Effects of *Thymus kotschyanus* var. *glabrescens* Boiss. extract on mitomycin-C induced chromosomal aberrations and sister chromatid exchanges in human lymphocytes

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Abstract In this investigation, clastogenic effects of *Thymus kotschyanus* var. *glabrescens* Boiss. extract (TE) and anticlastogenic effects of this extract against Mitomycin C (MMC) induced chromosome damage have been evaluated in human peripheral blood lymphocytes in vitro. Two series of experiments were conducted. In the first, only 10^{-5} , 10^{-4} , 10^{-3} and 10^{-2} $\mu\text{l ml}^{-1}$ concentrations of TE were used for 48 h to detect potential clastogenicity. In the second, MMC ($0.38 \mu\text{g ml}^{-1}$) plus 10^{-5} , 10^{-4} , 10^{-3} and 10^{-2} $\mu\text{l ml}^{-1}$ concentrations of TE were used for 48 h to determine anticlastogenic effects. TE did not increase sister chromatid exchanges (SCEs) (except 10^{-2} $\mu\text{l ml}$ concentration) and chromosome aberrations (CAs) significantly compared with negative and solvent controls. However, it

decreased the frequency of MMC induced chromosome aberrations. Decreasing was significant at 10^{-4} , 10^{-3} and 10^{-2} $\mu\text{l ml}^{-1}$ concentrations. On the other hand, TE significantly increased MMC-induced SCEs for all treatment groups compared with positive control.

Keywords *Thymus kotschyanus* var. *glabrescens* extract · Mutagenicity · Antimutagenicity · Sister chromatid exchanges · Chromosome aberrations · Human lymphocyte culture

Introduction

The medicinal usage of plants is probably as old as human kind itself. In addition, many essential oils and their ingredients have been shown to exhibit a range of biological activities including antibacterial, antimutagenic, anticlastogenic and antifungal activity (Nakatani 1994). Although plant extracts have been used in the treatment of some diseases, scientific researches indicated that some of these extracts have a potential toxicity.

Thymus is a polymorphic genus and belongs to the family Lamiaceae. Some of *Thymus* species are used in folk medicine. For example, *Thymus vulgaris* L. and *Thymus zygis* L. extracts have been used orally to treat dyspepsia and other gastrointestinal disturbances; coughs due to colds, bronchitis and pertussis; and laryngitis and

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tonsillitis (as a gargle). *Thymus kotschyanus* var. *glabrescens* known as a member of thyme species is used as traditional medicine among people. The chemical composition of TE reveal that carvacrol represent the most important compounds in the oil, that is found as 44.2% by Meriçli (1986) and, as 53–70% by Başer (2002). This extract also contains *p*-cymene (7%), γ -terpinene (6%) and thymol (6%) (Başer 1993).

Thyme essential oil did not have any mutagenic activity in the *Bacillus subtilis* rec-assay or the *Salmonella*/microsome reversion assay (Zani et al. 1991; Azizan and Blevins 1995). Recent investigations suggest that thyme extracts are antimutagenic (Natake et al. 1989) and that luteolin, a constituent of thyme, is a strong anti-mutagen against the dietary carcinogen Trp-P-2 (3-amino-1-methyl-gamma-carboline) (Samejima et al. 1995). İpek et al. (2003) reported that carvacrol did not increase the formation of SCE, in contrast, it inhibited the rate of SCE induced by Mitomycin-C (MMC). In another study major constituent of thyme, i.e thymol and γ -terpinene did not seem to induce DNA strand breakage at lower concentrations in human lymphocytes, but at higher concentrations, they caused DNA damage (Ayдын et al. 2005).

The objective of the present study was to evaluate both clastogenic properties and anti-clastogenic activity of TE against MMC. For that purpose, we used chromosome aberration (CA) and sister chromatid exchange (SCE) tests in human lymphocytes in vitro, because they are reliable and most widely used bioassays.

Material and method

Plant material

The flowering parts of *Thymus kotschyanus* var. *glabrescens* was collected from Ahırdağ Mountain, Kahramanmaraş, Türkiye, at altitude 1600 m in July, 2000. A voucher specimen has been deposited in the herbarium of the Department of Biology, Science Faculty, KSU University, Kahramanmaraş, Türkiye.

Isolation of the essential oils: The flowering parts of the plant were dried at room temperature

and hydro distilled for 3 h using a Clevenger-type apparatus according to the standard procedure described in the European Pharmacopoeia (1975).

