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Possible modulating action of plant infusion of *Ocimum sanctum* L. on chromosomal aberrations and sister chromatid exchanges induced by chlormadinone acetate in human lymphocytes *in vitro*

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Abstract: *Chlormadinone acetate (CMA) is a synthetic progesterone analogue. It has its usage in oral contraceptives formulations and also for estrous synchronization of animals. The aim of the present study is to study the anti- genotoxic activity of the plant infusion against the CMA induced genotoxic damage on cultured human lymphocytes, using chromosomal aberrations and sister chromatid exchanges (SCFs) as parameters. For chromosomal aberration analysis, the treatment of 40 μM of CMA was associated with 4.33% abnormal metaphases. The treatment of 40 μM of CMA, separately with 1.075 x10^{-4}, 2.125 x10^{-4} and 3.15x10^{-4} gm l^{-1} of plant infusion results in the reduction of the number of abnormal metaphases i.e. 2.67%, 2.00% and 1.67% respectively. For sister chromatid exchange analysis, the frequency of sister chromatid exchange per cell (SCE$_S$ / Cell) for the treatment of 40 μM of CMA was 6.43. The treatment of 40 μM of CMA, separately with 1.075 x10^{-4}, 2.125 x10^{-4} and 3.15 x10^{-4} gml^{-1} of plant infusion results in the significant reduction of the frequency of SCE$_S$/Cell i.e. 3.76, 3.01 and 2.94, respectively, as compared to the CMA (40 μM) treatment alone(6.43) .The used dosages of plant infusion did not increase chromosomal aberrations and sister chromatid exchanges at significant level as compared to the untreated. The results of the present study suggest that the plant infusion per se does not have genotoxic potential, but can modulate the genotoxicity of chlormadinone acetate in human lymphocytes in vitro.*

Key words: *Ocimum sanctum, Chlormadinone acetate, Aqueous extract, Human lymphocytes, Genotoxicity*
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Introduction

Ocimum sanctum L. is considered as a sacred plant by Indians (Wagner *et al*., 1994). It is known as the holybasil in English and as tulsi or tulasi in Hindi. It belongs to family Lamiaceae (Labiatae) and is found throughout India and in many parts of the old world tropics and also occurs in the warmer parts of Australia (Dastur, 1962). Aqueous extract of *O. sanctum* leaves has been used for the treatment of variety of conditions including vomiting, fever, bronchitis, earache, diseases of the heart and blood, diabetes, arthritis and asthma since ancient times (Maity *et al*., 2000). Pharmacological evidences show that *Ocimum sanctum* also known as sacred basil possesses immunomodulating (Mediratta *et al*., 2002), hepatoprotective (Prashar *et al*., 1998), chemopreventive (Sharma *et al*., 2005), antidiabetic (Grover *et al*., 2002), anticancer (Aruna and Sivaramakrishnan, 1992), antioxidant (Kedlaya and Vasudevan, 2004; Yapallewar *et al*., 2004; Shymala and Devaki, 1996; Ganasoundari *et al*., 1997), antimutagenic (Vrinda and Devi, 2001; Ganasoundari *et al*., 1998; Ganasoundari *et al*., 1997b), antiulcerogenic (Dharmani *et al*., 2004), anticataract (Sharma *et al*., 1998), anti-inflammatory (Singh *et al*., 1996) and antihelmintic properties (Asha *et al*., 2001). Chlormadinone acetate is a synthetic progestin. It is used as oral contraceptives and in the treatment of sexual and metabolic disorders (IARC, 1987). Earlier reports suggest that synthetic progestins have genotoxic potential (Siddique and Afzal, 2004 a,b; 2005a; Siddique *et al*., 2005a). The genotoxic effects of steroids can be reduced by the use of antioxidants (Ahmad *et al*., 2002; Siddique *et al*., 2005b,c) and natural plant products (Ahmad *et al*., 2004; Siddique and Afzal, 2004c; Siddique *et al*., 2006, 2007). In our earlier study it was reported that chlormadinone acetate induced genotoxic damage in cultured human lymphocytes by generating reactive oxygen species (Siddique and Afzal, 2004d). Since the chlormadinone acetate have genotoxic potential and have also been reported to induce mammary tumors in dogs (IARC, 1987), the present study is to investigate the antigenotoxic effect of *O. sanctum* infusion at different dosages on chromosomal aberrations and sister chromatid exchanges induced by chlormadinone acetate in human lymphocytes *in vitro*.

