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. The treatment of 10 μM of ethinylestradiol along with 1.02×10^{-4}, 2.125×10^{-4}, 3.15×10^{-4} and 4.17×10^{-4} g/mL of *Eclipta alba (E. alba)* extract in culture medium results in a significant dose dependent decrease in the genotoxic effects induced by the treatment of 10 μM of ethinylestradiol. The results of the present study suggest that the plant extract *per se* does not have genotoxic potential, but can modulate the genotoxicity of ethinylestradiol in cultured human lymphocytes.

Introduction

Eclipta alba (L.) (*E. alba)* is commonly known as false daisy or bhringraj. It belongs to the Family Asteraceae and is widely distributed throughout India, China, Thailand and Brazil.[1] It has a great traditional importance because of being used as a medicinal resource in India for centuries.[2] It is used as a promoter of hair growth and blackener of hair.[3] The leaf extract of *E. alba* is a powerful liver tonic, rejuvenative and hepatoprotective.[4] The extract is also used as anti-venom against snake bites in China and Brazil.[5]

Estrogens are used for treating many types of sexual disorders and as part of various oral contraceptive formulations.[6] There is sufficient evidence for estrogen carcinogenicity and genotoxicity in various experimental models.[7] Ethinylestradiol is commonly used in oral contraceptives and in other drug formulations.[8] The prolonged use of oral contraceptives has been reported to induce various types of cancers.[7] There are also reports of the genotoxicity of ethinylestradiol in various experimental models.[9-15] In the present study we have evaluated the effect of a leaf extract of *E. alba* on the ethinylestradiol induced genotoxicity in the presence of metabolic activation in cultured human peripheral blood lymphocytes using mitotic index (MI), chromosomal aberrations, sister chromatid exchanges and replication index (RI) as parameters.

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Key words: *Eclipta alba*, ethinylestradiol, human lymphocytes, genotoxicity, natural products.

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Materials and Methods

Chemicals

Ethinylestradiol (CAS: 57-63-6; Sigma); S9 mix from rat liver, Sprague-Dawley (Sigma); RPMI 1640, fetal calf serum, Phytohaemaglutinin-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 gm of 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 rotatory 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.[16]

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% phytohaemaglutinin 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 μl/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. 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 μl/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.[19] 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.[20]

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.[21-22] The RI, indirect measure of studying cell cycle progression was calculated by applying the following formula:

$$RI = \frac{M_1+2M_2+3M_3}{100}$$

where, M_1, M_2 and M_3 denote the number of metaphases in the first, second and third cycle, respectively.

Statistical analysis

Statistical analysis was performed by one way analysis of variance (ANOVA) and to study the dose response the regression analysis was also performed by using the commercial software program Stat Soft Inc (2007).

Results

The treatment of 10 μM of ethinylestradiol in the presence of S9mix reduced the MI significantly as compared to the untreated. A significant dose dependent increase in the MI was observed when 10 μM of ethinylestradiol treatment was given along with the different doses of *E. alba* extract, i.e. 1.075×10^{-4}, 2.125×10^{-4}, 3.15×10^{-4} and 4.17×10^{-4} g/mL. Ethinylestradiol induced a significant increase in chromosomal aberrations per cell as compared to untreated in the presence of S9mix. A significant dose dependent decrease in the chromosomal aberration per cell was observed when 10 μM of ethinylestradiol treatment was given along with the different doses of *E. alba* extract, i.e. 1.075×10^{-4}, 2.125×10^{-4}, 3.15×10^{-4} and 4.17×10^{-4} g/mL. A significant increase in SCE/chromosome was observed at 10 μM of ethinylestradiol as compared to untreated. A significant decrease in sister chromatid exchanges per chromosome was observed when 10 μM of ethinylestradiol treatment was given along with the different doses of extract of *E. alba* i.e. 1.075×10^{-4}, 2.125×10^{-4}, 3.15×10^{-4} and 4.17×10^{-4} g/mL (Table 1). A significant decrease in the RI was observed at the treatment of 10 μM of ethinylestradiol as compared to the untreated (Table 1). A significant dose dependent increase in the RI was observed at each of the concentrations of *E. alba* extract (Table 1). Regression analysis was also performed to determine the dose effects of *E. alba* extract on 10 μM of ethinylestradiol, for MI, chromosomal aberrations, sister chromatid exchanges and RI (Table 2). For MI the treatments of various doses of *E. alba* extract were associated with an r value of 0.921 (Table 2). For chromosomal aberrations per cell the treatments of various doses of *E. alba* extract were associated with an r value of -0.97 (Table 2). For SCE per cell the treatments of various doses of *E. alba* extract were associated with an r value of -0.98 (Table 2). For RI the treatments of various doses of *E. alba* extract were associated with an r value of 0.985 (Table 2). A decrease in the slope of linear regression lines for chromosomal aberration ($F=35.84$; $P<0.0047$) and sister chromatid exchange ($F=93.62$; $P<0.0005$) was observed as the dose of the *E. alba* extract increases. An increase in the slope of linear regression lines for MI ($F=11.27$; $P<0.0005$) and RI ($F=69.89$; $P<0.00001$) was observed as the dose of the *E. alba* extract increases.

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.[9] Medicinal plants and their products have been used for centuries to cure various ailments.[23] Many plant products protect against xenobiotics either by inducing detoxifying enzymes or by inhibiting oxidative enzymes.[24] The herb *E.*

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

Treatments	SCE/Chromosome±SE	RI±SE
EE (M)		
10	0.25±0.082[a]	1.63±0.53[a]
EE (M) + EAE(g/mL)		
$10+1.075\times10^{-4}$	0.20±0.064[b]	1.69±0.59[b]
$10+2.125\times10^{-4}$	0.18±0.053[b]	1.72±0.63[b]
$10+3.15\times10^{-4}$	0.17±0.049[b]	1.74±0.66[b]
$10+4.17\times10^{-4}$	0.15±0.043[b]	1.78±0.72[b]
EAE (g/mL)		
1.075×10^{-4}	0.11±0.032	1.90±0.92
2.125×10^{-4}	0.10±0.029	1.89±0.88
3.15×10^{-4}	0.12±0.036	1.85±0.83
4.17×10^{-4}	0.13±0.039	1.82±0.81
Untreated	0.10±0.031	1.94±0.96
Negative control	0.11±0.033	1.92±0.93
Positive control	0.52±0.12[a]	1.30±0.13[a]

A total of 250 cells were scored for the sister chromatid exchange analysis and 100 cells were scored for replication index; [a]P<0.005 significantly different from the untreated; [b]P<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.

S. No.	Parameters	Regression equation	Beta	Standard error	F	P
1.	Mitotic index	Y=3.664+0.09496X	0.921	0.82	11.27	<0.0005
2.	Chromosomal aberrations	Y=0.10216-0.0149X	-0.97	0.007	35.84	<0.0047
3.	Sister chromatid exchanges	Y=0.21699-0.0162X	-0.98	0.003	93.62	<0.0005
4.	Replication index	Y=1.6566+0.02930	0.985	0.007	69.89	<0.00001

alba contains mainly coumestans i.e. wedelolactone (I) and demethylwedelolactone (II), polypeptides, polyacetylenes, thiophene-derivatives, steroids, triterpenes and flavonoids.[25] The wedelolactones are reported to possesses 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 have 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 acts 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 been 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.

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