Genotoxicity test

In this research human peripheral blood cells were used as the test systems. Peripheral blood was obtained from two healthy (1 male and 1 female) non smoking donors (aged 23–24). Whole blood (0.2 ml) was added to 2.5 ml Chromosome Medium B (Biochrome) supplemented with $10\ \mu\text{g ml}^{-1}$ bromodeoxyuridine. The cultures were incubated at 37°C for 72 h. Two series of experiments were carried out. In the first, the cells were treated with 10^{-5} , 10^{-4} , 10^{-3} and $10^{-2}\ \mu\text{l ml}^{-1}$ concentrations of TE and in the second, MMC ($0.38\ \mu\text{g ml}^{-1}$) + 10^{-5} , 10^{-4} , 10^{-3} and $10^{-2}\ \mu\text{l ml}^{-1}$ concentrations of TE for 48 h. In addition, a negative (untreated control), a positive (MMC) and a solvent control (ethanol, 7%, $13.70\ \mu\text{l ml}^{-1}$) were included for each experiments to ensure validity of the assay. The test sample was dissolved with 70% alcohol and then diluted with sterile distilled water. Final concentrations of TE in lymphocyte cultures were 10^{-2} , 10^{-3} , 10^{-4} and $10^{-5}\ \mu\text{l ml}^{-1}$. The final concentration of ethanol in media was adjusted to 0.096%. $0.06\ \mu\text{g ml}^{-1}$ colchicine was added 2 h prior to harvesting of the culture. Cells were harvested by centrifugation ($923\times g$, 10 min), and pellet was resuspended in a hypotonic solution of 0.075 M KCl for 30 min at 37°C. Cells were centrifuged again and fixed in cold methanol:acetic acid (3:1) for 45 min at + 4°C. At the end of this procedure, cells were fixed for 2 more times and then slides were made by dropping and air drying.

For chromosome aberrations, slides were stained with 5% Giemsa (pH = 6.8) prepared in Sorensen buffer solution, for 20–25 min, and then washed in distilled water, dried at room temperature and mounted in depex. For the SCE study, slides were stained with Giemsa according to Speit and Houptner (1985)'s method with some modifications.

Chromosomal abnormalities were scored from 100 well-spread metaphases per donor (totally 200 metaphases per concentration). For the occurrence of the number of SCEs, a total of 50 cells (25 cells from each donor) under second

metaphases were scored for each treatment. In addition, a total of 200 cells (100 cells from each donor) were scored for determination of replication index (RI). The RI was calculated according to the following formula; $[1 \times M_1] + [2 \times M_2] + [3 \times M_3]/N$ (N = number of observed cells) where M_1 , M_2 , and M_3 represent the number of cells undergoing first, second and third mitosis (Schneider et al. 1981). The mitotic index (MI) was determined by scoring 1000 cells from each donor.

The significance between the percentage of abnormal cell, CA/cell, RI and MI in treated cultures and their controls were determined using the z -distribution test method. The significance between mean SCE in treated cultures and their controls were determined using the student's t -test.

Results

Results of the treatment with 10^{-5} , 10^{-4} , 10^{-3} and 10^{-2} $\mu\text{l ml}^{-1}$ concentrations of TE and MMC + 10^{-5} , 10^{-4} , 10^{-3} and 10^{-2} $\mu\text{l ml}^{-1}$ concentrations of TE in human peripheral blood lymphocytes in vitro are summarized in Table 1 and Table 2. While TE induced only sister union, MMC induced six types of chromosomal aberrations; chromatid and chromosome breaks, sister union, dicentric chromosome, chromatid exchanges and fragment. The percentage of CA induced by TE was not

statistically significant compared with negative and solvent controls. When TE was used together with MMC, the percentage of chromosome aberrations as well as CA/cell ratio decreased compared with positive control. This decrease was significant at 10^{-4} , 10^{-3} and 10^{-2} $\mu\text{l ml}^{-1}$ concentrations. Here, decreasing of abnormal cells and CA/cell were dose dependent ($r = -0.92$ and -0.94 , respectively) (Table 1).

10^{-5} , 10^{-4} and 10^{-3} $\mu\text{l ml}^{-1}$ concentrations of TE did not effect the mean number of SCE compared with negative and solvent controls. Only 10^{-2} $\mu\text{l ml}^{-1}$ concentration increased the number of SCEs in human lymphocytes. However, all concentrations of TE used together with MMC, increased the mean number of SCEs compared with positive control. This increase was dose dependent ($r = 0.90$) (Table 2).

Mitotic index was not effected by the treatment of TE compared with negative (except 10^{-2} $\mu\text{l ml}^{-1}$) and solvent controls. However, treatments of MMC + TE significantly decreased MI compared with negative control ($r = -0.96$). Replicative index was not effected significantly by TE or MMC + TE applications (Table 2).

