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

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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.

KEYWORDS
Andrographia paniculata; Chromosomal aberration; Sister chromatid exchange; Replication index; Clastogeny

Abbreviations: CA, chromosomal aberrations; SCE, sister chromatid exchanges; RI, replication index; AFB1, Aflatoxin B1; AE, extracts of Andrographia paniculata.
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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 bonduc cella, 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 (C20H30O5, 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 nitric oxide (42.8%) in vivo 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 hydroalcoholic extract was prepared by refluxing with the double distilled water (DDH2O) 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 Andrographis extract (AE), was re-dissolved in DDH2O 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 B1

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 S9 (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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AE (Y/kg.bw)</th>
<th>Cell with pulverized chromosome</th>
<th>Types of chromatic aberrations</th>
<th>Aberrant cell no. (%) (%) Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDH₂O</td>
<td>00</td>
<td>00</td>
<td>04 03 18 00</td>
<td>21 (2.1)</td>
</tr>
<tr>
<td>AFB₁</td>
<td>00</td>
<td>12</td>
<td>35 53 80 04</td>
<td>148 (14.8)</td>
</tr>
<tr>
<td>AE₂</td>
<td>00</td>
<td>05</td>
<td>05 02 16 00</td>
<td>18 (1.8)</td>
</tr>
<tr>
<td>AFB₁ + AE</td>
<td>AE₁</td>
<td>08</td>
<td>27 45 70 02</td>
<td>125 (12.5) 15.54*</td>
</tr>
<tr>
<td>AE₂</td>
<td>05</td>
<td>20</td>
<td>20 38 62 01</td>
<td>106 (10.6) 28.37</td>
</tr>
<tr>
<td>AE₃</td>
<td>03</td>
<td>22</td>
<td>22 32 54 00</td>
<td>89 (8.9) 39.86*</td>
</tr>
<tr>
<td>AE₄</td>
<td>01</td>
<td>16</td>
<td>16 26 50 00</td>
<td>77 (7.7) 47.97*</td>
</tr>
<tr>
<td>AE₅</td>
<td>00</td>
<td>05</td>
<td>05 21 47 00</td>
<td>68 (6.8) 54.05*</td>
</tr>
</tbody>
</table>

Note: AE₁–AE₅, concentrations of Andrographia paniculata extracts, DDH₂O; Double Distilled water, AFB₁; Aflatoxin B₁ 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.
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 (+S9 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 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.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AE/kg.bw</th>
<th>Cell with aberration</th>
<th>Total number of aberration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDH₂O</td>
<td>0</td>
<td>979 17 04 00 00 00 00 00 25</td>
<td></td>
</tr>
<tr>
<td>AFB₁</td>
<td>0</td>
<td>852 81 25 14 12 11 05 05 311</td>
<td></td>
</tr>
<tr>
<td>AE</td>
<td>AE₅</td>
<td>982 16 02 00 00 00 00 00 20</td>
<td></td>
</tr>
<tr>
<td>AFB₁ + AE</td>
<td>AE₁</td>
<td>875 79 18 10 09 07 07 02 231</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AE₂</td>
<td>894 73 14 07 06 06 06 00 176</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AE₃</td>
<td>911 62 16 05 02 04 04 00 137</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AE₄</td>
<td>923 55 13 03 03 03 03 00 117</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AE₅</td>
<td>932 50 14 01 02 01 01 00 94</td>
<td></td>
</tr>
</tbody>
</table>

*Note: AE₁-AE₅: concentrations of Andrographia paniculata extracts, DDH₂O: Double Distilled water, AFB₁: Aflatoxin B₁ 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.
of the mean range and the total SCEs and SCE per cell from 13.40 to 06.80 and in the presence of S9 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
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 p27. 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 interleukin-2 and interferon-γ [25]. Another study in the in vivo demonstrated that the 70% ethanol extract of A. paniculata and andrographolide 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 Bel-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 NH2-terminal kinase (JNK) activation.
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.

Table 5 In vitro analysis of cell cycle kinetics after treatment with aflatoxin B1 along with Andrographia paniculata extract, in the presence of S9 mix.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell scored</th>
<th>Percentage (%) cell in cell scored</th>
<th>Replication index</th>
<th>2 x 3 Chi square test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M1</td>
<td>M2</td>
<td></td>
</tr>
<tr>
<td>Aflatoxin B1</td>
<td>200</td>
<td>75</td>
<td>24</td>
<td>01</td>
</tr>
<tr>
<td>AFB1 + AE1</td>
<td>200</td>
<td>70</td>
<td>26</td>
<td>04</td>
</tr>
<tr>
<td>AFB1 + AE2</td>
<td>200</td>
<td>66</td>
<td>28</td>
<td>06</td>
</tr>
<tr>
<td>AFB1 + AE3</td>
<td>200</td>
<td>62</td>
<td>31</td>
<td>07</td>
</tr>
<tr>
<td>AFB1 + AE4</td>
<td>200</td>
<td>58</td>
<td>32</td>
<td>10</td>
</tr>
<tr>
<td>Control</td>
<td>DDH2O</td>
<td>200</td>
<td>37</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>AE3</td>
<td>200</td>
<td>38</td>
<td>50</td>
</tr>
</tbody>
</table>

Note: 2 x 3 Chi square (χ²) test was conducted, AE1–AE4 concentrations of Andrographia paniculata extracts, DDH2O; Double Distilled water, AFB1; Aflatoxin B1 5 µg/ml/culture. Calculations were made at < 0.05 probability level.

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.

Conflict of interest

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


