Protective role of *Eclipta alba* L. extract against ethinylestradiol induced genotoxic damage in cultured human lymphocytes

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Abstract

In India, natural preparations derived from plants are widely used for the treatment of various diseases. Hence it becomes necessary to assess the modulating action of the plant extracts when associated with other substances. Ethinylestradiol is not only a genotoxic agent but also a tumor initiating agent. It is widely used in oral contraceptive formulations and also for the treatment of various sexual and metabolic disorders. In the present study, the antigenotoxic effect of *Eclipta alba* was evaluated against the genotoxic effect induced by 10 μM of ethinylestradiol in the presence of metabolic activation using mitotic index (MI), chromosomal aberrations, sister chromatid exchanges and replication index (RI) as parameters.

Materials and Methods

Chemicals

Ethinylestradiol (CAS: 57-63-6; Sigma); S9 mix from rat liver, Sprague-Dawley (Sigma); RPMI 1640, fetal calf serum, Phytohaemagglutinin-M (*In vitrogen*); dimethylsulphoxide, colchicine, 5-bromo-2-deoxyuridine, methanol (SRL, India).

Preparation of plant extract

*E. alba* leaves were collected from the nursery of Forest Research Institute (FRI), Dehradun (UK) and were air dried and ground to fine powder. Extraction was performed by soaking samples (30 g/m dry weight) in 300 mL of acetone for 8-10 h at 40-60°C in a Soxhlet apparatus. After filtration, the excess solvent was removed by rotary evaporator. The extract concentrations of 1.075×10−4, 2.127×10−4, 3.15×10−4 and 4.17×10−4 g/mL of culture medium were established.

**Human lymphocyte culture**

Heparinized blood samples were collected from 10 healthy donors (5 males, 5 females, non-smokers, age range 20-25). Whole blood (0.5 ml) was added to 5 mL of culture medium (pH 6.8-7.0), supplemented with 10% fetal calf serum, 10% antibiotic-antimycotic mixture and 1% phytohaemagglutinin of the final volume of cell culture.17 The culture tubes were then placed in the incubator at 37°C for 24 h.

Chromosomal aberrations analysis

Following 24 h of incubation about 10 μM of ethinylestradiol (dissolved in DMSO, 5 μM/L) was given along with 1.075×10−4, 2.127×10−4, 3.15×10−4 and 4.17×10−4 g/mL of *E. alba* extract, supplemented with the 0.5 mL of S9 mix. The cells were incubated with the S9 mix for 6 h. The cells were then collected by centrifugation and washed in the pre-warmed medium to remove the excess traces of S9 mix and added drugs and were further incubated for the remaining 42 h. Treatment of 0.2 mL of colchicine (0.2 μg/mL) was given to the culture tubes, 1h prior to harvesting. Cells were then centrifuged at 1000 rpm for 10 min. The supernatant was removed and 8 mL of pre-warmed (37°C), 0.075 M KCl (hypotonic solution) was added and the cells were then re-suspended and incubated at 37°C for 15 min. The supernatant was removed after centrifugation at 1000 rpm for 10 min, and subsequently 5 mL of chilled fixative was added. The fixative was removed by centrifugation and the procedure was repeated twice. To prepare slides, 3-5 drops of the fixed cell suspension were dropped on a clean slide and air dried. The slides were then stained in a Giemsa solution in phosphate buffer (pH 6.8) for 15 min. The slides were coded before scoring and approximately 50 metaphases were scored for chromosomal aberrations according to the recommendations of EHC 51 for short term tests for mutagenic and carcinogenic chemicals.18

Sister chromatid exchange analysis

To study sister chromatid exchange analysis, bromodeoxyuridine (BrdU, 10 μg/mL) was added at the beginning of the culture. After 24 h of incubation, 10 μM of ethinylestradiol (dis-
solved in DMSO, 5 μM/mL) was given along with 1.075×10⁻⁴, 2.127×10⁻⁴, 3.15×10⁻⁴ and 4.17×10⁻⁴ g/mL of E. alba extract, supplemented with the 0.5 ml of S9 mix for 6 h. The cells were then collected by centrifugation and washed in the prewarmed medium to remove the traces of S9 mix and drugs. Mitotic arrest was subsequently triggered by the addition of 0.2 mL of colchicine (0.2 μg/mL). Hypotonic treatment and fixation were performed in the same manner as previously described for chromosomal aberration analysis. The slides were coded before scoring and the sister chromatid exchange average was calculated from an analysis of metaphases during the second cycle of division. A total of 25 well spread and complete (2n=46) second division metaphases were scored for SCE. The frequency of SCE/chromosome was recorded according to Carrano and Natarajan.

**Mitotic index and replication index**

The MI was calculated as the number of metaphases in 1500 cells analysed per culture for each dose group and donor. A total of 100 metaphases per culture for each dose group and donor were scored to calculate the RI. Metaphase divisions were detected by the BrdU-Harlequin technique for differential staining of metaphase chromosomes. Statistical analysis was also performed by using the commercial software program Stat Soft Inc (2007).

