PROTECTIVE EFFECT OF AEGLE MARMELOS BARK EXTRACT AGAINST CYCLOPHOSPHAMIDE INDUCED GENOTOXICITY IN MOUSE BONE MARROW CELLS

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ABSTRACT

Aegle marmelos, plant known for its medicinal importance since ancient times and every part of the plant has been used for curing different diseases. In the present set of investigation, antimutagenic effect of Aegle marmelos bark (AMB) extract against Cyclophosphamide (CP) has been evaluated using in vivo Micronucleus (MN) and Chromosomal aberrations (CA) assay. Mice were categorized in to 6 different groups. Three groups were administered intraperitoneally (i.p) with different doses of AMB i.e. 450, 675 & 900 mg/kg body weight (b.w) 24 hours prior to CP treatment. The CP treated group, vehicle alone group and AMB control group were also included. AMB pretreatment resulted in a decreased frequency of MN and CA, whereas CP treated groups showed significant increase in DNA damage in the bone marrow as evidenced by an increase in the number of micronuclei and CA. AMB extract alone group could not induce any kind of gentoxicity. The protective effect of Aegle marmelos bark extract was found to be more protective at the test dose of 900 mg/kg b.w, indicating a dose dependent protective effect against CP induced genotoxicity. Thus, the present study revealed the antigenotoxic activity of AMB extract on micronuclei formation and chromosomal aberrations induced by CP.

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INTRODUCTION
Cyto genetic assays are extensively used for genotoxicity assessment of test compounds under both in vivo and in vitro conditions. Micronuclei formation & chromosomal aberrations are important cytogenetic endpoints used for the evaluation of genotoxicity [1,2,3,4]. Micronuclei are formed due to chromosome breakage or fragmentation that is not incorporated in the main nucleus after cell division. The most probable reasons for micronuclei formation are clastogenic (chromosome breakage) and aneugenic (chromosome lagging and effects on spindle), whereas chromosomal aberrations arise due to chromosome breakage and exchange. For in vivo genotoxicity evaluation of test compounds, mouse model has been extensively used [5].

Throughout the history of mankind, medicinal plants play an important role in the treatment of infectious diseases. Many plant families have been highlighted for their important contribution in ethno medicinal properties i.e. Asteraceae, Liliacea, Apocynaceae, Solanaceae, Rutaceae, Sapotaceae and many more [6, 7]. Aegle marmelos Correa, commonly known as Bael, belongs to family Rutaceae, has been universally used in indigenous medicine due to its vast medicinal properties. The pharmacological functions such as antioxidant [8], hepatoprotective [9], antidiabetic [10] and radioprotective effects [11] have been well described. Reports have been also there for its anticarcinogenic effects [12, 13] and antigenotoxic effects against doxorubicin induced micronucleus [14]. However no studies have been done to examine the antigenotoxic activity of Aegle marmelos bark extract against Cyclophosphamide using in vivo Micronucleus and Chromosomal aberration assay. Therefore, the present study was planned to elucidate the antimutagenic activity of hydromethanolic extract of Aegle marmelos bark extract.

MATERIALS AND METHODS:
Collection and identification of plant:
Bark of Aegle marmelos were collected from local market of Bhopal, India and were identified by Dr Shaukat S Khan, Professor of Botany, Department of Botany, Saifia Science College, Bhopal, voucher no. provided was AM/003.

Extraction:
Bark was shade dried and finely powdered in a mixer. Known quantity of powdered drug was defatted with petroleum ether and was extracted with 50% methanol for 48 hours with occasional shaking. The filtrate was concentrated under reduced pressure at 50°C to yield brown residue, which was further dried in an oven at 37–38°C to eliminate the traces of solvent and store it in an air tight plastic container. The extract for dosing was prepared by dissolving it in solvent i.e. distilled water (d/w).

Animals:
Both sexes of Swiss Albino mice of 6-7 weeks old and weighing 20-25 gms were procured from the animal house of Jawaharlal Nehru Cancer Hospital & Research Centre, Bhopal. Animals were housed in temperature controlled rooms, 25±2°C with a 12 hour light/dark cycle and provided with standard pelleted diet and water ad libitum. The study was done with prior approval from IAEC (Institutional Animal Ethical Committee) protocol no. 500/01/a/2001/19/0/proj-1/27-07-09.

Chemicals:
Cyclophosphamide, May-Gruenwald and Giemsa were obtained from Sigma Chemicals, Co. (St. Louis, USA). Colchicine was purchased from HiMedia Lab Pvt. Ltd, Mumbai. All other chemicals and solvents used were of analytical grade.

