Protective role of *Plumbago zeylanica* extract against the toxic effects of ethinylestradiol in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ)Bg*^{9} and cultured human peripheral blood lymphocytes

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Abstract

In the present study the effects of *Plumbago zeylanica* extract were studied against the toxic effects induced by 10 μM of ethinylestradiol in cultured human peripheral blood lymphocytes using chromosomal aberrations as a parameter and in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ)Bg*^{9} (by 0.50 and 1.0 μl/mL of ethinylestradiol) using hsp70 expression as a parameter. The selected doses of *P. zeylanica* extract i.e. 1.075×10^{-4}, 2.125×10^{-4}, 3.15×10^{-4} and 4.17×10^{-4} g/mL were not toxic but were potent enough to reduce significantly the toxic effects induced by ethinylestradiol in cultured human peripheral blood lymphocytes. The selected doses for the estimation of hsp70 expression in the third instar larvae of transgenic *D. melanogaster (hsp70-lacZ) Bg*^{9}. i.e. 1, 2, 4 and 6 μl/mL were not toxic though reduced significantly the expression of *hsp70* induced by 0.5 and1.0 μl/mL of ethinylestradiol.

Introduction

Estrogens are used not only for the cure of many types of sexual disorders but also in oral contraceptive formulations.[1] There is sufficient evidence of estrogen carcinogenicity and genotoxicity in various experimental models.[2-3] The prolonged use of steroids has been reported to induce various types of cancer.[4] All living organisms under stressful conditions respond by synthesizing heat shock proteins (HSPs).[5-6] In recent years, *hsp70* has been considered to be one of the candidate genes for predicting the cytotoxicity against environmental chemicals.[7-9] *Plumbago zeylanica* is commonly known as white lead wort and is found abundantly in the plains of Bengal and Southern India.[10] In India, it has been used traditionally, against a number of ailments such as skin diseases, diarrhea, and leprosy.[11] Its crude extract has been reported to have antimicrobial, antimutagenic and antitumor properties.[12] The studies related to the antimutagenic effects of its extract are limited. The modulatory effects of some plant extracts have been studied using *Drosophila melanogaster* as a model.[13-15] The fruit fly *D. melanogaster* is a well established model for the study of antigenotoxic effects of different compounds and mixtures due to its well documented genetics and developmental biology.[16] *D. melanogaster* is also capable of activating promutagens and procarcinogens.[17]

In our earlier study the effect of ethinylestradiol was studied for the hsp70 expression at 0.25, 0.50, 1.0 and 2.0 μl/ml in the third instar larvae of transgenic *D. melanogaster (hsp70-lacZ)Bg*^{9} and was found to increase the *hsp70* expression significantly, as compared to the untreated at 0.50, 1.0 and 2.0 μl/mL.[18] In the present study we have evaluated the effects of leaf extract of *P. zeylanica* on ethinylestradiol induced hsp70 expression in the third instar larvae of transgenic *D. melanogaster (hsp70-lacZ)Bg*^{9} in order to verify the model to be used for antigenotoxicity studies. The effect of *P. zeylanica* extract was also studied against the genotoxicity of ethinylestradiol in the presence of metabolic activation (S9mix) in cultured human peripheral blood lymphocytes.

Materials and Methods

Chemicals

Ethinylestradiol (Sigma); RPMI 1640, Fetal calf serum, Phytohaemagglutinin-M, antibiotic-antimycotic mixture (In Vitrogen); Dimethylsulphoxide, 5-Bromo-2-deoxyuridine, Colchicine (SRL, India); Giemsa stain (Merck), S9 mix from rat liver Sprague-Dawley (Sigma); O-nitrophenyl-β-D-galactopyranoside, sodium carbonate, maize powder (Himedia).

Extract preparation

P. zeylanica leaves were collected from the nursery of Forest Research Institute (FRI), Dehradun (UK) and were air dried and grounded 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°C-60°C in Soxhlet's apparatus. After filtration, the excess of solvent was removed by rotatory evaporator. The extract concentrations of 1.075×10^{-4}, 2.127×10^{-4}, 3.14×10^{-4} and 4.17×10^{-4} g/mL in culture medium were established.[14]

Human lymphocyte culture

Heparinized blood samples were collected from 5 healthy donors (2 males, 3 females, non smokers, age range 22-28). 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 the cell culture.[19] The culture tubes were then placed in the incubator at 37°C for 24 hr.

