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Modulatory Effect of Distillate of *Ocimum sanctum* Leaf Extract (Tulsi) on Human Lymphocytes Against Genotoxicants

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Objective To study the modulatory effect of distillate of *Ocimum sanctum* (traditionally known as Tulsi) leaf extract (DTLE) on genotoxicants. **Methods** In the present investigation, we studied the antigenotoxic and anticlastogenic effect of distillate of Tulsi leaf extract on (i) human polymorphonuclear leukocytes by evaluating the DNA strand break without metabolic activation against mitomycin C (MMC) and hexavalent chromium (Cr^{+6}) and (ii) human peripheral lymphocytes (*in vitro*) with or without metabolic activation against mitomycin C (MMC), hexavalent chromium (Cr^{+6}) and B[a]P by evaluating chromosomal aberration (CA) and micronucleus assay (MN). Three different doses of DTLE, 50 μL/mL, 100 μL/mL, and 200 μL/mL were selected on the basis of cytotoxicity assay and used for studying DNA strand break, chromosomal aberration and micronucleus emergence. The following positive controls were used for inducing genotoxicity and clastogenicity: MMC (0.29 μmol/L) for DNA strand break, chromosomal aberration and 0.51 μmol/L for micronucleus assay; Potassium dichromate (Cr^{+6}) 600 μmol/L for DNA strand break and 5 μmol/L for chromosomal aberration and micronucleus assay; Benzo[a]pyrene (30 μmol/L) for chromosomal aberration and 40 μmol/L for micronucleus assay. The active ingredients present in the distillate of Tulsi leaf extract were identified by HPLC and LC-MS. **Results** Mitomycin C (MMC) and hexavalent chromium (Cr^{+6}) induced statistically significant DNA strand break of respectively 69% and 71% (P<0.001) as revealed by fluorometric analysis of DNA unwinding. Furthermore, the damage could be protected with DTLE (50 μL/mL, 100 μL/mL, and 200 μL/mL) on simultaneous treatment. Chromosomal aberration and micronucleus formation induced by MMC, Cr^{+6} and B[a]P were significantly protected (P<0.001) by DTLE with and without metabolic activation. **Conclusion** Distillate of Tulsi leaf extract possesses antioxidants contributed mainly by eugenol, luteolin and apigenin as identified by LC-MS. These active ingredients may have the protective effect against genotoxicants.

Key words: Distillate of Tulsi leaf extract (DTLE); Cytotoxicity; DNA strand break; Chromosomal aberration (CA); Micronucleus (MN); Hexavalent chromium (Cr^{+6}); Mitomycin C (MMC); Benzo[a]pyrene (B[a]P)

INTRODUCTION

Ocimum sanctum (traditionally known as Tulsi), found throughout the semi tropical and tropical parts of India, is well known for its medicinal value since time immemorial. Several recent investigations showed that *Ocimum sanctum* possesses significant anti-inflammatory, antipyretic, analgesic, anti-anesthetic, and chemo-preventive properties[1-2]. It has been reported that leaf distillate of this plant has significant ability to scavenge reactive free radicals[3].

It has been well established that biological effects of MMC, Cr^{+6}, and B[a]P induce formation of reactive oxygen species (ROS), which are known to cause oxidative stress to several cellular molecules (DNA, lipids, and proteins) and cause 8-hydroxydeoxyguanosine (8-OH-dG) formation, DNA strand breaks, lipid peroxidation, and p53 activation[4]. Phytoderived polyphenols and flavonoids can prevent injuries by directly scavenging of free radicals. *Ocimum sanctum*, has long been known for its antioxidant activity. However, information is scanty on modulatory effect of DTLE on genotoxicants.

The present study was, therefore, conducted to establish antigenotoxic/anticlastogenic effect of DLTE in the presence and absence of external metabolic activation (S9-mix), and to identify and quantify phytochemicals present in the distillate using

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HPLC and LC-MS.

MATERIALS AND METHODS

Chemicals and Media

Dulbacco's modified Eagle's medium (DMEM) for culturing human lymphocytes, mitomycin C (MMC), benzo[a]pyrene, apigenin, luteolin, cytochalasin-B (Cyt-B), colchicines, and dimethyl sulfoxide(DMSO), Arochlor-1254, β-nicotinamide adenine di-nucleotide phosphate, D-glucose -6-phosphate sodium salt were purchased from Sigma (St. Louis, MO, USA). Potassium dichromate was purchased from Merck, India. Penicillin, streptomycin, and phytohemagglutinin M were purchased from GIBCO, Invitrogen Corporation (UK). Ethidium bromide, sodium sulphate, D-glucose, magnesium sulphate, sodium hydrogen phosphate (dibasic and monobasic), and Giemsa stain were procured from Hi-Media Laboratories, India. Tris-HCl, ammonium chloride, potassium dichromate, trypan blue, sodium hydroxide and potassium chloride were obtained from Sisco Research Laboratories, India. Heat-inactivated fetal bovine serum was purchased from Life Technology (UK).

