Investigation of Antimutagenic Potential of *Embelia Ribes* Fruit Extract Against Genotoxicity and Oxidative Stress Induced by Cyclophosphamide

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Summary

The present study investigated the protective effects of *Embelia ribes* (*Emb*) against genotoxicity and oxidative stress induced by cyclophosphamide (CP). Mice bone marrow chromosomal aberration, micronucleus and sperm abnormality assay were employed to measure the genotoxicity, respectively. The activities of superoxide dismutase (SOD), glutathione (GSH), Catalase (CAT) and the malondialdehyde (MDA) content in liver were also investigated by spectrophotometric methods. The animals were administered 2 weeks intra gastric treatment of two different doses of *Emb* 100 and 200 mg/kg. The results showed that the CP produced significant increase in chromosomal aberrations (CA), and micronuclei (MN) in polychromatic erythrocytes (PCEs), produced cytotoxicity in mouse bone marrow cells and induced abnormal sperms in male germ line, markedly inhibited the activities of SOD, CAT and GSH, and increased the MDA content. *Emb* significantly inhibited the CAs, micronuclei formation and cytotoxicity in mouse bone marrow cells induced by CP, also produced significant reduction of abnormal sperm and antagonized the reduction of CP-induced SOD, CAT and GSH activities, and inhibited the increase in MDA content in liver. In conclusion, *Emb* have protective effect against mutagenicity induced by CP.

Key words *Embelia ribes*, Cyclophosphamide, Chromosomal aberration, Micronucleus, Sperm abnormality assay, Antioxidant enzymes

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Introduction

A majority of potential chemotherapeutic drugs have been demonstrated the limited clinical application owing to their significant cytotoxicity on organism. Among the anti-cancer drugs, Cyclophosphamide (CP) has been currently one of the most effective anti-cancer agents for the treatment of malignant and non-malignant disorders for over 40 years. However, despite its wide spectrum of clinical uses, CP also possesses wide spectrum of cytotoxicity to normal cells. Genotoxicity of anticancer drugs is one of their most serious side effects due to the possibility of inducing secondary malignancies. Its metabolites can interact with the big molecules such as proteins, membrane lipids, RNA, as well as DNA, which can cause cell DNA damage and induce cell apoptosis. Phosphoramide mustard and acrolein are the two active metabolites of CP. CP’s antineoplastic effects are associated with the phosphoramidemustard, while the acrolein is linked with its toxic side effects. Acrolein interferes with the tissue antioxidant defense system, produces highly reactive oxygen free radicals and is mutagenic to mammalian cells. CP-induced DNA or chromosomal damages in mammalian cells have been widely reported, and DNA breaks induced by CP are the important markers of genotoxicity and/or mutagenicity.

Several pharmacological studies proved that there are many possibilities to discover the new anticarcinogenic and antimutagenic from the plants possessing antioxidant property. A large number of recognized inhibitors of mutagens/carcinogens are basically of plant origin and of highly diverse chemical nature. Different antimutagenesis mechanisms mediated by compounds from foods and plants are known, some of these compounds having antioxidant activities which protect cells from the initiation and development of cancer. So there is an increasing interest in the protective biochemical function of natural antioxidants contained in medicinal herbs, which are candidates for the prevention of oxidative damage caused by oxygen-free radical species.

*Embelia ribes burm* (family, Myrsinaceae) known commonly as *vidanga* is widely distributed throughout India. It is highly esteemed in Ayurveda as a powerful anthelmintic, cures flatulence and colic, possess antimicrobial, antifertility, hepatoprotective, and antihyperglycemic activity. The fruit cures tumors, ascites, bronchitis and mental disorders. *Emb* also found to possess antidyslipidemic, cardioprotective and strong antioxidant activity. Chemically *Emb* is reported to contain embelin, quercitol (polyphenols), flavonoids, tannins, saponins and alkaloid. In the present study, effect of 14 days’ oral treatment with methanolic extract of *Emb* (100 mg/kg and 200 mg/kg) was investigated in order to evaluate the protective effect of *Emb* against the genotoxicity induced by CP.

