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Protective Effect of the *Spilanthes acmella* Extract Against the Genotoxic Damage Induced by Cyproterone Acetate in Cultured Human Peripheral Blood Lymphocytes

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Abstract: In the present study the effect of *Spilanthes acmella* extract was studied against the genotoxic damage induced by 30 µM of cyproterone acetate (CPA) in cultured human peripheral blood lymphocytes. The extract was evaluated at $1.02x10^{-4}$, $2.125x10^{-4}$ and $3.15x10^{-4}$ and $4.17x10^{-4}$ g/ml of culture medium. The treatment of all the selected doses of *S. acmella* extract results in a significant dose dependent decrease in the genotoxic effect induced by the CPA treatment alone. The results suggests that the plant extract *per se* do not have genotoxic effect, but can modulate the effect of CPA in cultured human peripheral blood lymphocytes.

Key words: Cyproterone acetate · Human lymphocytes · Mitotic index · Chromosomal aberrations · Sister chromatid exchanges · Replication index · *Spilanthes acmella*

INTRODUCTION

Spilanthes acmella is a flowering herb belongs to a family Asteraceae [1]. It has been used traditionally as a medicine for toothache, headache and treatment of asthma, rheumatism, fever, sore throat and haemorrhoids [2]. The crude extract of the flower head of the plant shows a potent ovicidal, larvicidal and pupicidal activities [3].

Synthetic progestins are not only used in the treatment of sexual and metabolic disorders, but also in oral contraceptives formulations [4]. Earlier studies have shown that the synthetic progestins have DNA damaging potential in the presence or absence of metabolic activation [5-11] and their genotoxic effects can be reduced by the use of antioxidants and natural plant products [12-21]. In India, the majority of population uses traditional natural preparations derived from the plant materials for the treatment of various diseases and for that reason it becomes necessary to assess the modulating potential of the plants extract when associated with other substances. Cyproterone acetate (CPA) is not only a tumor initiating agent [22, 23], but also a genotoxic agent [23-25]. An increase in the genotoxicity is associated with the increase in the risk of developing cancer [26]. Studies of genotoxicity and antigenotoxicity of natural plant extracts can help us to evaluate the safety and effectiveness of herbal health products [27]. In present work we have studied the effect of *Spilanthes acmella* extract against the genotoxic dose of CPA, in cultured human peripheral blood lymphocytes.

MATERIALS AND METHODS

Chemicals: Cyproterone acetate (CAS No: 427-1-0, Sigma); RPMI 1640, Fetal calf serum, Phytohaemagglutinin-M, antibiotic-antimycotic mixture (In vitrogen); Dimethylsulphoxide, 5-Bromo-2-deoxyuridine, Colchicine (SRL), India); Giemsa stain (Merck).

Extract Preparation: *S. acmella* 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 8-10h at 40°C-60°C in Soxhlet's apparatus. After filteration, the excess of solvent was removed by rotatory evaporator.

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The extract concentrations of $1.075x10^{-4}$, $2.127x10^{-4}$, $3.15x10^{-4}$ and $4.17x10^{-4}$ g/ml of culture medium were established [18].

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 the cell culture [28]. The culture tubes were then placed in the incubator at 37°C for 24 h.

Chromosomal Aberration Analysis: Following a 24h, incubation, 30 μM of cyproterone acetate (dissolved in DMSO, 5 μl/ml) was given along with $1.075x10^{-4}$, $2.127x10^{-4}$, $3.15x10^{-4}$ and $4.17x10^{-4}$ g/ml of *S. acmella* extract of final dose of culture medium. The cells were allowed to incubate for the remaining 48h. Treatment of 0.2 ml of colchicine (0.2 μg/ml) was given to the culture tubes prior to 1 h of the harvesting. Cells were then centrifuged at 1000 rpm for 10 min. The supernatant was removed and 8 ml of prewarmed (37°C) 0.075M KCl (hypotonic solution) was added and the cells were 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 with 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 per dose per treatment per donor according to the recommendations of EHC 51 for short term test for mutagenic and carcinogenic chemicals [29].

Sister Chromatid Exchange Analysis: Bromodeoxyuridine (BrdU, 10 μg/ml) was added at the beginning of the culture. After 24 h, 30 μM of cyproterone acetate (dissolved in DMSO, 5 μl/ml) was given along with $1.075x10^{-4}$, $2.127x10^{-4}$, $3.15x10^{-4}$ and $4.17x10^{-4}$ g/ml of *S. acmella* extract. The cells were kept for the remaining 48h. 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 way as previously described for the 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 [30]. A total of 25 well spread and complete (2n=46) second division metaphases were scored per dose per treatment per donor. The frequency of SCE/chromosome was recorded according to Carrano and Natarajan [31].