Chromosomal aberration analysis

Following 24 hr incubation, the treatment of 10 μM of ethinylestradiol (dissolved in DMSO, 5 μl/mL) was given along with 0.5 ml of S9 mix. A separate treatment of 10 μM of ethinylestradiol (dissolved in DMSO, 5 μl/mL) was also given along with 1.075×10^{-4}, 2.127×10^{-4}, 3.15×10^{-4} and 4.17×10^{-4} g/mL of *P. zeylanica* extract along with 0.5 mL of S9 mix. The cells were incubated with S9 mix for 6hr. The cells were then collected by centrifugation and washed in the prewarmed medium to remove the excess traces of S9 mix and added drugs and were further incubated for the remaining 42 hr. The negative (DMSO, 5 μl/mL) and positive (cyclophosphamide, 0.16 μg/mL) controls were also run simultaneously. Treatment of 0.2 ml of

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colchicine (0.2 μg/mL) was given to the culture tubes prior to 1 hr of cell 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 resuspended 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 to 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 50 metaphases were scored for chromosomal aberrations (TCAs) per dose per treatment per donor according to the recommendations of EHC 51 for short term tests for mutagenic and carcinogenic chemicals.[20]

Soluble O-nitrophenyl-β-D-galactopyranoside (ONPG) assay

A transgenic *D. melanogaster* line that expresses a bacterial-β-galactosidase as a response to stress was used in the present study.[21] In the said strain of fly, the transformation vector is inserted with a P-element, the line contains wild type *hsp70* sequence up to the lacZ fusion point. The flies and larvae were cultured on standard *Drosophila* food containing agar, cornmeal, sugar and yeast at 24°C.[22] Ethinylestradiol was dissolved in dimethylsulphoxide and 0.50 and 1.0 μl/mL of food concentrations were established. 0.50 μl/mL of ethinylestradiol was mixed separately with 1, 2, 4 and 6 μl/mL of *P. zeylanica* extract in food. The similar food concentrations were established with 1 μl/mL of ethinylestradiol. The third instar larvae were allowed to feed on them for different time intervals (6, 24 and 48 hr). We followed the method as described by Nazir *et al.*[22] Briefly, after washing in phosphate buffer the larvae were taken in a microcentrifuge tube (20 larvae/tube, 5 replicates /group), permeabilized for 10 min in acetone, and incubated overnight at 37°C in 600 μL of ONPG staining buffer. Following incubation, the reaction was stopped by adding 300 μL of Na_2CO_3. The extent of reaction was quantified by measuring the absorbance at 420 nm using systronics UV/VIS spectrophotometer 118, India. Dimethylsulphoxide at the dose of 2 μl/ml of food was used as a negative control and the third instar larvae were allowed to feed for different durations.

Statistical analysis

Statistical analysis was performed by one way analysis of variance (ANOVA) using the commercial Software Programme Stat Soft Inc (2007).

Results

The treatment of 10 μM of ethinylestradiol in the presence of S9 mix results in a significant increase in TCA / Cell (Table 1). A significant decrease in the TCA/ cell was observed when the treatment of 10 μM of ethinylestradiol was given along with 1.075×10^{-4}, 2.127×10^{-4}, 3.14×10^{-4} and 4.17×10^{-4} g/mL of *P. zeylanica* extract (Table 1). The treatment of 10 μM of ethinylestradiol in the presence of S9 mix was associated with 0.16±0.153 TCA/Cell. The treatment of 1.075x10-4, 2.125x10-4, 3.15×10^{-4} and 4.17×10^{-4} g/mL of plant extract along with the 10 μM of ethinylestradiol in the presence of S9 mix was associated with 0.075±0.091, 0.056±0.084, 0.044±0.073 and 0.032±0.041 TCA/Cell, respectively (Table 1). The exposure of the third instar larvae to 0.50 μl/mL of ethinylestradiol for 6, 24 and 48 hr was associated with the mean absorbance values of 0.2802±0.0070, 0.2931±0.0083 and 0.3146±0.0077, respectively. The exposure of the third instar larvae of transgenic *D. melanogaster (hsp70-lacZ)Bg*^{9} to 0.50 μl/mL of ethinylestradiol along with different concentrations of plant extract i.e. 1, 2, 4 and 6 μl/ml for 6 hr were found to be associated with mean absorbance values of 0.2511±0.0069, 0.2433±0.0053, 0.2401±0.0044 and 0.2346± 0.0041, respectively (Table 2). The exposure of the third instar larvae of transgenic *D. melanogaster(hsp70-lacZ)Bg*^{9} to 0.50 μl/mL of ethinylestradiol along with different concentrations of plant extract i.e. 1, 2, 4 and 6 μl/mLfor 24 hr were associated with the mean absorbance values of 0.2703±0.0072, 0.2614± 0.073, 0.2533±0.0052 and 0.2421± 0.0046, respectively (Table 2). The exposure of the third instar larvae of transgenic *D. melanogaster (hsp70-lacZ)Bg*^{9} to 0.50 μl/mL of ethinylestradiol along with different concentrations of plant extract i.e. 1, 2, 4 and 6 μl/ml for 48 hr were associated with the mean absorbance values of 0.2915±0.0081, 0.2833± 0.0073, 0.2767±0.0070 and 0.2704±0.0063, respectively (Table 2). The exposure of the third instar larvae to 1.0 μl/mL of ethinylestradiol for 6, 24 and 48 hr was associated with the mean absorbance value of 0.1303±0.0073, 0.3094±0.0089 and 0.3277± 0.0086, respectively (Table 2). The exposure of the third instar larvae of transgenic *D. melanogaster* (hsp70-lacZ)Bg9 for 6 hr to 1.0 μl/mL of ethinylestradiol along with the different concentrations of plant extract i.e.1, 2, 4 and 6 μl/mL were associated with the mean absorbance values of 0.2947±0.0089, 0.2721±0.0084, 0.2634±0.0079 and 0.2441±0.0066, respectively (Table 2). The exposure of the third instar larvae of transgenic *D. melanogaster* (hsp70-lacZ)Bg9 for 24 hr to 1.0 μl/mL of ethinylestradiol along with the different concentrations of plant extract i.e.1, 2, 4 and 6 μl/mL were associated with the mean absorbance values of 0.2843±0.0059, 0.2711±0.0063, 0.2653±0.0071 and 0.2601± 0.0064, respectively (Table 2). The exposure of the third instar larvae of transgenic *D. melanogaster (hsp70-lacZ)Bg*^{9} for 48 hr to 1.0 μl/ml of ethinylestradiol along with the different concentrations of plant extract i.e.1, 2, 4 and 6μl/ml were associated with the mean absorbance values of 0.2997±0.0053, 0.2819±0.0072, 0.2784±0.0073 and 0.2723± 0.0068, respectively (Table 2).