Material and methods

Drugs and chemicals

Cyclophosphamide (Endoxan-N) was purchased from Cadila Health Care Limited, Goa, India, Colchicine, Bovine albumin Fraction-V, Geimsa’s stain, May-grunwald’s stain and (Hi Media Laboratories Pvt Limited, Mumbai, India), were used for the study. All other chemicals were of reagent grade and purchased from commercial sources.
Preparation of the extract

Dried Emb fruits were purchased locally from Nimbank Ayurvediv Pharmacy, Mehsana, Gujarat (in India, it is commonly available) and authenticated by Dr. Tariq Hussain, Taxonomist, National Botanical Research Institute, Lucknow. 200 g dried fruit powder of Emb was extracted in Soxhlet with 90% methanol for 72 hours. The solvent was removed under reduced pressure to give a dried extract, 12.5% yield w/w (with respect to the crude material).

Determination of phenolics and flavonoids

Total Phenolic content in extract was determined using the Folin-Ciocalteu’s reagent, expressed as mg Gallic acid equivalents per g dry weight of sample as a standard. Determination of total flavonoids was done using colorimetric method, modified from the procedure reported by Woisky and Salatino and result expressed as mg Quercetine equivalents per g dry weight of sample using calibration curve of Quercetine as a standard.

Experimental design

Swiss albino mice (8-12 weeks) procured from institutional animal house of S K Patel College of Pharmaceutical Education and Research, Mehsana. They were acclimatized for 7 days under standard husbandry conditions, i.e.; room temperature of 25 ± 10° C; relative humidity 45-55% and a 12:12h light/ dark photoperiod, with ad libitum access to food (commercial mouse pellets) and water throughout the experiments. For the animal experimentation approval from Institutional Animal Ethical Committee (IAEC) of the institution was taken prior to the experiments. All the protocols and the experiments were conducted in strict compliance according to ethical principles and guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Healthy mice, of approximately 25g b.w. each, were selected and divided randomly into 5 groups (n=5) for each study. The group distribution was as follows

Group 1: Negative control (1% tween 80)

Group 2: Positive control (CP)

Group 3: Emb 200 mg/kg

Group 4: Emb (100 mg/kg + CP).

Group 5: Emb (200 mg/kg + CP).

The extracts were administered for 2 weeks, orally. Mutagenicity was induced by administering CP (40 mg/kg, I.P) 24 hrs prior to tissue sampling.

Chromosomal aberration assay

Animals were given 0.4 ml of 0.05% colchicine intraperitoneally 90 min before scarify to each mouse in order to stop the mitotic process in metaphase. At the time of death both femurs were
dissected out, bone marrow extracted in 0.075M KCl and the cell suspension incubated for 20 min at 37 °C. Cells were collected by centrifugation at 1000 rpm for 10 min and fixed three times with methanol/acetic acid (3:1). Chromosome slides were prepared by dropping the cell suspension onto cleaned slides, which were flame dried and all slides were coded and stained in dilute Giemsa solution. The microscopic observations, performed with a magnification of 100× oil immersion. Hundred well spread metaphase were scored per animal (500 metaphase per treatment group) at random. The types of aberration were scored and recorded strictly in accordance with the method of Tice et al. All aberrations (chromatid gaps, chromosomal gaps, chromatid breaks, chromosomal breaks, deletion, ring and fragmentation) were considered equal regardless of the number of breakages involved. Chromosomal aberrations cells (CA/cell) were calculated including and excluding gaps.

**Micronucleus assay**

All the animals were sacrificed and bone-marrow cells from the both femur of each animal were flushed with bovine serum albumin, for the estimation of the frequency of micronucleated polychromatic erythrocytes in 1000 polychromatic erythrocytes (MNPCE per 1000 PCE). The obtained cell suspension was centrifuged (1000×g, 5 min), the supernatant was removed and the pellet re-suspended in bovine serum albumin. Then, a drop of the suspension was smeared on a clean slide, air-dried, fixed in methanol and stained with May-Gruenwald and Giemsa as originally described by Schmid for microscopic examination with a magnification of 100X. About 1000 PCE were scored for the presence of micronuclei for each animal. To evaluate bone marrow toxicity, the ratio polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) was calculated by counting 1000 erythrocytes.