Materials and Methods

Chemicals: Chlormadinone acetate (CMA) (CAS No: 302-22-7) was purchased from Wyeth Lab. RPMI 1640, phytohaemagglutinin-M, antibiotic-antimycotic mixture, fetal calf serum was purchased from Gibco. Dimethylsulphoxide and giemsa solution were purchased from Merk. 5-bromo-2-deoxyuridine was purchased from SRL, India.

Chlormadinone acetate: Chlormadinone acetate (6-chloro-17-hydroxypregna-4, 6-diene-3, 20-dione acetate) is a synthetic progesterone analogue. It is a derivative of 17α-hydroxy progesterone (Pregnanes) having chlorine atom (Cl) at carbon-6 (Schindler *et al*., 2003). It is used in oral contraceptives formulations either single entity or with estrogens (estradiol 17-β ethinylestradiol / mestranol) and also for estrous synchronization of animals (IARC, 1987). After oral intake it is readily absorbed and undergoes nearly no first pass metabolism and its bioavailability is nearly 100% (Schindler *et al.,* 2003).

Infusion preparation: The infusion was prepared by chopping the *Ocimum sanctum* leaves into small pieces with a pair of scissors.

Chopped leaves (1 g) were placed in 100 ml boiling distilled water and covered for 5 min without heating. The material was then shaken for 5 min and filtered for sterilization and placed in a refrigerator. The infusion concentrations of $1.075x10^{-4}$, $2.125x10^{-4}$ and $3.15x10^{-4}$ gml^{-1} of culture medium were established (Roncada *et al.,* 2004).

Human lymphocytes culture: Duplicate peripheral blood cultures were prepared according to Carballo *et al*. (1993). Briefly, heparinized blood samples (0.5 ml) were obtained from healthy female donor and were placed in sterile flasks containing 7 ml of RPMI-1640 medium supplemented with fetal calf serum (1.5 ml), antibiotic-antimycotic mixture (1.0 ml) and phytohaemagglutinin (0.1 ml). The cultures were placed in an incubator for 24 hr at 37°C.

Chromosomal aberration analysis: After 24 hr, 40 mM of chlormadinone acetate dissolved in dimethylsulphoxide was treated with $1.075x10^{-4}$, $2.127x10^{-4}$ and $3.15x10^{-4}$ g ml^{-1} of plant infusion separately, and kept for another 48 hr at 37°C in the incubator. After 47 hr, 0.2 ml of colchicine (0.2 μg ml^{-1}) was added to the culture flask. Cells were centrifuged at 1000 rpm for 10 min. The supernatant was removed and 5 ml of prewarmed (37°C) KCl hypotonic solution (0.075 M) was added. Cells were re-suspended and incubated at 37°C for 15 min. The supernatant was removed by centrifugation at 1000 rpm for 10 min, and 5 ml of chilled fixative (methanol: glacial acetic acid; 3:1) was added. The fixative was removed by centrifugation and the procedure was repeated twice. The slides were stained in 3% Giemsa solution in phosphate buffer (pH 6.8) for 15 min 300 metaphases were examined for the occurrence of different types of abnormality (Cid *et al.,* 1995). Criteria to classify the different types of aberrations were in accordance with the recommendation of Environmental Health Criteria 46 for Environmental Monitoring of Human Population (IPCS, 1985).

Sister chromatid exchange analysis: For the analysis of sister chromatid exchanges bromodeoxyuridine (10 μg ml^{-1}) was added at the beginning of the culture. After 24 hr, 40 mM of chlormadinone acetate dissolved in dimethylsulphoxide, along with $1.075x10^{-4}$, $2.127x10^{-4}$ and $3.15x10^{-4}$ g ml^{-1} of plant infusion was added separately and kept for another 48 hr at 37°C in an incubator. Mitotic arrest was done 1 hr prior to harvesting by adding 0.2 ml of colchicine (0.2 μg ml^{-1}). Hypotonic treatment and fixation were performed in the same way as described in chromosomal aberrations analysis. The slides were processed according to Perry and Wolff (1974). The average of sister chromatid exchanges was taken from an analysis of the metaphase during second cycle of division.