Treatment phase:
The mice were divided in to 6 groups comprising of 6 mice in each group as follows (all these treatments were followed in both the screening models):
- **Group I. Treatment group**: Received single i.p dose of 450 mg/kg body weight of AMB extract.
- **Group II. Experimental group**: Treated with a single i.p dose of CP (50 mg/kg b.w.) 24 hours after the treatment with 450 mg/kg body weight of AMB extract.
- **Group III. Experimental group**: Treated with a single i.p dose of CP (50 mg/kg b.w.) 24 hours after the treatment with 675 mg/kg body weight of AMB extract.
- **Group IV. Experimental group**: Treated with a single i.p dose of CP (50 mg/kg b.w.) 24 hours after the treatment with 900 mg/kg body weight of AMB extract.
- **Group V. Positive Control Group**: Received a single i.p dose of CP (50 mg/kg b.w.) in saline.
- **Group VI. Negative control Group**: Received a single i.p dose of solvent i.e. distilled water (d/w) 0.2 ml.

Micronucleus Assay:
The Micronucleus assay was performed as per the method reported by Schmid (1975) [15] & modified by Aron et al (1989) [16]. The mice were sacrificed at proper intervals according to their treatment phase by cervical dislocation and the femur was dissected out. Bone marrow was aspirated by flushing with HBSS solution (Hanks Balanced Salt solution). Cell suspension was centrifuged at 1000 rpm for 10 min. & the supernatant was discarded. The cells were dispersed by gentle pipetting and again suspended in HBSS solution. Centrifuge it again at 1000 rpm for 10 min,, supernatant was again discarded and a small volume of viscous suspension should be retained.

The slides were prepared by smearing, was then dried and fixed for 2-5 min. in methanol. Subsequent staining was done for 5 min. in May-Gruenwald and 10 min in Giemsa. Then the slides were rinsed in distilled water. Total 0f 1000 (PCE) polychromatic erythrocyes were scored per mouse and the no. of micronucleated PCE (MnPCE) was recorded. To compare the frequencies of
MnPCE and normal PCE between treated and control group, the results were expressed as Mean ± Standard deviation and analysed statistically by Student’s t’ test.

**Chromosomal Aberration Analysis:**

The Chromosomal Aberration assay was performed as per the protocol reported by Preston *et al* (1987). After subsequent treatment phase, the animals were sacrificed by cervical dislocation after 2 hours of intraperitoneal Colchicine (4 mg/kg bw) injection. The femur bone was excised and the bone marrow was aspirated by flushing it with normal saline. The suspension was centrifuged at 1000 rpm for 10 min. Discard the supernatant and treated with 0.56% KCl solution at 37°C for 20 min. The pellet was fixed with freshly prepared Cornoy’s fixative and was again centrifuged. The pellet was washed and finally resuspended in 1-2 ml of fixative. The slides were prepared by standard air drop method and stained with Giemsa. The slides were observed under 100X magnification. The chromosomal aberrations were observed and recorded in standard format. Aberrations such as Centromeric association (CA), Chromosomal fragment (CF), Chromatid breaks (CB), Chromatid gap (CG) and Ring formation (RF) were observed.

**RESULT AND DISCUSSION:**

**Micronucleus Assay:**

The protective effect of intraperitoneal treatment of AMB extract on the frequencies of micronucleated erythrocytes in the bone marrow of normal and exposed mice to CP are described in Table 1. The results revealed that CP when given at a single dose of 50 mg/kg, b.w.,(Group. VI) caused a high incidence of micronucleus induction in Swiss albino mice. The CP dose of 50 mg/kg caused bone marrow toxicity as evidenced by a decrease in the proportion of PCE/NCE ratio. The treatment group i.e. Aegle marmelos bark extract treated group had no statistically significant MNPCE values, reiterating the fact that the extract has no pro-mutagenic components. On the other hand, the % of reduction in the frequency of CP-induced DNA damage was significantly increased in a dose dependent manner.

**Table1. Results showing the protective effect of AMB in micronucleus formation induced by CP.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>Treatment</th>
<th>MnPCE (Mean±SD)</th>
<th>PCE/NCE (Mean±SD)</th>
<th>% Reduction in the frequency of CP induced DNA damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Treatment group</td>
<td>450 mg/kg AMB</td>
<td>0.33±0.20</td>
<td>0.67±0.05</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>Experimental group</td>
<td>450+CP</td>
<td>1.8±0.4*</td>
<td>0.55±0.02</td>
<td>61%</td>
</tr>
<tr>
<td>III</td>
<td>(AMB+CP)</td>
<td>675+CP</td>
<td>0.62±0.05*</td>
<td>0.71±0.08</td>
<td>76%</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td>900+CP</td>
<td>0.70±0.03*</td>
<td>0.98±0.08</td>
<td>80%</td>
</tr>
<tr>
<td>V</td>
<td>Control</td>
<td>Negative (Solvent alone)</td>
<td>0.16±0.16*</td>
<td>0.67±0.05</td>
<td>-</td>
</tr>
<tr>
<td>VI</td>
<td></td>
<td>Positive (CP alone)</td>
<td>4.5±0.4</td>
<td>0.51±0.02</td>
<td>-</td>
</tr>
</tbody>
</table>

Data presented as Mean and standard Error (SD) among mice (n = 6). (*) Denotes statistically significant value at p<0.05

**FIG 1.** Showing Micronucleus (MN) in Polychromatic erythrocytes (PCEs).

NCEs: Normochromatic erythrocytes
Graph 1. Showing the % Reduction in the frequency of CP induced DNA damage by Aegle marmelos Bark extract.

