Antigenotoxic effects of Aegle marmelos Fruit Extract in Cyclophosphamide Induced Chromosomal Aberrations and Aberrant Sperms in Germ Cells of Swiss Albino Mice

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ABSTRACT
The present investigation was attempted to study the possible anti-genotoxic activity of crude Aegle marmelos (AMF) fruit extract in cyclophosphamide-induced genotoxicity in mouse by using chromosomal aberrations (CA’s) in germ cells and sperm morphology essay (SMA). Three different doses of Aegles marmelos (200, 400, 600 mg/kg) were orally administered for their modulatory capacity on the mutagenecity exerted by CP (50 mg/kg, i.p.). AMF alone did not induce any significant variation in the incidence of CA’s and frequency of aberrant sperms. Pretreatment of mice with AMF for 7 days and simultaneously with a single dose of cyclophosphamide significantly reduced the frequency of CA’s and frequency of aberrant sperms, suggesting that the Aegles marmelos fruit extract modulates the CP induced genotoxicity in a dose dependent manner. Thus the results clearly indicate the protective nature of Aegles marmelos fruit extract on cancer chemopreventive strategy.

Key words: Aegle marmelos, Chromosomal Aberrations, Aberrant Sperms, Germ Cells.

INTRODUCTION
Cyclophosphamide is an anti-cancerous alkylating agent. The metabolites of this compound can alkylate nucleophilic sites in DNA, RNA and protein4,10. It induces DNA single strand breaks at molecular level in rat embryos19,24 in testicular cells31. Further cyclophosphamide is capable of inducing structural chromosomal aberrations in Chinese Hamster cells, in human chorionic villae and various stages of spermatogenesis in germ cells11. Further chromosomal damage and a decrease in mitotic index were reported in somatic cells1,22,27 comet tail length in swissmice26,28.

Herbs are gaining additional focus because of their less toxicity and high efficacy against a number of ailments. Epidemiological studies have shown that fruits, vegetables, spices, tea and medicinal herbs rich in antioxidants and other micronutrients protect against diverse forms of chemically induced carcinogenesis, inhibit DNA-damage, mutagenesis and lipid peroxidation5,37. Aegle marmelos, known as bael grows in tropical and subtropical parts of the world. Various parts of the Aegles marmelos are used in Indian system of medicine for treatment of many diseases, including diarrhoea, dysentery and dyspeptic symptoms29,30.

Cite this article: Sushma, Ch. and Rudrama, D.K., Antigenotoxic effects of Aegle marmelos Fruit Extract in Cyclophosphamide Induced Chromosomal Aberrations and Aberrant Sperms in Germ Cells of Swiss Albino Mice, Int. J. Pure App. Biosci. 3 (5): 178-183 (2015), http://dx.doi.org/10.18782/2320-7051.2119
Marmelosin, isolated from the AM, has been reported to have anti-helminthic, anti-bacterial, antioxidant activity and anticarcinogenic activity. Hence in the present investigation a study was undertaken to observe the efficacy of AMF extract against Cyclophosphamide induced chromosomal aberrations and aberrant sperms in germ cells of mice.

MATERIALS AND METHODS

Chemicals
Trisodium citrate (Merck), NaCl (Loba Chemie), Methanol (s.d fine chemie), acetic acid (Qualingens), and 2% Giemsa stain solution in phosphate buffer (pH 6.8) were all purchased from E. Merck, India. Cisplatin, garlic extract, ethanol, mitomycin-C and eosin were purchased from Cipla. However, all other chemicals used in the experiments were of analytical grade.

Extract Preparation
The identification of the plant Aegle marmelos was done by botanist Prof. Prathibadevi, Department of Botany, Osmania University, Hyderabad, Andhra Pradesh, India. The fruit Aegle marmelos were collected. The pieces of fruits were taken and cut in to small pieces. After that paste was taken in a separating funnel and added double distilled water and extracted with double distilled water by refluxing for 36 hrs. at 60°C. On the day of experimentation, the desired amount of powder was dissolved in double distilled water for the final administration.