Plant Material

Fresh leaves of *Ocimum sanctum* were collected from the botanical garden of National Environmental Engineering Research Institute (NEERI), Nagpur, Maharashtra located in central India.

Extraction and Sample Preparation

The freshly collected leaves were washed with distilled water and the excess water was wiped out. About 10 gm of these leaves was ground in a homogenizer and the homogenate was distilled in a temperature-controlled distillation apparatus. The distillate was collected and filter sterilized at room temperature (24℃-28℃) by using 0.45 μm-membrane filter.

Instruments

Active compounds present in the distillate of Tulsi leaf extract (DTLE) were identified and quantified using HPLC and LC-MS system (Waters, UK).

HPLC analysis The HPLC system is comprised of a Water LC 1525 pump, a UV-Vis detector (Waters 2487), a column of C-18 [(5 μm) and 3.9 mm (id) × 300 mm (length)], a chart recorder at the chart of 1 cm/min, a mobile phase of MeOH:H_2O. Initial conditions were deionized water (A) 75% and (B) 25% with linear gradient reaching B=100% at 20 min. This was followed by isocratic elution (B=100%) for 24 min, after which the programme returned to its initial solvent composition. Sample volume used was 10 μL. Column temperature was maintained at 35℃, flow rate of mobile phase was maintained at 1 mL/min throughout the experiment. Retention time and peak heights of flavonoids were compared with those of standards for the purpose of their identification and quantification.

HPLC With Electro Spray Ionization Mass Spectrometry (ESI LC-MS)

The LC-MS used in the present investigation involved a quadrupole mass spectrometer (MicroMass Quattro Ultima UK) with the sample ionized by electrospray ionization (ESI) source at 250℃ with a flow rate of 0.15 mL/min. Masslynx 3.5 software was programmed to record mass spectra for positive identification.

Cells

Peripheral blood samples were collected by veinpuncture in heparinized syringes from non-smoking healthy volunteers (aged 25-30 years) using established blood-borne pathogen/biohazard safety protocols.

Cytotoxicity Test

The cytotoxicity of DTLE to human polymorphonuclear leukocytes (HPNLs) was determined by trypan blue dye exclusion assay[5]. Around 1×10^6 cells/mL were treated with different concentrations of DTLE (10 μL/mL, 50 μL/mL, 100 μL/mL, 200 μL/mL, and 500 μL/mL) at different time intervals (up to 12 h).

Test Chemicals

The dose of potassium dichromate (Cr^{+6}) for DNA strand break was 600 μmol/L[6] and 5 μmol/L[7] for CA and MN. The dose of MMC for DNA strand break and CA was 0.29 μmol/L, and 0.51 μmol/L for MN assay[8-9]. The dose of benzo[a]pyrene was 30 μmol/L for CA, and 40 μmol/L for MN assay[10]. MMC and Cr^{+6} were used as a direct acting positive control and B[a]P as a metabolic activated positive control.

Fluorimetric Analysis of DNA Unwinding (FADU) Assay

The modified procedure for FADU was followed with minor modifications[11-12]. Human polymorphonuclear leukocytes (HPNLs) at the concentration of 5×10^6 cells/mL, were treated in test solution with a final concentration of Cr^{+6} (600 μmol/L) and MMC (0.29 μmol/L), different concentrations of DTLE (50 μL/mL, 100 μL/mL, and

200 µL/mL), 0.1% DMSO and sterile distilled water (negative control) in a final volume of 1 mL for 1 hour. To study the ameliorative effect of DTLE on DNA strand breaks induced by test chemicals, the cells were treated with MMC and Cr^{+6} along with DTLE simultaneously and incubated for 1 hour in 5% CO_2 incubator at 37℃. The treatment was terminated by the addition of 5 mL ice-cold saline (0.9 % NaCl). The treated and control cells were centrifuged at 400×g for 10 minutes at 4℃, pellet was obtained and resuspended in solution B and the volume was made up to 2.0 mL. The suspended HPNLs were processed for FADU assay.

