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The effects of thymol on sister chromatid exchange, chromosome aberration and micronucleus in human lymphocytes

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ABSTRACT

The genotoxic effects of thymol were investigated in human peripheral lymphocytes treated with 25, 50, 75, and 100 μg/ml concentrations of thymol for 24 and 48 h treatment periods by using sister chromatid exchange (SCE), chromosome aberration (CA), and micronucleus (MN) tests. Nuclear division index (NDI), replication index (RI), and mitotic index (MI) were also calculated in order to determine the cytotoxicity of thymol. Thymol significantly increased the SCE, especially at the lower concentrations. Thymol also increased the SCE at the highest concentrations without statistical significance. Thymol induced both the structural CA and frequency of MN at all concentrations. Thymol dose-dependently decreased the NDI for two treatment periods. Thymol decreased the RI for the 24 h treatment time without any statistical significance. However, thymol decreased the RI for the 48 h treatment time in a dose-dependent manner. Thymol also decreased the MI at the higher concentration without dose-dependent effect.

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1. Introduction

Thymol, a phenol derivative, is a major component of the essential oils of thyme (*Thymus vulgaris* and *Thymbra spicata*). Phenol is a very hazardous pollutant for the environment. Natural sources of phenol include the degradation of natural plant products such as carbonization of thymol-containing phenol rings (Huma et al, 1999). Consumption of phenol-containing water by human beings result in symptoms such as gastrointestinal illness, nausea, omitting, diarrhea, and abdominal pain (Kim et al., 1994; OPSW, 1994). Thymol has an antimicrobial effect on *bacteria*, fungi, and yeasts. Thymol and the other phenol compounds, carvacrol and the saponins have expectorant properties that help to relieve bronchitis and lung conditions. Thymol can also relax smooth muscles, thereby aiding digestion, easing menstrual cramps, and alleviating respiratory conditions. Thymol is a component found in many different products including soaps, toothpastes, shampoos, deodorants, and mouthwashes (Shapiro et al., 1994; Manou et al., 1998). Aeschbach et al., (1994) reported that thymol has also potent antioxidant properties.

Most of the essential oils were found to have been mutagenic, genotoxic, and cytotoxic substances (Whysner and Williams, 1996; Franzios et al., 1997; Karpouhtsis et al., 1998; Gomes-Carneiro et al., 1998; Stammati et al., 1999; Ipek et al., 2005) while the negative evidence for *in vivo* genotoxic effects of eugenol were also shown (Maura et al., 1989; Abraham, 2001). It was also reported that some of the essential oils had anti-genotoxic effects, (Ipek et al., 2005; Aydin et al., 2005). Azirak and Rencuzogullari (2008) reported that both carvacrol and thymol were capable of inducing structural, numerical, and total CA in rat bone marrow cells and carvacrol and thymol have cytotoxic effect by decreasing the MI. However, there is no study available on the genotoxicity of thymol in human lymphocytes. Madle et al. (1993) reported that using human lymphocytes for the mutagenicity studies could explain the best results for humans. To consider the genotoxicity of substances, genotoxicity of them should be investigated in human lymphocytes (Madle et al., 1993). For this reason, the aim of this study was to investigate the genotoxic effects of thymol using SCE, CA, and MN tests in human peripheral lymphocytes.

2. Material and methods

In this study, thymol was used as the test substance in in vitro test system. Test substance was purchased from Sigma. The properties of thymol are shown as follows:

Synonym: 6-isopropyl-*m*-cresol; 3-hydroxy-*p*-cymene; isopropyl-cresol; 5-methyl-2-(1-methylethyl) phenol; 5-methyl-2-isopropyl-1-phenol; 3-*p*-cymenol; 2-isopropyl-5-methyl phenol
Chemical name: 5-methyl-2-(1-methylethyl) phenol
CAS no.: 89-83-8
Chemical formula: $C_{10}H_{14}O$
Purity: 99.6%

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The methods of Evans (1984) and Perry and Thompson (1984) were followed in preparation of SCE and CA tests with minor modifications. This study was conducted according to IPCS guidelines (Albertini et al., 2000).

Whole blood (0.2 ml) from four healthy donors (at a day intervals) (two male and two female, non-smokers, age: 23–26), immediately was added to 2.5 ml chromosome medium B (Biochrom, F5023) supplemented with 10 μg/ml bromodeoxyuridine (Sigma, B5002). The culture media per each liter was consisted as follows:

Mem Joklik with non-essential amino acids 850 ml
Fetal calf serum 150 ml
Heparin 25000 E
Penicillin G, sodium salt 75,000 E
Streptomycin sulfate 50 mg
Phytohaemagglutinin M 2.5 mg

The cultures were incubated at 37 °C for 72 h. The cells were treated with 25, 50, 75, and 100 μg/ml concentrations of thymol (Sigma, T0501) dissolved in dimethyl sulfoxide (DMSO) (Sigma, D8418) for 24 h (thymol was added 48 h after initiating the culture) and 48 h (thymol was added 24 h after initiating the culture). A negative control (untreated cultures), a solvent control (4 μl/ml DMSO) and a positive control (0.7 μg/ml ethyl methanesulfonate (EMS, Sigma, M0880) were also used. The cells were exposed to colchicine (0.06 μg/ml, Sigma C9754) 2 h before harvesting. The cells were harvested by 0.4% KCl as hypotonic solution and methanol: glacial acetic acid (3:1) as fixative. The staining of air-dried slides were performed following the standard methods using 5% Giemsa stain for CA and modified fluorescence plus Giemsa method for SCE (Speit and Haupter, 1985). The slides were irradiated with 30 W, 254 nm UV lamp at 15 cm distance in Sorensen buffer, then incubated with 1 × SSC (standard saline citrate) at 60 °C for 45–60 min and stained with 5% Giemsa prepared with Sorensen buffer.