**Sperm abnormality assay**

Mice from each group were killed by cervical dislocation and their cauda epididymis were removed. Sperm suspensions were prepared by mincing the caqda in 2 ml of phosphate buffered physiological saline, pipetting the resulting suspension and filtering it through an 80 gm stainless steel mesh to remove tissue fragments. A fraction of each suspension was then mixed (10:1) with 1% Eosin Y (H2O) and 30 min later smears were made, allowed to dry in air, and were mounted under a cover slip with permount mounting medium. For each animal 1000 sperm were examined at 1000X magnifications with blue-green filters; a total of 5000 sperm were thus examined for each group. 1000 sperm per animal were assessed according to Bruce and, Wyrobek for morphological abnormalities, which included without hook, amorphous, folded, banana shape, and tail abnormality.

**Determination of SOD, CAT, GSH activities and MDA content**

The livers were excised and perfused with ice cold saline 0.9% sodium chloride. A 10% liver homogenate was prepared with fresh tissue in 0.1 M Tris HCl buffer pH 7.4. The tissue homogenate was used for the estimation of protein content, Malondialdehyde, glutathione GSH, and the activities of superoxide dismutase SOD and catalase CAT. The reaction products were determined by spectrophotometry.
Statistical analysis

The calculated average data generated at different end points of the treated groups of mice were compared with the respective data of vehicle/negative control and positive control group. For statistical analysis the one-way ANOVA was applied followed by turkey’s test for multiple pairwise comparisons using Prism software (PRISM, 1997) as “a posteriori” test were used in all the experiments. The significance of differences was examined at the \( p \)-value of 0.05.

Results

Determination of phenolics and flavonoids

The maximum quantity of phenolic compound was found in methanolic extract of \textit{Emb} is 15.9\%. The flavonoid content of \textit{Emb} was found to be 8.43\%.

Chromosomal aberration assay

The aberrations include chromosomal and chromatid gaps and breaks, deletions, ring, dicentric chromosomes and fragmentations, are the common abnormalities induced by CP (Fig 1). The results of current study revealed that the mice of negative control group showed 0.19±0.05 aberrations per cell with 0.16±0.05 aberrations per cell (excluding gaps), respectively (Table: 1). In the positive control group the animals showed 1.15±0.16 aberrations with 0.99±0.06 aberrations (excluding gaps) per cell, respectively. The increased percentages of aberrant metaphases and CAs in the positive control group are statistically significant \((p<0.001)\) when compared to that of the respective negative control group of mice. Animals treated with Embelia 200 alone were comparable to negative control group regarding to the total CA and CA excluding gap, while rings were more frequent than other types of aberration compare to control. \textit{Emb} 100 and 200 mg/kg induced average no. of aberrant metaphases in the group of mice were 0.52±0.07 and 0.44±0.07, 0.30±0.11 and 0.27±0.11, respectively after induction of clastogenicity by CP. The total aberrations per cell and total aberrations per cell (excluding gap) induced by CP in both the tested doses of \textit{Emb} in mice are significantly \((p<0.001)\) lower than its respective positive control group of mice (Table: 1).

Mice of the negative control group showed 9.59±1.06 average percentage of mitotic index. Their counter parts in the positive control group showed 3.31±1.02 average percentage of mitotic index, respectively, with significant difference from that of the negative control mice (Table: 1). In the group of mice that received \textit{Emb} 200 mg/kg alone, the average percentages of dividing cells was 10.08±1.14, respectively which is insignificant compare to negative control. Average percentages of dividing cells in both the \textit{Emb} (100 or 200 mg/kg) treated groups combined with CP were increased (6.64±1.11 & 7.11±1.91) from that of the positive control mice and are statistically significant.
Micronucleus assay

Table 2, indicates the results of analysis of micronucleus (MN) in bone marrow cells of mice. Average percentage of MN in thousand PCEs of the negative control group of mice was 3.0±1.87. Whereas, their counterparts of the positive control group showed 25.80±7.19 average MN per thousand PCEs, respectively, which are significantly (p<0.001) higher than that of the negative control mice. Emb 200mg/kg induced 2.80±1.92 average MN per thousand PCEs in the female and male groups of mice, respectively. The decreases in induced MN in both the Emb +CP treated groups of mice from that of the respective positive control group were observed which were statistically highly significant (p≤0.01).

In this study, following 14 days administration of different doses of Emb, with 40 mg/kg CP did not induce erythropoietic cell toxicity. Cyclophosphamide induced clastogenicity was higher 0.34±0.11 in the positive control animals, while in the animals that were pre-treated with the different doses of Emb (100 or 200 mg/kg) before injection with CP, a significant reduction (0.86±0.18 or 1.03±0.29) was seen in PCE/NCE ration when compared to positive control and the negative control. From our results, we conclude that the Emb extract protects mouse bone marrow cells against CP-induced lesions.