In vitro Human Lymphocyte Metaphase Analysis

Tests were performed according to the OECD Guideline 473[13] for chromosomal aberration assay and Guideline 487 for micronucleus assay[14].

Metabolic Activation System

The procedures of Garner *et al.*[15] and Ames *et al.*[16] were used for the preparation of rat liver homogenate (S9). Sprague-Dawley rat liver was stimulated following an i.p. injection of Aroclor-1254 (dissolved in peanut oil) at a dose of 500 mg/kg per day for five consecutive days. On the sixth day of induction, following an overnight fasting, animals were sacrificed by cervical dislocation for rat liver homogenate (S9). The Bradford assay (595 nm) was used to measure the protein concentration in the S9 fraction. All steps of the preparation were performed at 0℃-4℃ with cold sterile solutions and glassware. The S9 fraction was distributed in 2 mL aliquots in small sterile plastic tubes and stored at -80℃. The S9 mix was prepared following the method of Ames *et al.*[16].

Sample Preparation for Clastogenic Assay

Around 0.5 mL of human venous blood was added to 3.5 mL of DMEM (human lymphocyte culture media) supplemented with 20% of fetal bovine serum to which phytohemagglutanin (PHA) (50 µg/mL), antibiotics (Penicillin 100 IU/mL and streptomycin 50 µg/mL) and heparin sodium salt, 5000 IU (0.4 mL/100 mL) were added and incubated at 37℃ for up to 72 h. After 24 hours of culture initiation, lymphocytes were treated with DTLE at different doses (50 µL/mL, 100 µL/mL, and 200 µL/mL) and clastogens MMC, Cr^{+6}, and benzo(a)pyrene to study their effects on chromosomes. For protective effect, studies were carried out in two sets: lymphocytes were simultaneously treated (i) with DTLE and MMC for 1 hour, with DLTE and Cr^{+6} for 3 hours without metabolic activation, and (ii) with DTLE and B[a]P for 2 hours with metabolic activation using S9-mix (i.e. 2% of S9, final concentration).

Chromosomal Aberration Assay

After treatment, the cultures were washed, complete medium was added and further incubated in a 5 % CO_2 incubator at 37℃ for 72 hours. Cells were treated with colchicine (0.1 µg/mL) 2 hours before harvesting. The cultures were processed and the slides were prepared for CA according to the method of Api and San[17]. Hundred well spread metaphases were scored for aberration study *viz* chromatid and chromosome breaks, fragments, exchanges, rings, and gaps.

Micronucleus Assay

After treatment, the cultures were washed, complete medium was added and further incubated in a 5 % CO_2 incubator at 37℃ for 72 hours. At the 44th hour, the cultures were treated with 0.6 µg/mL of cytochalasin B to arrest the cells in a binucleated state and incubated till the completion of assay (at the 72th hour). At the end of the incubation period, the cultures were processed and the slides were stained with 4% Giemsa stain for 10 min. About 2000 binucleated cells with well-preserved cytoplasm were scored for the presence of micronucleus[18].

Statistical Analysis

The $\bar{x} \pm s$ was calculated for each parameter. The data were subjected to one-way ANOVA test using "Analyze It Software". To evaluate the protective property of distillate, the simultaneously treated cultures (DTLE + clastogens) were compared with the cultures exposed to clastogens only.

RESULTS

Figure 1A-C shows the ingredients present in distillate of DTLE, that were identified as eugenol, luteolin and apigenin by comparing the elution profile of distillate of DTLE with the respective standards. Molecular weight of each component was determined by electrospray ionization mass spectrometry and matched with that of standard. Quantification of luteolin (4.8 mg/g) and apigenin (4.9 mg/g) was carried out on dry weight basis and eugenol was identified on the basis of mass spectra using HPLC and LC-MS.

Before studying the antigenotoxic and anticlastogenic property of DTLE, the cytotoxic potential of DTLE was determined by trypan blue dye exclusion assay for different doses at different time intervals. As shown in Fig. 2, distillate could not induce statistically significant level (P>0.05) of cell

death at all doses tested (1-500 µL/mL) up to 12 hours exposure.

Figures 3-5 show the antigenotoxic effect of DTLE on human polymorphonuclear leukocytes. When the cells were treated with DTLE at the doses of 50 µL/mL, 100 µL/mL, and 200 µL/mL, they showed 29%, 32%, and 33% of DNA strand breaks respectively. These were not significant when compared to the control showing 24%. DNA stand breaks induced by MMC and Cr^{+6} observed during the investigation were 69% and 71% respectively. The maximum protection by DTLE was observed at 200 µL/mL showing 63% protection with MMC and 67% protection with Cr^{+6}.