Discussion

In this study, clastogenic effects and anticlastogenic effects of TE against MMC induced

Table 1 The chromosome aberrations in cultured human lymphocytes treated with MMC, TE and MMC + TE

Test substance	Treatment		Aberrations						Abnormal cell $\pm$ SE (%)	CA/Cell $\pm$ SE
	Period(h)	Dose	B'	B''	SU	DC	CE	F		
Negative control			–	–	3	–	–	–	1.5 ± 0.86	0.015 ± 0.010
MMC	48	$0.38 \mu\text{g ml}^{-1}$	38	5	13	4	32	3	46.0 ± 3.52	0.475 ± 0.035
Solvent control	48	$13.70 \mu\text{l ml}^{-1}$	1	–	2	–	–	–	1.5 ± 0.86	0.015 ± 0.010
TE	48	$10^{-5} \mu\text{l ml}^{-1}$	–	–	6	–	–	–	3.0 ± 1.20	0.030 ± 0.012
		$10^{-4} \mu\text{l ml}^{-1}$	–	–	5	–	–	–	2.5 ± 1.10	0.025 ± 0.011
		$10^{-3} \mu\text{l ml}^{-1}$	–	–	6	–	–	–	3.0 ± 1.20	0.030 ± 0.012
		$10^{-2} \mu\text{l ml}^{-1}$	–	–	–	–	–	–	0.0 ± 0.00	0.000 ± 0.000
MMC + TE	48	$10^{-5} \mu\text{l ml}^{-1}$	41	14	1	–	31	3	43.0 ± 3.50	0.450 ± 0.035
MMC + TE		$10^{-4} \mu\text{l ml}^{-1}$	6	2	6	–	30	4	$21.5 \pm 2.90^*$	0.240 ± 0.030
MMC + TE		$10^{-3} \mu\text{l ml}^{-1}$	22	4	4	–	20	–	$24.0 \pm 3.02^*$	$0.250 \pm 0.030^*$
MMC + TE		$10^{-2} \mu\text{l ml}^{-1}$	12	1	2	–	18	–	$16.5 \pm 2.62^*$	$0.165 \pm 0.030^*$

B': Chromatid break, B'': Chromosome break, SU: Sister union, DC: Dicentric chromosome, CE: Chromatid exchange, F: Fragment

* Significant from the positive control $P < 0.001$ (z test)

chromosome damage were assessed in human peripheral lymphocytes. Used concentrations of TE itself did not show any clastogenic effect. TE did not effect mitotic index and cell proliferation either. Only the highest concentration ($10^{-2} \mu\text{l ml}^{-1}$) decreased MI. Thymus extract did not increase the mean number of SCEs significantly at three concentrations, however, the highest concentration increased this number. Previous reports showed that carvacrol and thymol, components of TE, significantly increased DNA damage at high concentration levels (İpek et al. 2003; Aydın et al. 2005). In the microbial and mammalian short-term assays, thymol and carvacrol showed very weak genotoxic potential, however, the possibility of their action at DNA level cannot be excluded because, at least carvacrol caused nuclear fragmentation (Stammati et al. 1999). TE, in this study, significantly reduced the clastogenicity of MMC at all concentrations (except $10^{-5} \mu\text{l ml}^{-1}$). MMC plus TE treatment, in contrary, increased the mean number of SCEs compared with positive control. This increase was nearly two fold of positive control at the highest concentration. İpek et al. (2003) reported that carvacrol strongly reduced MMC-induced SCE frequencies in a dose dependent manner. The differences between our results and İpek et al. (2003)'s may be due to different concentrations used or direct application of plant

extract instead of pure carvacrol. In the present study, MMC + all concentrations of TE significantly decreased MI compared with negative control. Replication index was effected by neither TE alone nor MMC + TE treatments compared with negative and positive controls. Similarly, mitotic index was not effected by chamomile oil alone but it was decreased by chamomile oil when used together with daunorubicin and methyl methanosulfonate. These results indicate a lack of cytotoxic protection against mutagens (Hernandez-Ceruelos et al. 2002).