Chromosomal Aberration Assay:
The protective effects of AMB in CP-induced CA observed in the bone marrow of Swiss albino mice are given in Table 2.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Group</th>
<th>Percentage incidence of aberrant cells</th>
<th>Percentage of different chromosomal abnormalities</th>
<th>Suppression % of chromosomal aberration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>CP alone</td>
<td>57.4±4.5</td>
<td>CF 19%, CB 16%, CG 10%, CA 12%, RF 1%</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>Drug alone (AMB)</td>
<td>17.40±3.3</td>
<td>CF 4%, CB 4%, CG 5%, CA 3%, RF Nil</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>450 mg/kg AMB + 50 mg/kg CP</td>
<td>29.3±2.5*</td>
<td>CF 11%, CB 6%, CG 6%, CA 4%, RF 2%</td>
<td>71%</td>
</tr>
<tr>
<td>4.</td>
<td>675 mg/kg AMB + 50 mg/kg CP</td>
<td>24.16±2.8*</td>
<td>CF 8%, CB 5%, CG 7%, CA 4%, RF Nil</td>
<td>76%</td>
</tr>
<tr>
<td>5.</td>
<td>900 mg/kg AMB + 50 mg/kg CP</td>
<td>22.8±2.9*</td>
<td>CF 7%, CB 4%, CG 9%, CA 3%, RF Nil</td>
<td>77%</td>
</tr>
</tbody>
</table>

(*) denotes statistically significant value at p<0.05

The data shows that the suppression percentage in AMB extract at the dose of 450, 675, 900 mg/kg b.w. were found to be 71%, 76%, 77%. The percentage incidence of aberrant cells in CP treated group was 57.4±4.5. However, AMB alone failed to induce chromosomal aberrations significantly confirming its non-mutagenicity. All types of chromosomal aberrations induced by CP include break, fragments, gap, centromeric association and ring formation were found to be significantly reduced by AMB. As the concentration of AMB was increased, the chromosomal aberration was reduced in a dose dependent manner, induced by CP.
FIG. 3. (A & B) Showing different types of chromosomal aberrations.

The present study revealed the response of AMB extract against CP induced genotoxicity. CP generally used to treat cancer malignancies and also an immunosuppressive agent. But, CP causes various physiological side effects and induces genotoxicity, apoptosis in non-tumor cells and induces alterations of male spermatozoa leading to sterility [17,18,19]. This study with CP showed a good correlation between the two measures of cytotoxicity because treatment with CP increased the frequency of MnPCE and the frequency of nucleated cells with chromosomal aberrations in bone marrow cells of Swiss Albino mice. It may be concluded with the results obtained that high number of chromosomal fragments and chromatid break results in an increased proportion of micronucleated PCE and it is due to CP exposure. Consequently from clinical point of view, major emphasis has been laid down on the use of dietary constituents preventing mutagen induced cytogenetic damage due to their non-toxic effects [20].
The results of the present study clearly show that hydromethanolic extract of AMB have a dose dependent preventing effect on CP induced micronuclei and chromosomal aberrations. Mice treated with different doses of AMB before treatment with CP reduced the frequency of MnPCEs and chromosomal aberration. And it was noted that Aegle marmelos extract alone group did not induce any kind of genotoxicity and had no statistically significant MnPCE and chromosomal aberration values, reiterating the fact that the extract has no pro-mutagenic components. It may be attributed that the protective effect was due to the potential involvement of the phytomolecules of the extract to interfere with the enzyme participating in the biotransformation of CP to cytotoxic metabolites. Free radical scavenging activity represents the most important strategies in antimutagenesis and anticarcinogenesis and certain evidences suggests that Aegle marmelos extracts contain rich amount of antioxidants [21]. It was also reported that extracts of Aegle marmelos contain tannin, saponins, flavonoids, glycosides and terpenoids [22]. Antioxidant vitamins, flavonoids, glucosinolates and organo-sulfur compounds have been proven to have antimutagenic potential [23]. Thus, our findings suggest that AMB extract have a protective effect against CP induced genotoxicity.

CONCLUSION
The study concludes the protective effect of Aegle marmelos bark extract against Cyclophosphamide induced Micronucleus formation and chromosomal aberration in mouse bone marrow cells. The protective effect of AMB extract towards CP induced cytogenetic damage implicit it as a god marker of its antimutagenic activity. Further investigations are needed to elucidate the active constituents responsible for its antimutagenicity and its interaction with genotoxic compounds at genetic level.

Conflict of interest statement:
There are none.

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20. Renner HW. *In vivo* effect of single or combined dietary antimutagens on mutagen induced chromosomal aberrations. Mutation Research, 1990, 244, 185-188.