Tables 1 and 2 show the anticlastogenic effect of DTLE on human lymphocyte culture (HLC) against MMC and Cr^{+6} without metabolic activation and that of B[a]P with metabolic activation. When human lymphocytes were treated with DTLE alone at different doses of 50 µL/mL, 100 µL/mL, and 200 µL/mL, no increase in the number of chromosomal aberration and significant decrease in mitotic index were observed when compared with negative control. As DTLE at 500 µL/mL showed a reduction in Mitotic index (MI), further studies were carried out with lower doses on the basis of mitotic index as it is an

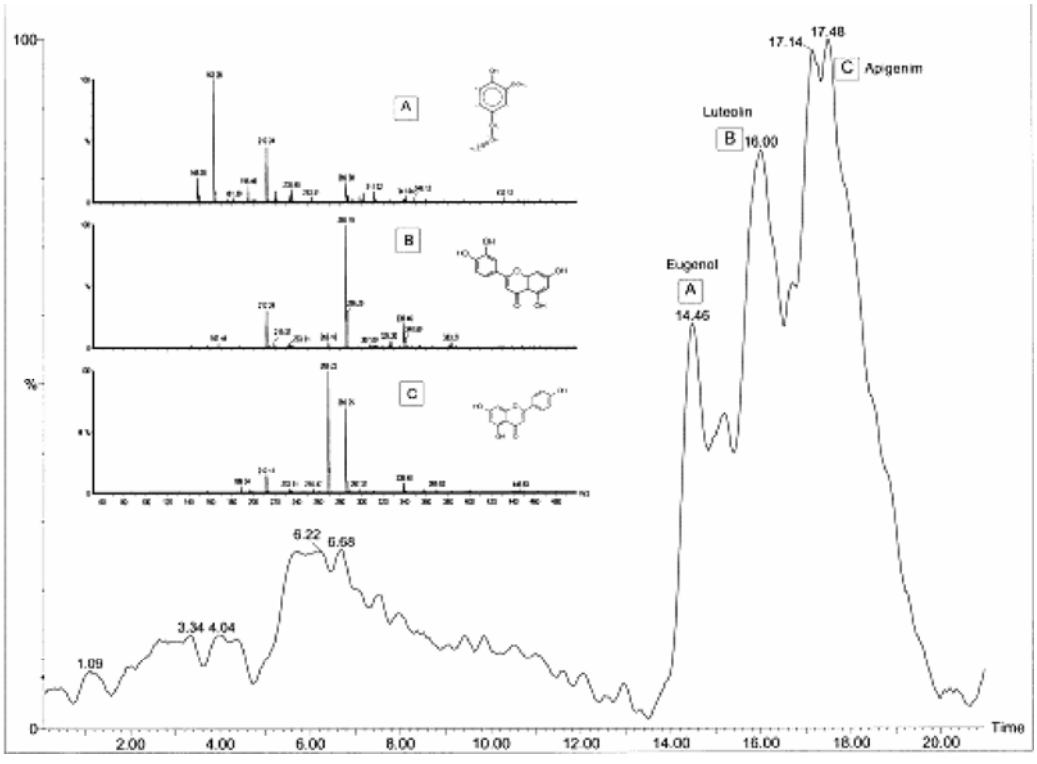

FIG. 1. Reconstituted total ion chromatograph and mass fragmentation pattern of the compounds present in distillate of *Ocimum sanctum* (Tulsi) leaf extract (DTLE).

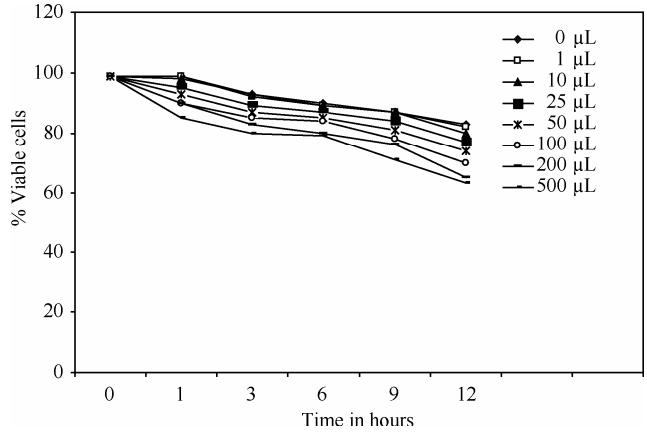

FIG. 2. Effect of distillate of Tulsi leaf extract (DTLE) on cell viability determined by trypan blue dye exclusion assay in the human lymphocytes. The results are average of five sets of experiments ± standard deviation.