Experimental animals
Eight weeks old random bred male Swiss albino mice (Mus musculus) average body weight of 25 ± 2 gms were purchased from National Institute of Nutrition, Hyderabad, were maintained in the departmental animal house under an absolute hygienic conditions as per the recommended procedures by fulfilling all the necessary ethical standards. They were housed in polypropylene shoe box type cages dimensions were 13.5" L x 7.0" W x 6.5" to 8.5"H cages, bedded with rice husk (rice husk procured locally and autoclaved to free from microorganisms) and kept in AC room at the temperature 25°C (± 2°C) and RH 65 ± 5% and a photo-cycle of 12:12 h light and dark periods, were fed with pelleted diet (from National Institute of Nutrition, Hyderabad) composed of 20.0% crude protein, 4.0% crude fiber, 1.0% calcium, 0.6% phosphorus, 8% fish meal, 20% ground nut cake and enriched with stabilized vitamins A, B, C, D3, K, thiamine, riboflavin, pantothenic acid, niacin, folic acid, minerals & trace elements and water.

Treatment
Aegle marmelos (200, 400 and 600 mg/kg) extractions were given in orally for 7 consecutive days and 50mg/ kg of cyclophoshaide was administrated on day 7 one hour after regular exposure to antimutagen as a single intraperitonal dose. This is repeated for four weeks. Control (H2O) and positive control (0.1 ml of mitomycin-C) group of animals were also maintained simultaneously. 5 animals were used in each treatment and control group. Slides were screened with Leica CW 4000 Image analyzer.

Chromosome aberration analysis from germ cells
The mice were killed on 28th day, 24 h after administration of last dose of the drug. Seminiferous tubules from testis were collected in 5ml of isotonic 1.2% trisodium citrate solution and incubated at the temperature 37°C for 45 min. The cell suspension was centrifuged in 120x17 mm conical centrifuge tubes for 10 min at 1000 rpm. To the pellet 5 ml of freshly prepared pre-chilled fixative (3:1 methanol and acetic acid) added and centrifuged. This process repeated for 4 to 5 times. The Chromosomal preparations were made by the air drying technique and stained with 2 ml of 2% Giemsa (2 ml of 2% Giemsa in 46 ml of double distilled water plus 2 ml of phosphate buffer pH 6.8) for 7-8 min. Approximately 500 meiotic metaphases screened for numerical (Autosomal Univalents, Sex- Autosomal Univalents, euploids and aneuploids) and structural (translocations) Aberrations.

Sperm morphology assay
All the control and treated animals were sacrificed on 35th day. This is because spermatogenesis takes about 34.5 days to complete in mice. Sperms were sampled from the caudal epididymis after the animals had been sacrificed by cervical dislocation. Sperm suspension was prepared from the caudal of each testis by mincing the caudal in physiological saline. To the suspension 2- 3 drops of 1% aqueous eosin was added and kept for about 20 min undisturbed. Smears were made on clean slides and allowed to dry in air.
1000 sperm cells/mouse were assessed for morphological abnormalities according to the criteria of Wyrobek and Bruce.

**Statistical analysis**

The significance for the differences between control and treated groups was statistically analyzed by using x^2^ test. Data are expressed as mean ± SES in the Tables 1 and 2. Results were considered statistically significant at \( P < 0.05 \).

**RESULTS AND DISCUSSION**

Cyclophosphamide is widely used drug among alkylating agents in cancer chemotherapy. Acrolein and phosphoramidate are active compounds of CP and further these compounds reduce the growth of cancerous cells by acting at DNA level. There are many studies showing chemotherapeutic agents and CP cause gene mutations, CA and aneuploidy and rearrangements in somatic and germ cells of mice in vivo and in vitro test systems and an elevated frequency of secondary treatment related tumors in human cancer survivors. Earlier studies have shown that post meiotic germ cells are specifically sensitive to cyclophosphamide treatment. The administration of low doses of cyclophosphamide to male rats for 6 week produced greater than 95% post implantation loss among their progeny. This loss caused to male rat with cyclophosphamide was characterized by early pre implantation embryonic death. Some abnormalities in progeny outcome caused by cyclophosphamide treatment persisted to a subsequent generation. Thus the effects of cyclophosphamide exposure were both specific and heritable. Further chronic low dose exposure to cyclophosphamide produced adverse effects on progeny by altering sperm nuclear components. The results of present study showed that there is a significant increase in the frequency of chromosomal aberrations when compared with control values (\( p<0.05 \)). The mutagenicity of CP is clearly related to the formation of ultimate cytotoxic metabolite phosphoramidate mustard through intermediate agent is hydroxyl cyclophosphamide and deschloroethyl cyclophosphamide which is responsible DNA cross links and strand lesions.

