40

Note: PA; concentrations of *Phyllanthus amarus* extracts, AFB1 x/kg bw; Aflatoxin B1 5 μg/ml/culture, SE; standard error, DMSO; dimethyl sulphoxide. Calculations were significant at <0.05 probability level.

Table 11 Analysis of cell cycle kinetics after treatment with Aflatoxin B1 along with *Phyllanthus amarus* extract *in vitro*, in the absence of S_9 mix.

Treatment	Cell scored	(%) cell in			Replication index	2 × 3 chi square test
		*M*1	*M*2	*M*3		
Aflatoxin B1	200	68	29	03	1.35	
AFB1 + PA_1	200	66	31	03	1.37	
AFB1 + PA_2	200	61	35	04	1.43	Significant
AFB1 + PA_3	200	56	39	05	1.49	Significant
AFB1 + PA_4	200	51	42	07	1.56	Significant
Normal	200	38	53	09	1.71	
DMSO	200	34	54	12	1.78	
DMSO + PA_3	200	35	52	13	1.78	

Note: 2 × 3 Chi square (χ^2) test were conducted, AE; concentrations *Phyllanthus amarus* extracts, AFB1 x/kg bw; Aflatoxin B_1 5 μg/ml/culture, DMSO; dimethyl sulphoxide. Calculations were made at <0.05 probability level.

Table 12 Analysis of cell cycle kinetics after treatment with Aflatoxin B1 along with *Phyllanthus amarus* extract *in vitro,* in the presence of S_9 mix.

Treatment	Cell scored	(%) cell in			Replication index	2 × 3 chi square test
		M_1	M_2	M_3		
Aflatoxin B1	200	78	21	01	1.23	
AFB1 + PA_1	200	72	25	03	1.31	
AFB1 + PA_2	200	65	28	07	1.42	Significant
AFB1 + PA_3	200	59	32	09	1.50	Significant
AFB1 + PA_4	200	55	36	09	1.54	Significant
Normal	200	37	53	10	1.73	
DMSO	200	34	54	12	1.78	
DMSO + PA_3	200	35	53	12	1.77	

Note: 2 × 3 Chi square (χ^2) test were conducted, AE; concentrations *Phyllanthus amarus* extracts, AFB1 x/kg bw; Aflatoxin B1 5 μg/ml/culture, DMSO; dimethyl sulphoxide. Calculations were made at <0.05 probability level.

decreased WBC count and bone marrow cellularity. Administration of *P. amarus* extract at doses 250 and 750 mg/kg body weight significantly reduced the myelosuppression and improved the WBC count, bone marrow cellularity as well as the number of maturing monocytes that accounted for its chemoprotective activity [20], All these works were supporting our finding. Methanolic extract of *P. amarus* 50, 200, and 1000 mg/kg body weight significantly inhibited gastric lesions, induced by intragastric administration of absolute ethanol (8 ml/kg). Biochemical analysis indicated that reduced glutathione (GSH) of gastric mucosa produced by ethanol administration was significantly elevated by treatment with *P. amarus* extract. Aqueous and methanol extracts of *P. amarus* produced an inhibition of rat paw edema up to