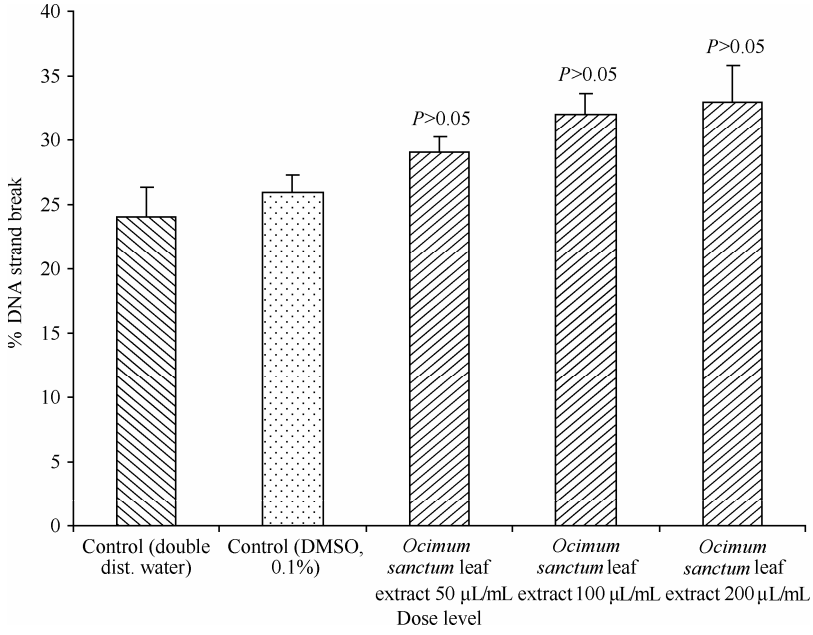

FIG. 3. Effect of distillate of *Ocimum sanctum* leaf extract on DNA in human polymorphonuclear leukocytes. The results are average of five sets of experiments ± standard deviation.

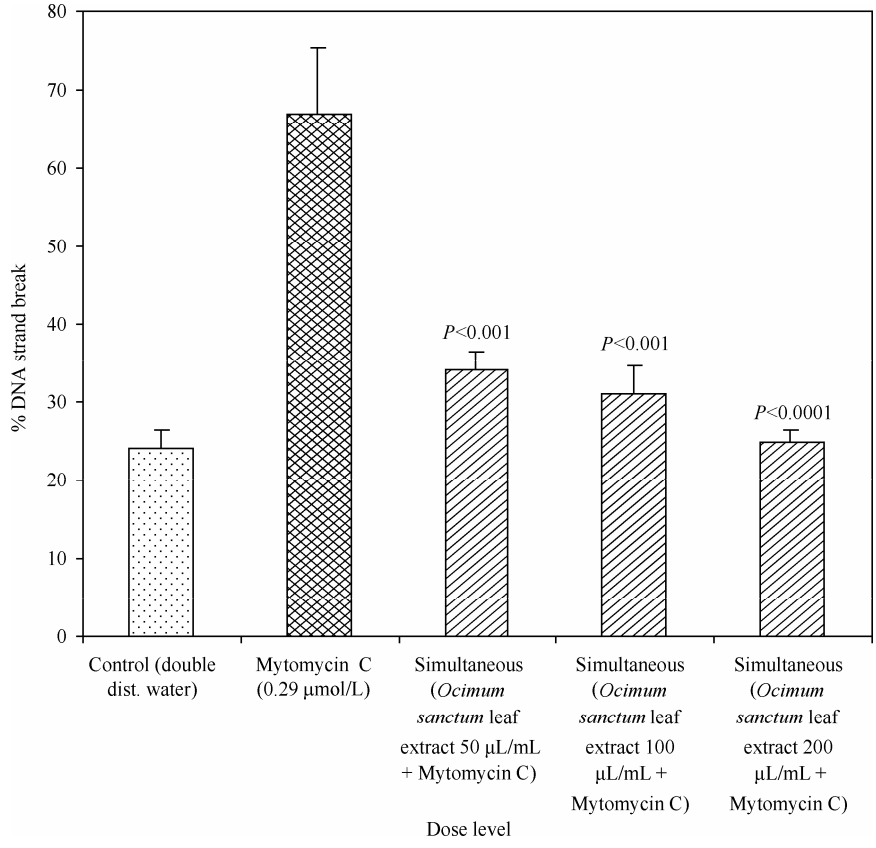

FIG. 4. Protective effect of simultanous treatment on HLC with DTLE against mitomycin C induced DNA strand break (0.29 µmol/L). The results are average of five sets of experiments ± standard deviation.