The present results clearly indicate the fruit extracts of *Aegle marmelos* showed antimutagenic in germ cells of mice. The percentage of CA in CP treated animals showed stastically significant when compared with controls (Table 2), whereas the frequency of CA’s were very near to controls in meiotic cells of mice treated with AMF (Table 1 \( P<0.05 \)). However when animals are co-administer with CP+AMF groups, the percentage of CA’s decreased from 22.40(CP treated group) to 16.60, 10.60 and 7.50 in CP+AMF groups (Table 2 \( P>0.05 \)). Further the AMF extract failed to induced sperm head abnormalities and values were near to control (\( P>0.05 \) Table 1). A significant increase in the percentage of sperm head abnormalities observed in cyclophosphamide treated group similar observations the incidence of aberrant sperms has been reported after treatment of male mice with irradiation chemical agents. Further the agents that accumulate in the testes can cause alterations in testicular DNA responsible for disruption of spermatozoa differentiation. It is clear the obtained results showed protective nature of *Aegle Marmelos* fruit extract against CP induced DNA damage in germ cells of mice.

*Aegle marmelos* (L.) Corr. commonly known as *Bael* (Family: Rutaceae) is described in the *Ayurveda* for its use in various illness such as fever, hyperlipidemia, hypertension, analgesic, anti-inflammatory, heart disease, etc. It is an important medicinal plant and compounds purified from *Bael* have proven biologically active against several major diseases in experimental animal models and have shown activities including antispermatogenic, antimicrobial, and antioxidant. The protective effects of *Aegle marmelos* fruit and leaf extract against doxoyrubicin induced micronuclei in bone marrow cells has been reported. The present results are comparable with who reported the protective effects of *Aegle marmelos* in mouse bone marrow cells at CP induced CAs. Earlier we have reported to protective effects of *phyllanthus emblica* fruit extract on adriamycin induced genotoxicity in mice. The protective against CP induced genotoxicity by *Aegle marmelos* may be due to inhibition of free radicals formed by Cyclophosphamide in cytoplasm of cells and increased antioxidiant status by addition of fruit extract. The fruit of *Aegel marmelos* contains marmelosin, luvangetin, aurapten, psorale, marmelide, tannins and phenols. The AMF extract has been used in for treating diarrhea, diabetic, constipation heart disease, ulcers woodhealing because of its medicinal properties. Lupeol, a compound present in *A. marmelos*...
possess antineoplastic effects on various human neoplastic cell lines. Marmelin (1-hydroxy-5, 7-
dimethoxy-2-naphthale necarboxy aldehyde) present in A. marmelos inhibiting growth of epithelial cancer
cells but not normal cells (mouse embryo fibroblasts) further it decreases cell survival, proliferation and
invasiveness. It is well known that consumption of fruits and vegetables is associated and are known to
prevent chromosomal and DNA damage in animals. Usually antimutagens acting in rodents are active
in human too. Our results have a practical decline of genotoxic effects of cyclophosphamide in drug
exposed population and pharmaceutical lead plant workers handle this drug which may alternate the
higher risks for development of secondary malignancy and for abnormal reproductive outcomes.

Table 1: Incidence of CAs in meiotic spermatocytes of mice treated with Aegle marmelos fruit extract

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Normal sperms</th>
<th>Changes in chromosome number</th>
<th>Structural changes</th>
<th>Abnormal metaphase scores %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Autosomal urivalents</td>
<td>Sex chromo urivalents</td>
<td>Polyploidy</td>
</tr>
<tr>
<td>Control-I</td>
<td>477 (95.40)</td>
<td>11 (2.10)</td>
<td>8 (1.60)</td>
<td>2 (0.40)</td>
</tr>
<tr>
<td>200mg/kg</td>
<td>476 (95.20)</td>
<td>10 (2.00)</td>
<td>8 (1.60)</td>
<td>1 (0.20)</td>
</tr>
<tr>
<td>400mg/kg</td>
<td>474 (94.90)</td>
<td>10 (2.00)</td>
<td>8 (1.60)</td>
<td>2 (0.40)</td>
</tr>
<tr>
<td>600mg/kg</td>
<td>469 (93.80)</td>
<td>10 (2.00)</td>
<td>11 (2.20)</td>
<td>3 (0.60)</td>
</tr>
</tbody>
</table>