Sperm abnormality assay

The data of sperm abnormality are shown in table 3. The different types of abnormal sperms observed were amorphous, hook less, banana, folded and double tailed (Fig 1). Results showed that abnormalities such as amorphous, banana sperm and sperm without the usual hook are the most frequently observed after CP administration. On the contrary, the two tails and the folded form are the least common morphological abnormalities but more than other groups. The average percentage of abnormal sperm was 4.05±0.94 in the negative control mice and it was increased significantly to (p<0.001) 19.29±2.08 in the positive control mice. Emb 100, and 200 mg/kg induced average percentages of abnormal sperm were 8.92±1.11 and 7.66±1.22, respectively, which are found decreased than that of the positive control mice and is said decreases are statistically significant. While slight decreased 3.58±0.47 value was obtained in animals treated with Emb 200 alone compare to negative control but it was not significant.

Effects of Emb on CP-induced changes in hepatic SOD, GSH, CAT activities and MDA content in mice

As shown in Fig: 2. A, B, C, the activities of SOD, CAT and GSH were substantially reduced and the MDA content (Fig: 2D) was significantly increased in the CP-treated group compared with the negative control group (P < 0.001). In the groups given Emb extract in combination with CP, the SOD, CAT and GSH activities were significantly increased (P < 0.01 to P < 0.001), and the MDA content was significantly reduced compared with that in the CP group (P < 0.01 to P < 0.001). There were no significant changes in the activities of SOD, GSH and MDA content were seen in mice given Emb 200 mg/kg alone.
Table 1: chromosomal aberration test in mice bone marrow cells treated with methanolic extract of *Embelia ribes*. (2 weeks continuous treatment)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose mg/kg</th>
<th>No. of metaphase analysed</th>
<th>chromatid gap</th>
<th>chromatid break</th>
<th>chromosomal gap</th>
<th>chromosomal break</th>
<th>del</th>
<th>ring</th>
<th>dc</th>
<th>frag</th>
<th>CA/cell Including gap</th>
<th>CA/cell Excluding gap</th>
<th>Mitotic index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>--</td>
<td>500</td>
<td>9</td>
<td>16</td>
<td>8</td>
<td>21</td>
<td>11</td>
<td>14</td>
<td>9</td>
<td>8</td>
<td>0.19±0.05</td>
<td>0.16±0.05</td>
<td>9.59±1.06</td>
</tr>
<tr>
<td>CP</td>
<td>40</td>
<td>500</td>
<td>43</td>
<td>62</td>
<td>63</td>
<td>41</td>
<td>84</td>
<td>155</td>
<td>81</td>
<td>19</td>
<td>1.15±0.16</td>
<td>0.99±0.06</td>
<td>3.31±1.02</td>
</tr>
<tr>
<td>Emb 200</td>
<td>200</td>
<td>500</td>
<td>5</td>
<td>10</td>
<td>11</td>
<td>4</td>
<td>17</td>
<td>41</td>
<td>9</td>
<td>5</td>
<td>0.21±0.05</td>
<td>0.17±0.04</td>
<td>10.08±1.13</td>
</tr>
<tr>
<td>Emb 100+CP</td>
<td>100+40</td>
<td>500</td>
<td>15</td>
<td>20</td>
<td>6</td>
<td>7</td>
<td>39</td>
<td>67</td>
<td>51</td>
<td>5</td>
<td>0.52±0.07</td>
<td>0.44±0.07</td>
<td>6.64±1.11</td>
</tr>
<tr>
<td>Emb 200+CP</td>
<td>200+40</td>
<td>500</td>
<td>8</td>
<td>16</td>
<td>8</td>
<td>4</td>
<td>38</td>
<td>34</td>
<td>35</td>
<td>7</td>
<td>0.30±0.11</td>
<td>0.27±0.11</td>
<td>7.11±1.91</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD (*n* = 5). Abbreviations: del, deletion; dc, dicentric; frag, fragmentation; *Emb*, Embelia extract; CP, Cyclophosphamide.