Statistical analysis: Student's *t*-test was used for the analysis of chromosomal aberrations and sister chromatid exchanges. Regression analysis was also performed for the dose effect of infusion on chlormadinone induced genotoxic damage. The level of significance was tested from standard statistical table of Fisher and Yates (1963).

Results and Discussion

The result of the present investigation reveals that the plant infusion results in the reduction of the genotoxic damage. For chromosomal aberration analysis the chromosome breaks were completely eliminated when the treatment of 40 μM of chormadinone acetate, separately, was given with $2.127x10^{-4}$ and $3.15x10^{-4}$ g ml^{-1} respectively. The tested dosages of plant infusion were associated with only chromatid types of breaks that were not significant, when compared with the untreated (Table 1).

The frequency of sister chromatid exchanges/ cell was found to be 6.43 when the treatment of 40 μM of chlormadinone acetate was given alone. It was significant when compared to the untreated (1.83). The treatment of 40 μM of chlormadinone acetate separately with the $1.075x10^{-4}$, $2.127x10^{-4}$ and $3.15x10^{-4}$ gml^{-1} of plant infusion showed a significant decrease in the frequency of sister chromatid exchanges/cell *i.e.* 3.76, 3.01 and 2.94 respectively, as compared to the chlormadinone treatment alone (6.43) (Table 2). Regression analysis was also performed to determine the dose effect of *O. sanctum* L. infusion on 40 μM of chlormadinone acetate for a number of abnormal metaphases and sister chromatid exchanges. For abnormal metaphases ($F=29.14$; $p<0.0414$) and SCE ($F=4.52$; $p<0.066$) decrease in slope of linear regression lines were observed as the dose of infusion was increased, in each of the treatment showing that the selected doses of infusion are potent in reducing the genotoxic damage induced by chlormadinone acetate (Table 3). Table 3 shows the regression equation obtained to show the dose effect of plant infusion on abnormal metaphases ($y = 9.3975-1.448x$) and sister chromatid exchanges ($y = 4.0759- 0.3965x$) induced by the treatment of 40 μM of chlormadinone acetate.

In our present study the treatment of the infusion reduced the frequency of sister chromatid exchanges and chromosomal aberrations in the system, thereby indicating the possibility of reducing the chances of carcinogenesis during the chlormadinone acetate therapy in patients. Chlormadinone acetate generates free radicals that are responsible for the genotoxic damage (Siddique *et al.,* 2004d). Chromosomal aberrations are the changes in chromosome structure resulting from a break or an exchange of chromosomal material. Most of the chromosomal aberrations observed in the cells are lethal, but there are many corresponding aberrations that are viable and can cause genetic effects, either somatic or inherited (Swierenga *et al.,* 1991). These events may lead to the loss of chromosomal material at mitosis or due to the inhibition of accurate chromosomal segregation at anaphase. The synthetic progestins have been classified as the possible carcinogens (Group 2) by the International Agency on Cancer (Martelli *et al.,* 2003). As the plant infusions are used as an alternative medicine it becomes necessary to detect one or more active principles present in the infusions that are potentially useful for the mankind. Sacred basil contains a volatile oil consisting of about 70% eugenol as well as methyleugenol and caryophyllene (Agrawal *et al*., 1996). Other constituents with likely pharmacological activity include the triterpenoid ursolic acid, rosmarinic acid, alkaloids, saponins, flavonoids, phenylpropane glucosides and tannins (Wagner *et al*., 1994; Devi *et al*., 1998; Kelm *et al*., 2000). Phenolic compounds like eugenol, rosmarinic acid and apigenin are excellent antioxidants (Ganasoundari *et al*., 1997a; Ganasoundari *et al*., 1998; Ganasoundari *et al*., 1997b). In liver microsomes ursolic acid prevents the lipid peroxidation *in vitro* (Balanehru and Nagarajan, 1991). The leaf extract of *O. sanctum*

Table - 1: Effect of *Ocimum sanctum* L. infusion on chromosomal aberrations induced by chlormadinone acetate in human lymphocytes