The number of CA was obtained by calculating the percentage of metaphases from each concentration and treatment period that showed the structural and/or numerical alterations. The CA was classified according to the International System for Human Cytogenetic Nomenclature (ISCN) (Paz-y-Miño et al., 2002). Chromosome aberrations were evaluated in 100 well-spread metaphases per donor (totally 400 metaphases per concentration). Gaps were not evaluated as CA according to Mace et al. (1978). The scoring of SCE was carried out according to the IPCS guidelines (Albertini et al., 2000). In order to score SCE, a total of 100 second division metaphases (25 cells per sample) were analyzed. The results were used to determine the mean number of SCE (SCE/cell). In addition, a total 400 cells (100 cells from each donor) were scored for replication index (RI). The mitotic index (MI) was also determined by scoring 3000 cells from each donor. The MI explained the effects of the chemicals on G2 stage of cell cycle, and the RI reflects the effects of chemicals on S and G2 stages of the cycles. The RI was calculated according to the formula as follows: $RI = (M1 \times 1)+(M2 \times 2)+(M3 \times 3)$/total scored cells. M1, M2, and M3 are the fraction of cells undergoing the first, second and third mitosis during the 72 h cell culture period.

For the analysis of micronucleus in binucleated lymphocytes, 0.2 ml of fresh blood was used to establish cultures. The cells were treated with 25, 50, 75, and 100 μg/ml concentrations of thymol for 24 and 48 h treatment periods. Cytochalasin B (Sigma, C6762) was added at 44 h of incubation to a final concentration of 6 μg/ml to block cytokinesis. After the additional 24 h incubation at 37 °C, cells were harvested by centrifugation and processed for micronucleus test in peripheral lymphocytes (Fenech, 2000; Kirsch-Volders et al., 2003). In all subjects, 2000 binucleated lymphocytes were scored from each donor (8000 binucleated cells were scored per concentration). For each donor, totally 1000 viable cells were scored to determine the frequency of cells with 1, 2, 3, or 4 nuclei and calculate the NDI (nuclear division index) for cytotoxicity of thymol using the formula: $NDI = (M1)+(2 \times M2)+(3 \times M3)+(4 \times M4)/N$; where M1–M4 represent the number of cells with 1–4 nuclei and N is the total number of viable cells scored (Fenech, 2000).

The t-test was used for the statistical significance of all the parameters. Dose–response relationships were determined from the correlation and regression coefficients for the percentage of structural and total CA, mean SCE, MN, RI, MI, and NDI.

3. Results

Thymol significantly increased the mean sister chromatid exchange (SCE) at all concentrations when compared with both control and DMSO control except 100 μg/ml concentration for 24 h and 75 and 100 μg/ml concentrations for 48 h treatment periods that thymol showed excessive toxicity at these concentrations. Thymol increased the mean SCE without dose-dependent effect (Table 1). Thymol significantly decreased the RI at the highest concentration for 48 h treatment time in a dose-dependent manner ($P = 0.03$) (Table 1).

Thymol induced the structural chromosome aberrations (CA) at all concentrations and treatment periods when compared with controls without dose-dependent manner. Thymol showed the similar effects with the positive control EMS on the induction of the percentage of structural CA at the highest concentrations (100 μg/ml) for 24 h treatment period and displayed a higher-level effect than the positive control (EMS) on induction of the structural CA at the highest concentration for 48 h treatment periods (Table 2). The chromatid-type abnormalities (chromatid breaks) were more common abnormalities than the chromosome type ones (chromosome breaks, sister union, disentric chromosome). Thymol did not induce the numerical chromosome abnormalities such as aneuploidy and euploidy (Table 2).

Thymol induced the frequency of micronucleus (MN) at all concentrations and treatment periods when compared with controls without a dose-dependent effect (Table 3). Thymol

Table 1
The mean sister chromatid exchange and replication index in cultured human lymphocytes treated with thymol.

Test substance	Treatment time (h)	Conc. (μg/ml)	Min–Max SCE	SCE/Cell ± SE	RI ± SE
Control	–	–	2–15	8.52 ± 0.85	2.54 ± 0.08
DMSO	24	4 μl/ml	2–16	8.91 ± 0.23	2.48 ± 0.05
EMS	24	0.7	6–57	22.77 ± 7.49	2.31 ± 0.03
Thymol	24	25	2–25	10.40 ± 0.46[a1b1c3]	2.48 ± 0.02[c3]
Thymol	24	50	2–17	11.31 ± 0.14[a3b3c3]	2.40 ± 0.06[c1]
Thymol	24	75	2–24	10.69 ± 0.45[a1b1c3]	2.35 ± 0.09
Thymol	24	100	3–28	9.97 ± 1.32[c2*]	2.36 ± 0.17
DMSO	48	4 μl/ml	2–16	9.73 ± 0.96	2.49 ± 0.08
EMS	48	0.7	4–66	25.07 ± 4.38	2.09 ± 0.10
Thymol	48	25	2–17	10.78 ± 0.70[a1b1c3]	2.43 ± 0.09[c1]
Thymol	48	50	2–21	12.62 ± 1.09[a1b1c2]	2.14 ± 0.16
Thymol	48	75	2–23	10.96 ± 0.97[c2]	2.08 ± 0.23
Thymol	48	100	3–23	12.49 ± 1.84[c2*]	1.92 ± 0.17[a1b1]

[a] Significant from control.
[b] Significant from solvent control, DMSO.
[c] Significant from positive control, EMS.
[1] $P \leqslant 0.05$.
[2] $P \leqslant 0.01$.
[3] $P \leqslant 0.001$.
* 91 and 96 cells were scored, respectively due to excessive toxicity.