a *p* < 0.001; b *p* < 0.01; significant when compared with the control.
c *p* < 0.001; d *p* < 0.01; significant when compared to positive control group (CP).
Table 2: Percentage of micronucleated polychromatic erythrocytes (MNPCE) in 1000 polychromatic erythrocytes and the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE ratio) in bone-marrow cells after treatment with *Embelia ribes*

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose mg/kg</th>
<th>MNPCE</th>
<th>PCE/NCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>--</td>
<td>3.0±1.87</td>
<td>1.21±0.26</td>
</tr>
<tr>
<td>CP</td>
<td>40</td>
<td>25.80±7.19a</td>
<td>0.34±0.11a</td>
</tr>
<tr>
<td><em>Emb</em> 200</td>
<td>200</td>
<td>2.80±1.92c</td>
<td>1.32±0.26c</td>
</tr>
<tr>
<td><em>Emb</em> 100+CP</td>
<td>100+40</td>
<td>11.60±3.21b,c</td>
<td>0.86±0.18d</td>
</tr>
<tr>
<td><em>Emb</em> 200+CP</td>
<td>200+40</td>
<td>5.80±1.79c</td>
<td>1.03±0.29c</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD (n = 5). Abbreviations: *Emb*, Embelia extract; CP, Cyclophosphamide.

- \( a_p < 0.001; b_p < 0.01; \) significant when compared with the control.
- \( c_p < 0.001; d_p < 0.05; \) significant when compared to positive control group (CP).

Five animals per group (representing a total of 5000 PCE) were analyzed for the presence of MNPCE and for the ratio PCE/NCE.
Table 3: Percentage frequencies of different types of abnormal sperm induced by Cyclophosphamide in different groups. Frequency of different types of abnormal sperm in mice at week 2 pre-treatment with different doses of *Embelia ribes*.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose mg/kg</th>
<th>Normal</th>
<th>Hook less</th>
<th>Banana</th>
<th>Amorphous</th>
<th>Folded</th>
<th>Two tailed</th>
<th>Total abnormal sperm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>--</td>
<td>4845</td>
<td>103</td>
<td>53</td>
<td>22</td>
<td>23</td>
<td>4</td>
<td>4.05±0.94</td>
</tr>
<tr>
<td>CP</td>
<td>40</td>
<td>4078</td>
<td>540</td>
<td>212</td>
<td>112</td>
<td>89</td>
<td>21</td>
<td>19.29±2.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Emb 200</td>
<td>200</td>
<td>4840</td>
<td>98</td>
<td>30</td>
<td>26</td>
<td>20</td>
<td>6</td>
<td>3.58±0.47&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Emb 200+CP</td>
<td>200+40</td>
<td>4558</td>
<td>171</td>
<td>71</td>
<td>66</td>
<td>59</td>
<td>12</td>
<td>7.66±1.22&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Emb 100+CP</td>
<td>100+40</td>
<td>4569</td>
<td>156</td>
<td>107</td>
<td>75</td>
<td>47</td>
<td>14</td>
<td>8.92±1.11&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD (n = 5). Abbreviations: Emb, Embelia extract; CP, Cyclophosphamide. 
<sup>a</sup>p < 0.001; <sup>b</sup>p < 0.01; significant when compared with the control. 
<sup>c</sup>p < 0.001; significant when compared to positive control group(CP). 
Five animals per group (representing about 5000 sperm cells) were analyzed for the presence of sperm abnormalities.
Fig. 2. Effects of *Embelia ribes* on hepatic (A) Catalase, (B) SOD (C) GSH and (D) MDA in mice. Data are expressed as mean±SD (*n* = 5). Abbreviations: EMB, Embelia extract; CP, Cyclophosphamide.

a *p* < 0.001; significant when compared with the control.

b *p* < 0.001; *cp* < 0.01; *dp* < 0.05; significant when compared to positive control group (CP).
Fig: 1. Photomicrographs of Cyclophosphamide-induced (A-E) Chromosomal aberrations in bone marrow metaphase showing chromosomal and chromatid gaps and breaks, fragmentation, dicentric, chromosomes and deletions, (F) micronucleated PCE, (G-L) Abnormal sperm cells, like hook less, two tailed, banana shaped, amorphous, and folded in mice at 24 h post-treatment.