Mitotic Index (MI) and Cell Cycle Kinetics (CCK): The mitotic index was calculated as the number of metaphases in 1500 cells analysed per culture for each dose a group and donor. At least 100 metaphases per culture for each dose group and donor were scored to calculate the replication index. Metaphase divisions were detected by the BrdU-Harlequin technique for differential staining of metaphase chromosomes [32-33]. The replication index (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 responses the regression analysis was also performed by using commercial, software programme Stat Soft Inc.

RESULTS

The results of the present study reveal that the doses of *S. acmella* extract are not genotoxic itself, but reduce the genotoxic effects of cyproterone acetate. A significant dose dependent decrease in the chromosomal aberration per cell was observed when the treatment of 30 μM of CPA was given along with the different doses of *S. acmella* extract i.e. $1.075x10^{-4}$, $2.127x10^{-4}$, $3.15x10^{-4}$ and $4.17x10^{-4}$ g/ml (Table 1). A significant dose dependent increase in the mitotic index was also observed when the treatment of 30 μM of CPA was given along with the different doses of *S. acmella* extract i.e. $1.075x10^{-4}$, $2.127x10^{-4}$, $3.15x10^{-4}$ and $4.17x10^{-4}$ g/ml (Table 1).The treatment of 30 μM of CPA results in the significant increase in the frequency of SCEs/chromosome as compared to the untreated (Table 2). The treatment of the different doses of the extract of *S. acmella* with the 30 μM of CPA results in the significant dose dependent decrease in the frequency of SCE/chromosome (Table 2).

Table 1: Effect of *Spilanthes acmella* extract on chromosomal aberrations and mitotic index induced by Cyproterone acetate

		Chromosomal abnormalities									
Treatment	MI±SE	CB	ICB	CG	ICG	CA	ICA	CE	P	DS	TCA/cell ±SE
CPA (µM)											
30	2.74±0.49[a]	35	25	19	16	13	3	1	5	3	0.240±0.103[a]
CPA (µM) + SAE(g/ml)											
30+1.075x10^{-4}	3.84±0.62[b]	20	13	10	9	6	0	0	0	0	0.116±0.083[b]
30+2.125x10^{-4}	3.98±0.65[b]	17	12	9	7	5	0	0	0	0	0.100±0.072[b]
30+3.15x10^{-4}	4.01±0.69[b]	15	10	9	6	5	0	0	0	0	0.090±0.006[b]
30+4.17x10^{-4}	4.27±0.71[b]	14	8	7	5	3	0	0	0	0	0.074±0.003[b]
SAE (g/ml)											
1.075x10^{-4}	4.59±0.82	9	7	8	7	6	0	0	0	0	0.074±0.003
2.125x10^{-4}	4.53±0.80	10	7	6	5	5	0	0	0	0	0.066±0.002
3.15x10^{-4}	4.50±0.73	11	6	6	5	6	0	0	0	0	0.068±0.002
4.17x10^{-4}	4.56±0.84	11	6	5	4	5	0	0	0	0	0.062±0.001
Untreated	4.80±0.90	8	5	5	4	6	0	0	0	0	0.056±0.001
Negative control (DMSO)	4.76±0.90[a]	9	6	5	5	4	0	0	0	0	0.058±0.001
Positive control (MMC)	1.36±0.19[a]	125	73	30	25	57	30	30	10	7	0.774±0.542[a]

A total of 500 cells were scored for chromosomal aberrations.
[a]P<0.005 significantly different from the untreated.
[b]P<0.005 significantly different from the CPA.
CB: Chromatid break; ICB: Isochromatid break; CG: Chromatid gap; ICG: Isochromatid gap; CA: Chromosome acentric fragment; ICA: Isochromatid acentric fragment; CE: Chromatid exchange; P: Pulverization; DS: Dicentric chromosome; DMSO: Dimethyl suphoxide (5µl/ml); MMC: mitomycin C (0.3 µg/ml) CPA: Cyproterone acetate; SAE: *Spilanthes acmella* extract.