The values in parenthesis are percentages *p>0.05

Table 2: Protective role of AMF extract in CP treated animal groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Normal metaphases</th>
<th>Changes in chromosome number</th>
<th>Structural changes</th>
<th>Abnormal metaphase scores %</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>Control - II</td>
<td>476 (95.20)</td>
<td>10 (2.00)</td>
<td>8 (1.60)</td>
<td>2 (0.40)</td>
</tr>
<tr>
<td>mitomycin</td>
<td>397 (79.40)</td>
<td>32 (6.40)</td>
<td>38 (6.90)</td>
<td>20 (4.00)</td>
</tr>
<tr>
<td>50mg CP</td>
<td>388 (77.60)</td>
<td>38 (7.60)</td>
<td>40 (8.00)</td>
<td>21 (4.10)</td>
</tr>
<tr>
<td>50+200mg/kg</td>
<td>420 (84.00)</td>
<td>30 (6.00)</td>
<td>32 (6.40)</td>
<td>10 (2.00)</td>
</tr>
<tr>
<td>50+400mg/kg</td>
<td>447 (89.40)</td>
<td>18 (5.65)</td>
<td>20 (4.00)</td>
<td>8 (1.60)</td>
</tr>
<tr>
<td>50+600mg/kg</td>
<td>463 (92.50)</td>
<td>14 (2.80)</td>
<td>16 (3.10)</td>
<td>4 (0.80)</td>
</tr>
</tbody>
</table>

The values in parenthesis are percentages

a* p<0.05 CP treated group compared with control group
b* p<0.05 Primed group compared with CP treated group

Table 3: Antigenotoxic effects of Aegles marmalos fruit extract on the incidence of aberrant sperms in mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Normal sperms</th>
<th>Aberrant sperms %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B A H hookless</td>
<td></td>
</tr>
<tr>
<td>Control -I</td>
<td>481 (96.20)</td>
<td>8 (1.60) 6 (1.20) 4 (0.80) 1 (0.20) 19 (3.80)</td>
</tr>
<tr>
<td>200mg/kg</td>
<td>480 (96.90)</td>
<td>9 (1.80) 5 (1.00) 4 (0.80) 2 (0.40) 20 (4.00)*</td>
</tr>
<tr>
<td>400mg/kg</td>
<td>479 (95.70)</td>
<td>9 (1.80) 6 (1.10) 4 (0.80) 2 (0.40) 21 (4.30)*</td>
</tr>
<tr>
<td>600mg/kg</td>
<td>478 (95.60)</td>
<td>9 (1.80) 7 (1.60) 4 (0.80) 1 (0.20) 22 (4.40)*</td>
</tr>
</tbody>
</table>

The values in parenthesis are percentages p>0.05
Table 4: Protective role AMF extract on CP induced aberrant sperms in mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Normal sperms</th>
<th>Changes in sperm morphology</th>
<th>Aberrant sperms %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Banana</td>
<td>Amor</td>
</tr>
<tr>
<td>Control -I</td>
<td>482(96.40)</td>
<td>6(1.10)</td>
<td>4(0.80)</td>
</tr>
<tr>
<td>Mitomycin-c</td>
<td>436(87.20)</td>
<td>26(3.80)</td>
<td>22(2.40)</td>
</tr>
<tr>
<td>CP 50 mg/kg</td>
<td>427(85.40)</td>
<td>28(5.40)</td>
<td>26(5.10)</td>
</tr>
<tr>
<td>50+200 mg/kg</td>
<td>458(91.60)</td>
<td>11(2.40)</td>
<td>16(3.10)</td>
</tr>
<tr>
<td>50+400 mg/kg</td>
<td>466(93.20)</td>
<td>14(2.80)</td>
<td>10(2.00)</td>
</tr>
<tr>
<td>50+600 mg/kg</td>
<td>474(94.80)</td>
<td>12(2.40)</td>
<td>8(1.60)</td>
</tr>
</tbody>
</table>

The values in parentheses are percentages
a* p<0.05 CP treated group compared with control group
b* p<0.05 Primed group compared with CP treated group

Acknowledgement

One of author Ch. Sushma is thankful to University Grant Commission of award of RGJRF and to Prof. G. Maruthi Ram, Former Head for providing laboratory facilities.

REFERENCES