Discussion

Cyclophosphamide (CP), the positive control chemical in the present study, is a covalent DNA binding agent. Its cytogenotoxicity has been reviewed and updated earlier. CP has been recommended to be used as a positive control chemical in genetic toxicity tests. The induction of significantly (p<0.001) high percentages of aberrant metaphases, CAs (including and excluding gaps) and MN per thousand PCEs in mouse bone marrow, and abnormal sperm by CP (40 mg/kg b.w. of mice), in the present study, is in complete agreement with its earlier reported clastogenicity.

Chromosomal analysis of bone marrow cells in vivo from mammals is a standard method for testing potential mutagenic or anti mutagenic effects of viruses, radiation, drugs and chemicals pollutants. Chromosomal analysis of bone marrow cells in vivo from mammals is based on the
ability of a test agent to induce chromosome structural or numerical alterations that can be visualized microscopically. The results obtained in present study indicate that Emb extract pre-treatment significantly reduced the frequency of structural chromosomal aberrations induced by CP in bone marrow cells. The relationship between cell cycle progression and inhibition of cell proliferation was examined by determining the mitotic index. Decrease in percentage of mitotic index in CP treated group shows that there was decrease in cell proliferation in bone marrow cells of mice, while pre-treatment with the extract in different groups given a significant improvement in mitotic activity in bone marrow cells. The improvement in mitotic activity of bone marrow cells of animals pre-treated with Emb may focus attention on the beneficial effect of Emb to overcome one of the most serious problems in cancer chemotherapy, which is the bone marrow suppression.

In anaphase, acentric chromatid and chromosome fragments lag behind when the centric elements move towards the spindle poles. After telophase the undamaged chromosomes, as well as the centric fragments, give rise to regular daughter nuclei. The lagging elements are included in the daughter cells, too, but a considerable proportion is transformed into one or several secondary nuclei which are, as a rule, much smaller than the principal nucleus and are therefore called micronuclei. The observed decrease in the incidence of Mn PCEs can be considered to indicate an inhibitory effect of Emb extract. The micronuclei test used in this study also detects cytotoxic effects by the PCE/NCE relationship. When normal proliferation of the bone marrow cells is affected by a toxic agent, the number of immature erythrocytes (PCE) is prejudiced in relation to mature erythrocytes (NCE). Thus, the PCE/NCE ratio may decrease. They also show not only antimutagenicity on mice bone marrow micronucleus model but also a significant protection against cytotoxicity induced by CP.

While evaluating the genotoxic effects of any agent in an organism it is very much relevant to study the genotoxic effects on the germinal cells also, because this will give information on transmissible genetic damage from one generation to another. Chemicals that showed positive response in the sperm abnormality tests are also proved to be carcinogenic. The change in sperm parameters probably arise from interference by the test substance with the genetically controlled differentiation of the sperm cells. Sperm shape test provides a direct measure of the quality of sperm production in chemically treated animals. Various reasons are given for the induction of abnormal sperms in mice. Perhaps they are the result of naturally occurring errors in differentiation process, or the consequence of an abnormal chromosome complement. The frequency of abnormal sperms induced by CP is significant increased, which indicated the reproductive genotoxicity of this drug. Data of sperm abnormality test shows that pre treatment with Emb at different dose levels prevents the induction of morphological changes induce by CP.

CP has a pro-oxidant character and its administration leads to generation of oxidative stress in liver, lungs and serum of mice and rats with a resulting reduction in the activities of anti-oxidant enzymes and increases in lipid peroxidation in these tissues. Several studies suggest that the important factor for the therapeutic and the toxic effects of CP is the requirement of the metabolic activation by the hepatic microsomal cytochrome P450 mixed functional oxidase system. It was reported that acroleine which is one of the active metabolite of CP produce toxic side effects by interfering with the tissue antioxidant defense system, produces highly reactive oxygen free radicals and suppresses SOD, GPx and CAT activities, which is
mutagenic to mammalian cells\textsuperscript{12}. As mentioned above, CP-induced oxidative stress which might be one of the mechanisms of its toxic actions. Therefore, in the present study, the effect of \textit{Emb} on CP-induced changes in the activities of anti-oxidant enzymes and the MDA content were investigated. The results showed that \textit{Emb} significantly inhibited the reduction in the activities of SOD, Catalase and GSH, and the increase in content of MDA induced by CP. These results suggest that the protective effects of \textit{Emb} on CP-induced changes in the anti-oxidation system might partially contribute to its protective effects against the genotoxicity induced by CP.