**Table 1. Effects of Eclipta alba extract on sister chromatid exchanges and replication index by ethinylestradiol.**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>SCE/Chromosome±SE</th>
<th>RI±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>EE (M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.25±0.08±</td>
<td>1.63±0.53±</td>
</tr>
<tr>
<td>(g/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EE (M) + EAE (g/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10+1.075×10⁻⁴</td>
<td>0.26±0.046±</td>
<td>1.69±0.59b</td>
</tr>
<tr>
<td>10+2.125×10⁻⁴</td>
<td>0.18±0.053±</td>
<td>1.72±0.63b</td>
</tr>
<tr>
<td>10+3.15×10⁻⁴</td>
<td>0.17±0.049±</td>
<td>1.74±0.66b</td>
</tr>
<tr>
<td>10+4.17×10⁻⁴</td>
<td>0.15±0.043±</td>
<td>1.78±0.72b</td>
</tr>
<tr>
<td>EAE (g/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.075×10⁻⁴</td>
<td>0.11±0.032</td>
<td>1.90±0.92</td>
</tr>
<tr>
<td>2.125×10⁻⁴</td>
<td>0.10±0.029</td>
<td>1.89±0.88</td>
</tr>
<tr>
<td>3.15×10⁻⁴</td>
<td>0.12±0.036</td>
<td>1.85±0.83</td>
</tr>
<tr>
<td>4.17×10⁻⁴</td>
<td>0.13±0.039</td>
<td>1.82±0.81</td>
</tr>
<tr>
<td>Untreated</td>
<td>0.10±0.031</td>
<td>1.94±0.96</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.11±0.033</td>
<td>1.92±0.93</td>
</tr>
<tr>
<td>Positive control</td>
<td>0.52±0.12a</td>
<td>1.30±0.13a</td>
</tr>
</tbody>
</table>

A total of 250 cells were scored for the sister chromatid exchange analysis and 100 cells were scored for replication index; aP<0.005 significantly different from the untreated; bP<0.005 significantly different from the ethinylestradiol EE, ethinylestradiol, EAE, eclipta alba extract.

**Table 2. Summary of regression analysis for the dose effects of Eclipta alba on mitotic index, chromosomal aberrations, sister chromatid exchanges and replication index after the treatment along with 10 μM of ethinylestradiol.**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Parameters</th>
<th>Regression equation</th>
<th>Beta</th>
<th>Standard error</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Mitotic index</td>
<td>Y=3.664+0.09496X</td>
<td>0.921</td>
<td>0.82</td>
<td>11.27</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>2.</td>
<td>Chromosomal aberrations</td>
<td>Y=0.10216–0.0149X</td>
<td>-0.97</td>
<td>0.007</td>
<td>35.84</td>
<td>&lt;0.0047</td>
</tr>
<tr>
<td>3.</td>
<td>Sister chromatid exchanges</td>
<td>Y=0.21699–0.0162X</td>
<td>-0.98</td>
<td>0.003</td>
<td>93.62</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>4.</td>
<td>Replication index</td>
<td>Y=1.6566+0.0293X</td>
<td>0.985</td>
<td>0.007</td>
<td>69.89</td>
<td>&lt;0.00001</td>
</tr>
</tbody>
</table>

**Discussion**

The results of the present study reveal that the extract of E. alba is potent enough to reduce the genotoxic effects of ethinylestradiol at all the selected doses. The selected doses of E. alba extract were not genotoxic. Our previous study with ethinylestradiol has shown that the metabolic activation and possible conversion of ethinylestradiol to a reactive species is responsible for the genotoxicity. Medicinal plants and their products have been used for centuries to cure various ailments. Many plant products protect against xenobiotics either by inducing detoxifying enzymes or by inhibiting oxidative enzymes. The herb E. alba has been used for centuries to cure various ailments.

\[
\text{RI} = \frac{M_1+2M_2+3M_3}{100}
\]

where, \(M_1\), \(M_2\) and \(M_3\) denote the number of metaphases in the first, second and third cycle, respectively.
E. alba contains mainly coumestans i.e. wedelolactone (I) and demethylwedelolactone (II), poly peptides, polyacetylenes, thiophene-derivatives, steroids, triterpenes and flavonoids.25 The wedelolactones are reported to possess a wide range of biological activities.26 The verification of the possible mutagenic and/or antimutagenic effects of medicinal plants, infusions/ extracts is an important factor in studies. Some plants may possess substances that can modulate the genotoxicity of the other compounds.26 The data obtained in the present study suggest that the compounds present in the extract of E. alba are not mutagenic. The protective effect of E. alba extract in the present study i.e. significant reduction in chromosomal aberrations and sister chromatid exchanges or increase in the MI and RI may be due to the direct action of the compounds present in the extract of E. alba on ethinylestradiol by inactivating it enzymatically or chemically. Our earlier studies with natural plant products and steroid toxicity are also encouraging.27 29 The compounds present in the extract may also scavenge electrophiles/nucleophiles.30 They may also enhance the DNA repair system or DNA synthesis or even may prevent the bioactivation of certain chemicals.31 The antigenotoxic potential of plant extracts has been attributed to their total phenolic content.30 Medicinal herbs contain complex mixtures of thousands of compounds that can exert their antioxidant and free radical scavenging effects either separately or in synergistic ways.32 Identification and characterization of these active principles in the plant extract may lead to strategies to reduce the risk for developing cancer in humans.33 A study on oral contraceptives and liver cancer has revealed that the oral contraceptives may enhance the risk of liver carcinomas.34 The present study shows that the extract of E. alba reduced the genotoxic effects of ethinylestradiol, and hence suggests the possibility of having lower risk of carcinomas in the patients undergoing ethinylestradiol therapy.

Flavonoids present in the extract may act as a blocking agent, thus preventing the metabolic activation of promutagens. They can also form adducts or scavenge free radicals, thus preventing tumor formation.35 The compounds present in the extract may act synergistically, as compared to an isolated compound and this supports the indigenous system of medicine namely, Ayurvedic, Siddha and Unani that have in existence for centuries.36 The identification and characterization of the compounds present in the E. alba extract to determine their particular function will be part of our future study.

References