Table 2: Effects of *Spilanthes acmella* extract on sister chromatid exchanges (SCEs) and replication index by Cyproterone acetate

Treatments	SCE/Chromosome±SE	RI±SE
CPA (µM)		
30	0.24±0.088[a]	1.61±0.36[a]
CPA (µM) + SAE(g/ml)		
30+1.075x10^{-4}	0.18±0.073[b]	1.73±0.60[b]
30+2.125x10^{-4}	0.16±0.065[b]	1.75±0.62[b]
30+3.15x10^{-4}	0.15±0.061[b]	1.76±0.64[b]
30+4.17x10^{-4}	0.13±0.056[b]	1.78±0.67[b]
SAE (g/ml)		
1.075x10^{-4}	0.10±0.036	1.89±0.80
2.125x10^{-4}	0.11±0.043	1.87±0.74
3.15x10^{-4}	0.11±0.040	1.86±0.72
4.17x10^{-4}	0.12±0.045	1.86±0.70
Untreated	0.09±0.021	1.94±0.89
Negative control (DMSO)	0.08±0.016	1.90±0.82
Positive control (MMC)	1.13±0.18[a]	1.34±0.16[a]

A total of 250 cells were scored for the SCE analysis and 100 cells were scored for RI
[a]P<0.005 significantly different from the untreated.
[b]P<0.005 significantly different from the cyproterone acetate.
CPA: Cyproterone acetate; SAE: *Spilanthes acmella* extract; DMSO: Dimethyl suphoxide (5µl/ml); MMC: mitomycin C (0.3 µg/ml)

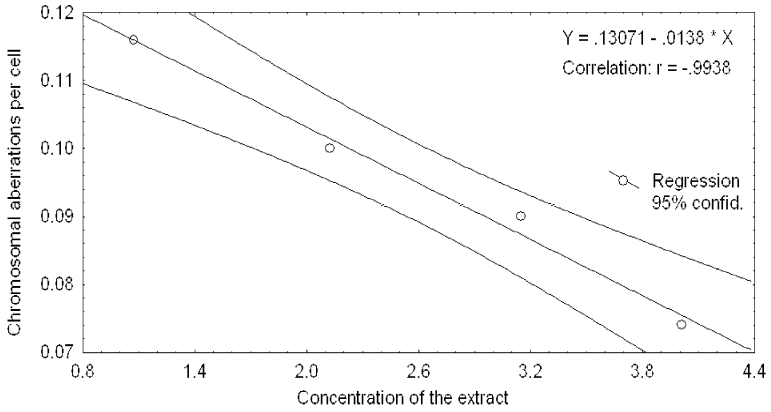

Fig. 1: Effect of *Spilanthes acmella* extract on the chromosomal aberrations per cell after the treatment along with 30 µM of Cyproterone acetate. The concentration of the extract is in (X 10^{-4} g/ml).

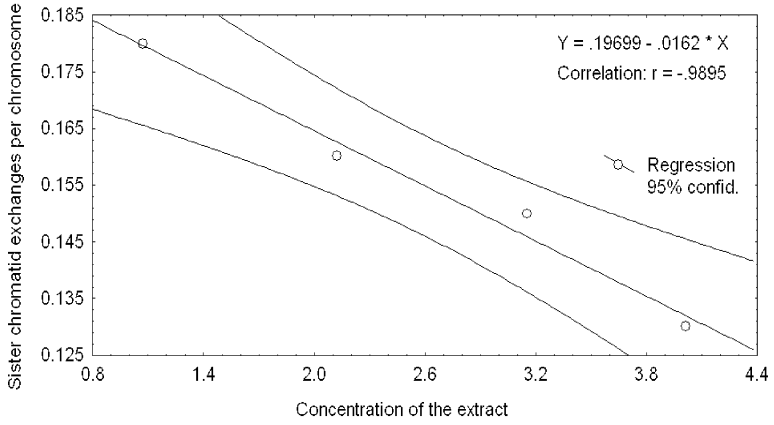

Fig. 2: Effect of *Spilanthes acmella* extract on the sister chromatid exchange per chromosome after the treatment along with 30 µM of Cyproterone acetate. The concentration of the extract is in (X 10^{-4} g/ml).