Table 1. Effect of Plumbago zeylanica extract on chromosomal aberrations induced by ethinylestradiol in cultured human lymphocytes.

Treatments	Chromosomal aberrations									TCA/Cell±SE
CB	ICB	CG	ICG	CA	ICA	CE	P	DS		
EE (M)										
10	14	9	5	3	3	2	1	2	1	0.16±0.153[a]
EE (M)+PZE (g/mL)										
10+1.075×$^{10-4}$	9	4	3	2	1	0	0	0	0	0.076±0.091[b]
10+2.25×$^{10-4}$	7	3	2	1	1	0	0	0	0	0.056±0.084[b]
10+3.15×$^{10-4}$	5	3	2	1	0	0	0	0	0	0.044±0.073[b]
10+4.17×$^{10-4}$	3	2	2	1	0	0	0	0	0	0.032±0.041[b]
PZE (g/mL)										
1.075×$^{10-4}$	5	0	2	1	0	0	0	0	0	0.032±0.049
2.125×$^{10-4}$	6	0	1	1	0	0	0	0	0	0.032±0.043
3.15×$^{10-4}$	5	0	1	1	0	0	0	0	0	0.028±0.031
4.17×$^{10-4}$	4	1	1	1	0	0	0	0	0	0.028±0.034
Untreated Negative control	3	1	1	0	0	0	0	0	0	0.02±0.023
(DMSO 5 l/mL) Positive control	4	2	1	2	1	0	0	0	0	0.04±0.021
(CP, 0.16 g/mL)	86	39	14	10	33	13	12	7	6	0.88±0.176

A total 250 cells were scored for chromosomal aberrations. [a]P<0.005 significantly different from the untreated. [b]P<0.005 significantly different from the EE; CB, chromatid break; ICB, isochromatid break; CG, chromatid gap; ICG, isochromatid gap; CA, chromosome accentric fragment; ICA, isochromatid accentric fragment; CE, chromatid exchange; P, pulverization; DS, dicentric chromosome; EE, ethinylestradiol; PZE, *Plumbago zeylanica* extract.