Several studies have also been conducted to determine mutagenic and antimutagenic effects of some compounds. L-ascorbic acid pretreatment caused a statistically significant increase in MMC-induced micronuclei (MN) and SCE frequencies for all treatment groups, but it did not show an increase in chromosome aberrations compared with MMC treatment alone (Krishnaja and Sharma 2003). This study indicates modulatory effect of L-ascorbic acid on MMC-induced chromosome damage. On the other hand, extracts from twigs/bark of *Rhamnus pirinoides*, twigs of *Diospyros whyteana*, *Prunus africana* and *Syzgium cordatum* significantly decreased the effect of the mutagen MMC in MN formation in human lymphocytes (Verschaeve et al. 2004). Hernandez-Ceruelos et al. (2002) reported that chamomile oil caused

Table 2 The frequency of the SCE, RI and the MI in cultured human lymphocytes treated with MMC, TE and MMC + TE

Test substance	Treatment		Min-max SCE	SCE/cell $\pm$ SE	M ₁	M ₂	M ₃	RI $\pm$ SE	MI $\pm$ SE (%)
	Period(h)	Dose							
Negative control			2–9	4.70 $\pm$ 0.24	40	57	103	2.32 $\pm$ 0.055	5.50 $\pm$ 0.51
MMC	48	0.38 $\mu\text{g ml}^{-1}$	19–58	34.82 $\pm$ 1.25	121	73	6	1.43 $\pm$ 0.039	3.00 $\pm$ 0.38
Solvent control	48	13.70 $\mu\text{l ml}^{-1}$	0–14	4.48 $\pm$ 0.46	45	64	91	2.23 $\pm$ 0.059	4.45 $\pm$ 0.46
TE	48	$10^{-5} \mu\text{l ml}^{-1}$	2–11	4.30 $\pm$ 0.24	31	58	111	2.40 $\pm$ 0.052	4.85 $\pm$ 0.48
		$10^{-4} \mu\text{l ml}^{-1}$	0–11	4.62 $\pm$ 0.37	35	68	98	2.33 $\pm$ 0.053	4.65 $\pm$ 0.47
		$10^{-3} \mu\text{l ml}^{-1}$	1–10	5.38 $\pm$ 0.34	24	71	105	2.41 $\pm$ 0.049	4.60 $\pm$ 0.47
		$10^{-2} \mu\text{l ml}^{-1}$	2–17	6.00 $\pm$ 0.51†	37	85	79	2.22 $\pm$ 0.051	3.60 $\pm$ 0.42**
MMC + TE	48	$10^{-5} \mu\text{l ml}^{-1}$	25–73	46.04 $\pm$ 1.64‡	104	90	6	1.46 $\pm$ 0.039	4.10 $\pm$ 0.44*
MMC + TE		$10^{-4} \mu\text{l ml}^{-1}$	10–76	42.94 $\pm$ 3.12‡	120	72	8	1.44 $\pm$ 0.040	3.55 $\pm$ 0.41**
MMC + TE		$10^{-3} \mu\text{l ml}^{-1}$	14–84	47.76 $\pm$ 3.50‡	92	93	15	1.56 $\pm$ 0.044	3.45 $\pm$ 0.41**
MMC + TE		$10^{-2} \mu\text{l ml}^{-1}$	30–94	60.28 $\pm$ 3.07‡	92	90	18	1.63 $\pm$ 0.045	3.15 $\pm$ 0.39**

* Significant from the negative control $P < 0.05$ (z test)

** Significant from the negative control $P < 0.01$ (z test)

† Significant from the negative control $P < 0.05$ (t test)

‡ Significant from the positive control $P < 0.05$ (t test)

dose dependent inhibitory effect on the SCEs formed by both daunorubicin and methyl methanesulfonate.

In this study, thymus extract at the concentrations tested has no clastogenic but anticlastogenic effects against MMC induced chromosomal damages. On the other hand, TE has no antimutagenic effect against MMC induced SCEs; it seems obvious that it enhanced the effect of the mutagen MMC. However, some chemicals have been reported to show anticlastogenic effect in the formation of SCEs in human lymphocytes (Gebhart and Kappauf 1980; Renner 1984).

In conclusion, the protective effect of TE seems to correlate with threshold doses. TE decreased MMC-induced chromosome aberrations significantly in all concentrations with the exception of the lowest dose. In contrast, TE did not exert protective effect against MMC induced SCE. There is a strong opinion that SCEs occur by reciprocal exchanges between sister chromatids in cells exposed to genotoxic agents. However, the sensitivity of SCE is lower than those of the CA test in detecting genotoxic effects related to xenobiotic exposure (Bolognesi 2003). Chromosomal aberrations in peripheral blood lymphocytes is a relevant biomarker for cancer risk in humans, reflecting either early biological effect of genotoxic carcinogens or individual cancer susceptibility while no association was seen between SCE frequency and subsequent cancer incidence/mortality (Hagmar et al. 1998). Although TE showed protective effect against MMC-induced CAs, enhancing the genetic effect of MMC in induction of SCEs raise some questions with regard to its safety, especially in case of its long term use in folk medicine. Further tests should be conducted by using other genetic endpoints in order to make any conclusion for the effect of TE.

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