indicator of cytotoxicity. Statistically significant reduction in the number of chromosomal aberration induced by MMC, Cr^{+6}, and B[a]P were observed on simultaneous treatment with DTLE at different doses, both with and without metabolic activation. However, better protection was observed in presence of metabolic

TABLE 1

Protective Effect of Simultaneous Treatment on Human Lymphocytes With DTLE Against Chromosomal Aberration Induced by Mitomycin C and Hexavalent Chromium Without S-9mix

Test Substance	No. of Metap Hases	Percentage of Aberrated Metaphases ($\bar{x} \pm s$)	No. of Aberrated Metaphases	Types of Aberration								Mitotic Index	% Protectio
				G	Ctb	Chb	R	M	F	Dic	Exc		
Cell Control Sterile double dist. Water)	100	3 ± 1.581	3	ND	ND	ND	ND	1	2	ND	ND	6.2	-
MMC (0.29 µmol/L)	100	34.4 ± 2.8**	35	4	12	5	3	ND	6	ND	4	2.9.	-
Cr^{+6} (5 µmol/L)	100	41.2 ± 1.8**	42	6	13	8	1	ND	6	2	4	2.6	-
DTL (50 µL/mL)	100	3 ± 1.5	3	ND	ND	ND	ND	ND	2	ND	1	5.8	-
DTL (100 µL/mL)	100	5 ± 1.5	5	ND	ND	ND	ND	ND	2	ND	1	5.7	
DTL (200 µL/mL)	100	7 ± 1.1	8	3	2	ND	ND	1	2	ND	ND	4.1	-
DTL (50 µL/mL) +MMC	100	20 ± 2.1*	20	2	6	2	ND	ND	5	5	1	4.6	42
DTL (100 µL/mL) +MMC	100	16 ± 3.3*	18	4	5	3	ND	ND	6	ND	2	4.8	53
DTL (200 µL/mL) +MMC	100	14 ± 3.9*	15	4	5	2	ND	ND	3	1	ND	4.9	59
DTL (50 µL/mL)+ Cr^{+6}	100	19 ± 1.3*	22	4	6	2	ND	ND	5	4	1	4.3	54
DTL (100 µL/mL) + Cr^{+6}	100	16.6 ± 3*	20	2	7	1	ND	ND	5	3	2	4.6	59
DTL (200 µL/mL) + Cr^{+6}	100	12.6 ± 2.9*	17	4	5	2	ND	ND	4	2	ND	4.8	68

Note. The results are average of five sets of experiments. ND: not detected; G: Gap; Ctb: chromatid break; Chb: Chromosome break; R: Ring; M: Minute; Exc: Exchange; F: Fragment; Dic: dicentric; (**represents $P<0.001$, when compared of cell control, *represents $P<0.001$, when compared to MMC and Cr^{+6} controls, respectively).