Discussion

The results of the present study reveal that the leaf extract of *P. zeylanica* is potent in reducing the genotoxic as well as cytotoxic effects induced by ethinylestradiol. Our earlier study showed that ethinylestradiol at 0.50, 1.0 and 2.0 μl/mL of food concentration induced significant expression of *hsp70*.[18] The selected doses of *P. zeylanica* are not genotoxic itself, but reduce the genotoxic effects of 10 μM of ethinylestradiol. The results also show that the selected doses of *P. zeylanica* extract are not potent in inducing the significant expression of *hsp70* but are potent enough to reduce the *hsp70* expression induced by 0.50 and 1.0 μl/ml of ethinylestradiol. Having distinct protective role in living systems, HSPs are being exploited by toxicologists.[23-24] Being effective biosensor to even a minor assault, *hsp70* expression is considered now-a-days to be an effective marker for toxicological evaluations.25 *Drosophila* is a well established animal model for geneticists and molecular biologists. In the past years a significant contribution has been made by successfully employing transgenic *D. melanogaster* as an alternative animal model for toxicological research.[26] Though there is no comparative data, the studies by Hirsch et al.[27] indicates that fly and human have similar dose response relationship with lead exposure. Our earlier study with ethinylestradiol has shown that the metabolic activation and possible conversion of it to a reactive species is responsible for the genotoxicity.3 HSPs are formed in response to stressors like lipid peroxidation assay, DNA damage, osmotic imbalance, protein misfolding, membrane perturbation, metal and heat shock etc.[8] A dose dependent decrease in the activity of β-galactosidase clearly demonstrates the protective effect of the plant extract. Medicinal plants and their products have been used for centuries for cure of various ailments.[28] The main component of the *P. zeylanica* extract i.e. Plumbagin at 4, 8, and 16 mg/kg.bw has already been reported to induce micronuclei in the bone marrow of Swiss albino mice.10 Again the root extract of *P. zeylanica* at 250 and 500 mg/kg.bw given orally for 5 days reduced significantly micronucleated polychromatic erythrocytes.10 The verification of the possible mutagenic and antimutagenic effects of medicinal plants, infusion / extract is an important factor in these studies. Some plants may possess substances that can modulate the genotoxicity of the other compounds.29 The leaf extracts and various natural plant products have been reported to reduce the genotoxic effects of synthetic steroids.30-33 An increase in the frequency of chromosomal aberrations in the peripheral blood lymphocytes is associated with an overall increase in the risk of having cancer and the reduction in the genotoxic effects by certain natural plant agents/ extracts may be helpful in reducing the possibility of having cancer at higher doses or by prolonged therapy of steroids.[31,34] The protective effect of *P. zeylanica* in causing significant reduction in chromosomal aberrations and reduction in the activity of *hsp70* in the present study is due to the direct action of these compounds present in the extract of *P. zeylanica* against ethinylestradiol by inactivating it enzymatically or chemically. The present study also supports the use of transgenic *D. melanogaster (hsp70-lacZ)Bg*[9] model for the antigenotoxicity studies. The compounds present in the extract may act synergistically, as compared to a compound in isolation and this supports the indigenous system of medicine namely Ayurvedic, Siddha and Unani, that have been in existence for several centuries.35 The identification and characterization of the compounds present in the *P. zeylanica* extract and to determine their particular functions will be the part of our future study.

Table 2. β-galactosidase activity measured in transgenic *Drosophila melanogaster (hsp70-lacZ)Bg*[9] third instar larvae exposed to different concentrations of ethinylestradiol and Plumbago zeylanica extract.

Treatments	After 6 hr O.D. (Mean±SE)	After 24 hr O.D. (Mean±SE)	After 48 hr O.D. (Mean±SE)
EE (μl/mL)			
0.50	0.2802±0.0070[a]	0.2931±0.0083[a]	0.3146±0.0077[a]
1.0	0.3103±0.0073[a]	0.3094±0.0089[a]	0.3277±0.0086[a]
EE (μl/mL) + PZE (μl/mL)			
0.50+1	0.2511±0.0069[ab]	0.2703±0.0072[ab]	0.2915±0.0081[ab]
0.50+2	0.2433±0.0053[ab]	0.2614±0.0073[ab]	0.2833±0.0073[ab]
0.50+4	0.2401±0.0044[ab]	0.2533±0.0052[ab]	0.2767±0.0070[ab]
0.50+6	0.2346±0.0041[ab]	0.2421±0.0046[ab]	0.2704±0.0063[ab]
1.0+1	0.2947±0.0089[ab]	0.2843±0.0059[ab]	0.2997±0.0053[ab]
1. 0+2	0.2721±0.0084[ab]	0.2711±0.0063[ab]	0.2819±0.0072[ab]
1.0+4	0.2634±0.0079[ab]	0.2653±0.0071[ab]	0.2784±0.0073[ab]
1.0+6	0.2441±0.0066[ab]	0.2601±0.0064[ab]	0.2723±0.0068[ab]
PZE (μl/mL)			
1	0.2153±0.0021	0.2176±0.0036	0.2170±0.0024
2	0.2163±0.0023	0.2201±0.0032	0.2212±0.0022
4	0.2122±0.0018	0.2174±0.0031	0.2281±0.0021
6	0.2169±0.0016	0.2231±0.0037	0.2243±0.0019
Control	0.2133±0.0013	0.2147±0.0019	0.2201±0.0025
Negative control (DMSO, 2 μl/mL)	0.2243±0.0030	0.2197±0.0032	0.2244±0.0019

[a]Significant at P<0.001 with respect to control. [b]Significant at P<0.001 with respect to Ethinylestradiol treatment

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