The preliminary phytochemical investigation of methanolic extract of \textit{Emb} is found to contain Polyphenols, tannins, flavonoids, proteins and saponins. Tannins and polyphenols are one such class of compounds which are suspected of possessing protective properties\textsuperscript{63, 64}. Several mechanisms have been proposed to explain the antimutagenic effect of polyphenols, eg. Scavenging of the reactive oxygen species or radicals, decreasing the generation of hydroxyl radicals, modifying the DNA repair pathway after DNA damage, inhibiting the formation of DNA adducts or methylation\textsuperscript{6,68,69}. Stich and Rosin proposed that phenolics had inhibitory effect on genotoxicity of several mutagens\textsuperscript{70}. It was found that such compounds were associated with low incidences of various cancers\textsuperscript{71-73}. According to results of our study it was found to have strong antimutagenic effect with high phenolic content (15.9 %). The major phenolic compound present in \textit{Emb} is embelin which possess anticancer, antitumor activity\textsuperscript{74} and also possess strong antioxidant activity in liver, intestine and kidney. Embelin has also been found to efficiently scavenge physiologically relevant oxidizing radicals like hydroxyl, glutathyl and peroxyl radical. Inhibition of lipid peroxidation, reduction of Fe (III) and restoration of Mn-superoxide dismutase by embelin in \textit{in vitro} studies suggest that it can be an efficient antioxidant in physiological system. Further, its free radical scavenging activity has been also found to be better than \(\alpha\)-tocopherol\textsuperscript{75}.

Flavonoid compounds were found to have the ability to reduce the production of reactive oxygen species (ROS), the inhibition of protein and DNA synthesis and the apoptosis caused by carcinogenic chemicals and showed good scavenging power\textsuperscript{76}. In our study, Embelia showed 8.43\% of total flavonoid contents equivalent Quercetine which suggest that the extract attenuated the CP-mediated decrease in the activities of GSH, Catalase and SOD. Moreover, Galvano et al. reported that flavonoid compounds at 0.5\% level caused a marked decrease in the ability of liver microsomes to metabolize different toxins thus preventing their activation toward DNA adduct formation\textsuperscript{77}. Furthermore, flavonoids were also found to induce cytosolic glutathione \(S\)-transferase activity, and in turn may be increasing the formation of glutathione conjugates with toxins. Moreover, the increasing in glutathione \(S\)-transferrase activity induced by these extract may be another way in the prevention of CP carcinogenicity through the increasing of CP-glutathione conjugates and consequently decreased the ability of hepatic microsomes to metabolize CP and preventing the formation of DNA adduct and prevent its cytogenetic effects. Also tannins and polyphenols are one such class of compounds which are suspected of possessing protective properties\textsuperscript{63}. Although the precise mechanism whereby the isolated compound acts is not clear, since CP has been reported to induce the oxidative stress including DNA damage. Similarly our results undoubtedly demonstrated that \textit{Emb} extract pretreatment may have blocked the oxidative stress in the liver, thus protecting against the CP induced DNA damage, probably due to its capability of trapping the CP-induced free radicals. This trapping is
due to its potent antioxidant activity, which might be due to presence high phenolic and flavonoid contents.

Based on the results obtained, we conclude that the protective effects of *Emb* against CP-induced geno- and cytotoxicity might be due to its protective action against CP-induced oxidative stress. Although the interactions may involve the metabolic or molecular pathways of *Emb*, the effects of CP and their metabolites on DNA damage and cell death requires further investigations, however, the present results suggest that *Emb* has potential as an adjuvant to CP for preventing the side effects associated with chemotherapeutic applications.

**Conflict of interest**

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of the manuscript entitled, “Investigation of antimutagenic potential of *Embelia ribes* fruit extract against genotoxicity and oxidative stress induced by cyclophosphamide”.

**References**

72. Kuo M, Lee K, Lin J, Genotoxicities of nitropyrenes and their modulation by epigenin, tannic acid, Ellagic acid and indole-3-carbinol in the Salmonella and CHO systems, Mutation Res 1995; 87-95.
76. Guerra MC, Galvano F, Bonsi L, et al. Cyanidin-3-O-beta-glucopyranoside, a natural free-radical scavenger against aflatoxin B1- and ochratoxin A-induced cell damage in a human hepatoma cell line (Hep G2) and a human colonic adenocarcinoma cell line (CaCo-2). British J Nutr 2005; 94: 211–220.