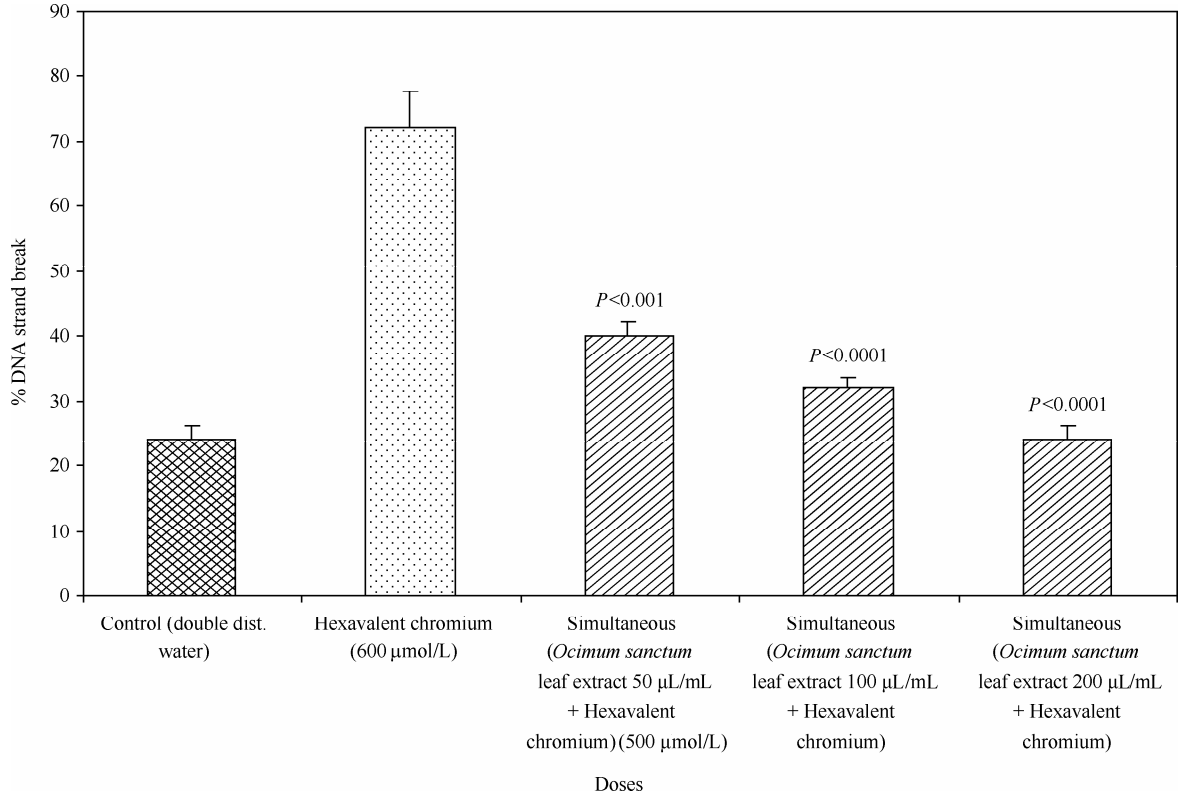

FIG. 5. Protective effect of simultanous treatment on HLC with DTLE against hexavalent chromium induced DNA strand break (5 µmol/L). The results are average of five sets of experiments ± standard deviation.

activation (S9).

Table 3 lists the number of MN/2000 binucleated HLC after treatment with only DTLE at doses 50 µg/mL, 100 µg/mL, and 200 µg/mL with and without metabolic activation, and the protective effect of DTLE against clastogens after simultaneous treatment. MMC, Cr^{+6}, and B[a]P showed a statistically significant level of micronuclei after treatment with human lymphocytes. The protective percentage after simultaneous treatment with DTLE was higher with metabolic activation (S9) showing a significant reduction in the number of micronuclei at 200 µg/mL.

TABLE 2

Protective Effect of Simultaneous Treatment on Human Lymphocytes With DTLE Against Chromosomal Abberation Induced by Benzo[A]Pyrene With S-9mix

Test Substance	No. of Metaphases	Percentage of Aberrated Metaphases ($\bar{x}$ ± SD)	No. of Aberrated Metaphases	Types of Aberration								Mitotic Index	% Protection
				G	Ctb	Chb	R	M	F	Dic	Exc		
Cell Control (0.1%DMSO)	100	4 ± 1.2	4	1	ND	ND	ND	1	2	ND	ND	6.0	-
B[a]P (30 µmol/L)	100	40 ± 3.2**	47	6	17	9	2	4	4	3	2	2.3	
DTL (50 µL/mL)	100	4 ±1.4	4	1	ND	ND	ND	2	1	ND	ND	5.9	
DTL (100 µL/mL)	100	6 ± 2.1	6	2	ND	ND	ND	1	3	ND	ND	5.8	
DTL (200 µL/mL)	100	6 ± 1.8	7	2	1	ND	ND	2	1	ND	1	5.8	
DTL (500 µL/mL)	100	7 ± 2.1	8	1	2	ND	ND	2	2	ND	1	5.0	
DTL (50 µL/mL) + B[a]P	100	15 ± 1.6*	16	3	7	3	ND	1	2	ND	1	4.9	65
DTL (100 µL/mL) + B[a]P	100	12 ± 1.3*	14	3	5	3	ND	2	1	ND	ND	5.0	70
DTL (200 µL/mL) + B[a]P	100	9 ± 2.1*	12	4	4	2	ND	2	ND	ND	ND	5.4	74

Note. The results are average of five sets of experiments. ND: not detected; G: Gap; Ctb: chromatid break; Chb: Chromosome break; R: Ring; M: Minute; Exc: Exchange; F: Fragment; Dic: dicentric; (**represents $P<0.001$, when compared of cell control, *represents $P<0.001$, when compared to B[a]P.