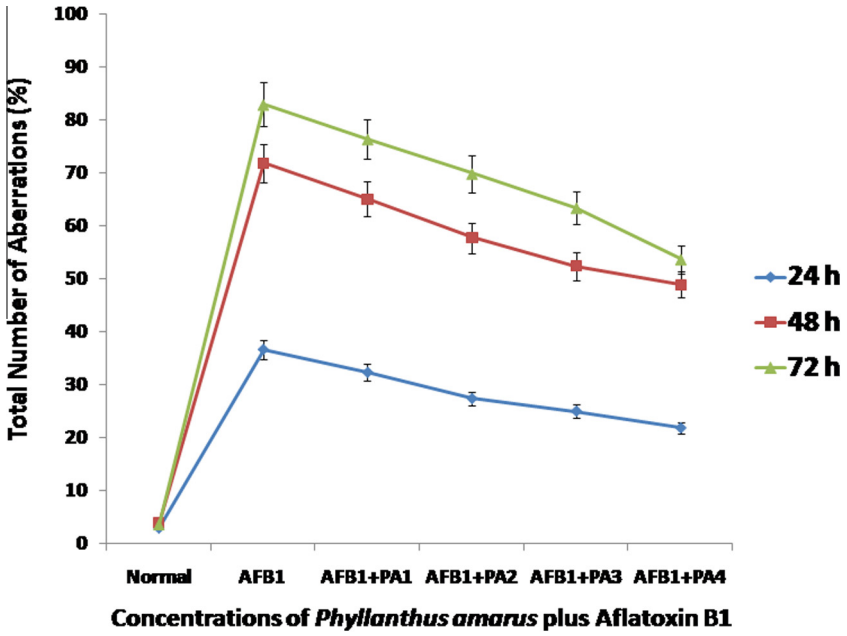

Figure 3 *In vitro* anticlastogenic effect of *Phyllanthus* extract in presence of S_9 mixture at 24 h, 48 h and 72 h of treatment duration.

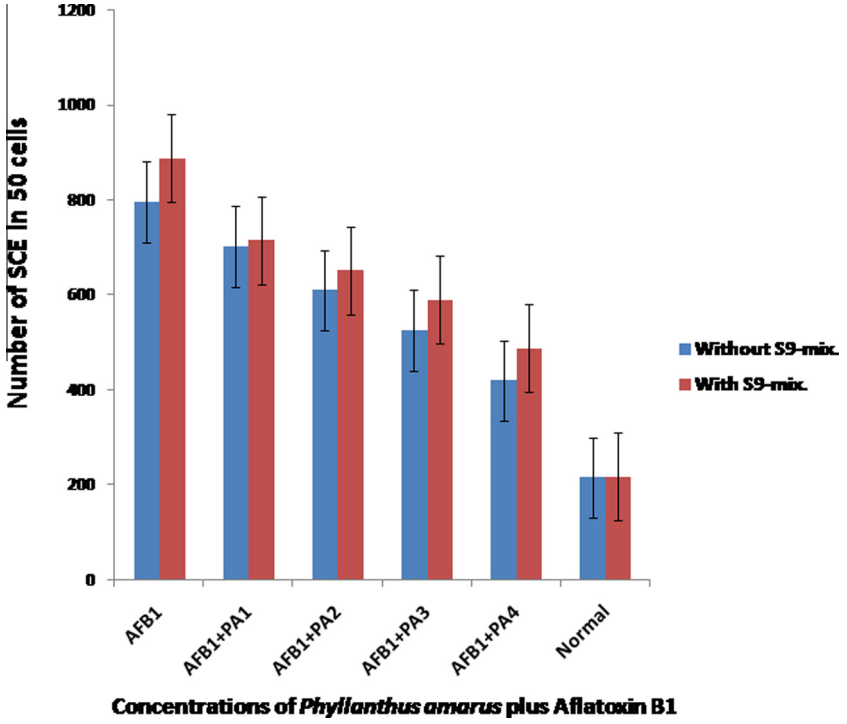

Figure 4 Antigenotoxic effect of *Phyllanthus* extract on sister chromatid exchanges in the absence as well as in the presence of metabolic activation system.

42% compared to control in 3 h and continued up to 8 h. Anti-oxidant activity of the extract as well as presence of tannins in the extract may be responsible for these observed activities [21].

The antimutagenic and anticarcinogenic potential of *P. amarus* using the bacterial pre-incubation mutation assay and an *in vivo* alkaline elution method for DNA single strand breaks in hamster liver cells have been studied. The aqueous extract of the entire plant showed an antimutagenic effect against induction by 2-aminofluorene (AF2), 2-aminoanthracene (2AA) and 4-nitroquinolone-1-oxide (4 NQO) in *Salmonella typhimurium* strains TA98 and TA100, and in *Escherichia coli* WP2 uvrA/pKM101. All the results were dose-dependent. Based on the alkaline elution method, the plant extract

prevented *in vivo* DNA single strand breaks caused by dimethylnitrosamine (DMN) in hamster liver cells. These results indicate that *P. amarus* possesses antimutagenic and antigenotoxic property [22] and that were parallel to our work.

5. Conclusion

P. amarus 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. These results indicate that *P. amarus* possesses antimutagenic and antigenotoxic property.

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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