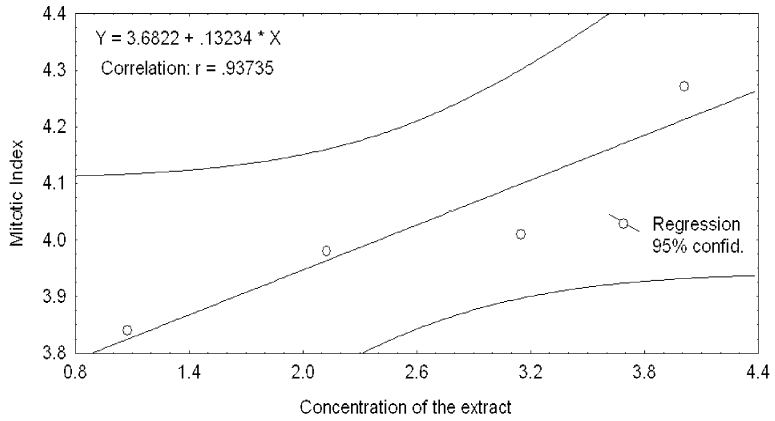

Fig. 3: Effect of *Spilanthes acmella* extract on the mitotic index after the treatment along with 30 µM of Cyproterone acetate. The concentration of the extract is in (X 10^{-4} g/ml).

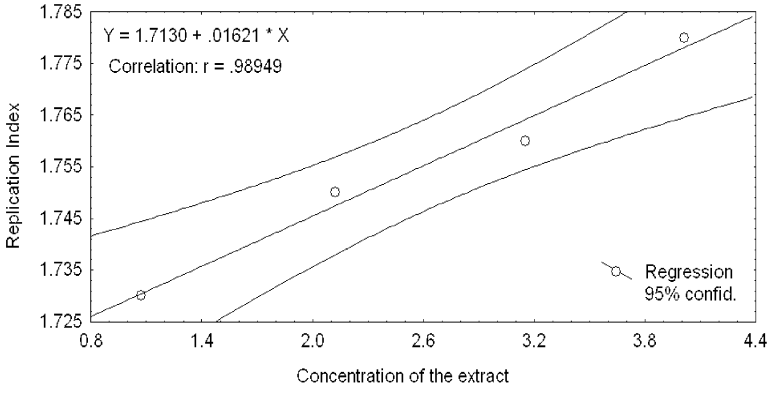

Fig. 4: Effect of *Spilanthes acmella* extract on the replication index after the treatment along with 30 µM of Cyproterone acetate. The concentration of the extract is in (X 10^{-4} g/ml).

The treatment of 30 µM of CPA results in the decrease in the replication index as compared to untreated (Table 2). A significant dose dependent increase in the replication index was observed when 30 µM of CPA treatment was given along with the different doses of the *S. acmella* extract (Table 2). Regression analysis was also performed to determine the dose effect of *S. acmella* extract on 30 µM of CPA, for mitotic index, chromosomal aberrations, sister chromatid exchanges and replication index. A decrease in

the slope of linear regression lines for chromosomal aberrations ($F=161.09$, $P<0.0005$) and sister chromatid exchange per chromosome ($F=93.62$; $P<0.0006$) was observed as the dose of the *S. acmella* extract was increased (Fig. 1 and 2). An increase in the slope of linear regression lines for mitotic index ($F=14.43$; $P<0.0007$) and replication index ($F=93.62$; $P<0.00001$) was observed as the dose of the *S. acmella* extract was increased (Fig. 3 and 4).

DISCUSSION

Our earlier study with cyproterone acetate shows that the generation of reactive oxygen species is responsible for the genotoxicity in cultured human peripheral blood lymphocytes [9]. The extract of *S. acmella* contain spilanthol, isobutylamide derivatives [34], α- and β-amyrin esters, stigmasterol, myricyl alcohol including sitosterol glucosides [35] and triterpenoidol saponins [36]. Besides the main active component spilanthol of *Spilanthes acmella* it has also stigmasteryl-3-o-β-D-glucopyranoside [37]. Identification and characterization of the active principles in the plant extract may lead to the strategies to reduce the risk for developing cancer in human [38]. The data obtained in the present study suggest that the compounds present in the extract of *S. acmella* are not genotoxic itself, but can reduce the genotoxicity of CPA in cultured human blood lymphocytes. The reduction in the genotoxicity of CPA by *S. acmella* extract is possibly due to the direct action of the compounds present in the extract that can scavenge the reactive oxygen species and thereby prevents the genotoxic damage of CPA. The compounds present in the extract may scavenge electrophiles/ nucleophiles [39] or they may enhance the DNA repair system or DNA synthesis or even may prevent the bio-activation of certain chemicals [40]. The compounds present in the extract may act synergistically as compared to the compound in isolation, as is evident in the present study and this supports the indigenous system of medicine namely Ayurvedic, Siddha and Unani, that have been in existence from centuries [1]. Hence, the present study also gives an indication that the extract of *S. acmella* may reduce the chances of having tumors in the patients undergoing CPA therapy. The identification and characterization of the compounds present in the *S. acmella* extract to determine their particular function will be the part of our future study.

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