TABLE 3

Protective Effect of Simultaneous Treatment on Human Lymphocytes With DTLE Against Micronucleus Formation Induced by Mitomycin C, Hexavalent Chromium, and Benzo[A]Pyrene With S-9mix ($\bar{x} \pm s$)

Test Substance	Total Number of Binucleated Cells	Total Number of Micronucleated Cells		% Protection
		Without S9-mix	With S9-mix Metabolic Activation	
Cell Control (Sterile Double dist. Water)	2000	5 ± 1.58	4 ± 1.21	
Cell Control (0.1% DMSO)	2000	6 ± 1.01	5 ± 1.16	
DTLE (50 µL/mL)	2000	5 ± 1.18	6 ± 1.15	
DTLE (100 µL/mL)	2000	7 ± 3.0	6 ± 2.01	
DTLE (200 µL/mL)	2000	9 ± 1.52	7 ± 1.96	
MMC (0.29 µmol/L)	2000	46 ± 6.66	–	
DTLE (50 µL/mL) +MMC	2000	23 ± 1.21**	–	50
DTLE (100 µL/mL) +MMC	2000	20 ± 3.21*	–	57
DTLE (200 µL/mL) +MMC	2000	17 ± 2.89*	–	63
Cr^{+6} (5 µmol/L)	2000	34 ± 4.67**	–	
DTLE (50 µL/mL) + Cr^{+6}	2000	18 ± 1.61*	–	47
DTLE (100 µL/mL) + Cr^{+6}	2000	15 ± 3.42*	–	56
DTLE (200 µL/mL) + Cr^{+6}	2000	11 ± 4.36*	–	68
B[a]P (40 µmol/L)	2000	–	41 ± 3.12**	
DTLE (50 µL/mL) + B[a]P	2000	–	15 ± 2.1*	63
DTLE (100 µL/mL) + B[a]P	2000	–	12 ± 1.3*	71
DTLE (200 µL/mL) + B[a]P	2000	–	9 ± 1.5*	78

Note. The results are average of five sets of experiments. **represents $P<0.001$, when compared of cell control, *represents $P<0.001$, when compared to positive controls.

DISCUSSION

It is now well documented that MMC, Cr^{+6}, and B[a]P could cause chromosomal aberrations *in vivo* and *in vitro*[19-20]. Cytochrome P450 and peroxidase metabolize to numerous oxidized bi-products. Although several B[a]P metabolites induce mutations, (±)-anti-benzo(a) pyrene-7,8-dihydrodiol-9, 10-epoxide (BPDE) is considered to be the most carcinogenic metabolite xenobiotics[21]. BPDE causes single and double–strand DNA breaks[22]. Chromosomal damage probably results from radical oxygen species[23]. Well-established studies are pointed to the involvement of redox-dependent mechanisms in the toxicity of Cr^{+6}. Formation of DNA or protein-DNA cross-links induced by Cr^{+6} may be viewed as the outcome of redox reactions coupled with formation of reactive oxygen species (ROS), antioxidant (e.g. GSH) depletion, and multifacetedmodulation of redox-related activities. Administration of DTLE in combination with Cr^{+6}, can result in significant protection to DNA strand breaks and dramatic decrease in the number of chromosomal aberrations and frequency of micronucleus in a dose- dependent manner.

Comparing the modulatory effect of DTLE in the absence and presence of S-9 mix, the maximum protection against DNA strand break, reduction in the number of chromosomal aberrations and micronucleus formation were in the presence of S-9 in a dose- dependent manner.

The protective effect of DTLE against Cr^{+6} and MMC induced DNA strand break, chromosomal aberrations and micronuclei formation were the highest at the maximum DTLE dose of 200 µg/mL showing 67% and 63% protection against DNA strand break, 68% and 59% protection against chromosomal aberrations and 63% and 68% protection against micronuclei formation respectively without metabolic activation. However, better protection was observed in the presence of metabolic activation, where on challenging the cells with benzo[a]pyrene, DTLE at the dose of 200 µg/mL, showed 74% protection against chromosomal aberrations and 78% protection against micronuclei formation.

In the present investigation, identification and quantification of active ingradients in DTLE, carried out by HPLC and LC-MS, showed the presence of phytoflavonoids such as eugenol, luteolin and apigenin in DTLE.

In conclusion, it appears that eugenol, apigenin and luteolin and other contents in DTLE (unidentified) are responsible for the presently observed protection against genotoxic and clastogenic effects. Distillate of Tulsi leaf extract can be fully used as a wonder drug against carcinogenic agents, however, further investigations are required to understand the exact mechanism of its protection against genotoxicity and clastogenicity induced by MMC, Cr^{+6}, and B[a]P.

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