Treatments	Abnormal metaphases without gaps		Chromosomal aberrations		
	Number	Percentage Ψ	Gaps	CTB	CSB
CMA					
40 μM	13	4.33 ± 1.17[a]	6	9	4
CMA + OSi					
40 μM + 1.075x10^{-4} g ml^{-1}	8	2.67 ± 0.93[b]	4	7	1
40 μM + 2.125x10^{-4} g ml^{-1}	6	2.00 ± 0.80[b]	3	6	-
40 μM + 3.15x10^{-4} g ml^{-1}	5	1.67 ± 0.73[b]	3	5	-
Untreated	2	0.67 ± 0.47	1	2	-
OSi					
1.075x10^{-4} g ml^{-1}	2	0.67 ± 0.47	1	2	-
2.125x10^{-4} g ml^{-1}	2	0.67 ± 0.47	1	2	-
3.15x10^{-4} g ml^{-1}	3	1.00 ± 0.57	1	3	-
Negative control (DMSO, 5 μl ml^{-1})	2	0.67 ± 0.47	1	2	-

[a]Significant with respect to untreated ($p < 0.01$), [b]Significant with respect to CMA ($p < 0.05$), OSi = *Ocimum sanctum* L. infusion, CTB = Chromatid break. CSB = Chromosome break, CMA = Chlormadinone acetate, DMSO = Dimethylsulphoxide, SE = Standard error, Ψ = Values are mean ± SE

Table - 2: Effect of *Ocimum sanctum* L. infusion on sister chromatid exchanges induced by chlormadinone acetate in human lymphocytes

Treatment	Cells scored	SCEs/Cell Ψ	Range
CMA			
40 μM	50	6.43 ± 0.41[a]	2 – 7
CMA + OSi			
40 μM + 1.075x10^{-4} g ml^{-1}	50	3.76 ± 0.24[b]	1 – 6
40 μM + 2.125x10^{-4} g ml^{-1}	50	3.01 ± 0.19[b]	1 – 6
40 μM + 3.15x10^{-4} g ml^{-1}	50	2.94 ± 0.18[b]	1 – 5
Untreated	50	1.83 ± 0.08	0 – 5
OSi			
1.075x10^{-4} g ml^{-1}	50	1.93 ± 0.12	0 – 5
2.125x10^{-4} g ml^{-1}	50	2.10 ± 0.14	1 – 5
3.15x10^{-4} g ml^{-1}	50	2.32 ± 0.16	1 – 5
Negative control (DMSO, 5 μl ml^{-1})	50	1.77 ± 0.07	0 – 5

[a]Significant with respect to untreated ($p < 0.01$), [b]Significant with respect to CMA ($p < 0.05$)
OSi = *Ocimum sanctum* L. infusion, CMA = Chlormadinone acetate, DMSO = Dimethylsulphoxide, SE = Standard error, Ψ = Values are mean ± SE

Table - 3: Regression analysis for the dose effect of *Ocimum sanctum* L infusion on abnormal metaphases and sister chromatid exchanges induced by chlormadinone acetate (40 μM)

S.No.	Parameters	Regression equation	r	R	R^2	F	p
1	Abnormal metaphases	Y = 9.3975 – 1.448x	-0.9833	0.9832	0.9668	29.14	<0.041
2	Sister chromatid exchanges	Y = 4.0759 – 0.3965x	-0.9049	0.9049	0.8189	4.52	<0.066

reduces the radiation induced damage in mouse bone marrow cells by scavenging free radicals (Ganasoundari *et al*., 1997a). Two water soluble flavonoids isolated from the holy basil *i.e.* orientin and vicenin showed a protection against radiation induced genotoxic damage in cultured human lymphocytes by scavenging free radicals in the system (Vrinda and Devi, 2001). The use of synthetic progestins cannot be completely eliminated as oral contraceptives, and the genotoxic damage caused by them can be reduced by the use of antioxidants (Ahmad *et al*., 2002; Siddique *et al*., 2005b; Siddique and Afzal, 2005b) and natural plant products (Ahmad *et al*., 2004; Siddique and Afzal, 2004c; Siddique *et al*., 2006). In this context natural plant products can take care of the genotoxic damage induced by chemical or physical agents. Chlormadinone acetate generates free radicals that are responsible for the genotoxic damage (Siddique and Afzal, 2004d). *O. sanctum* leaf extract infusions have two water soluble flavonoids *i.e.* orientin and vicenin that are potent free radical scavengers (Vrinda and Devi, 2001). The reduction in the genotoxic damage caused by the chlormadinone acetate might be due to the free radical scavenging property of these flavonoids present in the infusion. Therefore, the traditional methods should be employed in using the plant extracts taking utmost care with regard to its concentration and duration of treatments, so that the infusions have the desired pharmacological effects without causing any toxicity.

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