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ORIGINAL RESEARCH

Modulatory effects of *Thymbra spicata* L. different extracts against the mercury induced genotoxicity in human lymphocytes in vitro

Ebubekir Dirican · Hasan Turkez · Başak Toğar

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Abstract Mercury, a xenobiotic metal, is a highly deleterious environmental pollutant. Moreover, in any form mercury is reported to be toxic. On the other hand, *Thymbra spicata* L., a member of the Lamiaceae family, has long been investigated popularly of biological roles; mainly antimicrobial and antioxidant activities. However, there are very scarce data on the cytogenetic effects of thyme species. The purpose of this study was to investigate the genetic safety of different extracts from *T. spicata* (water extract, methanol extract, and ethanol extract) and the effects of *T. spicata* on mercury (as $HgCl_2$) induced genotoxicity. Sister chromatid exchange (SCE) and micronucleus (MN) assays were performed to assess DNA damages in cultured human lymphocytes (n = 5). Our results clearly revealed that, the SCE and MN rates induced by $HgCl_2$ were alleviated by the presence of *T. spicata*. As conclusion, this study demonstrated for the first time that the *T. spicata* provided increased resistance of DNA against $HgCl_2$ induced genetic damage in human lymphocytes. Based on the results of this study, it may be concluded that the *T. spicata* is a nontoxic material that could be used as a suppressor of heavy metal-induced genotoxicity.

Keywords *Thymbra spicata* · Mercuric chloride · Genotoxicity · Human lymphocyte culture · Antimutagenic activity

E. Dirican (✉)
Department of Microbiology, Medical Faculty, Kahramanmaraş Sütçü Imam University, 46100 Kahramanmaraş, Turkey
e-mail: ebubekirdirican44@gmail.com

H. Turkez
Department of Molecular Biology and Genetics, Faculty of Science, Erzurum Technical University, 25240 Erzurum, Turkey

B. Toğar
Department of Biology, Faculty of Science, Atatürk University, 25240 Erzurum, Turkey

Introduction

In the environment, animals and humans are exposed to numerous chemical forms of mercury (Hg), including elemental mercury vapor, inorganic mercurous (Hg(I)), mercuric (Hg(II)) and organic mercuric compounds (Fitzgerald and Clarkson 1991). Hg, one of the most widely diffused and hazardous organ-specific environmental contaminants, exists in a wide variety of physical and chemical states, each of which with unique characteristics of target organ specificity (Aleo et al. 2002). Elemental, inorganic, and organic forms of mercury exhibit toxicologic characteristics including nephrotoxicity, neurotoxicity, and gastrointestinal toxicity with ulceration and hemorrhage (Zalups and Koropatnick 2000). Besides, the genotoxicity of mercuric compounds have been reported in

both in vitro and in vivo model systems (Silva-Pereira et al. 2005; Grotto et al. 2010; Turkez et al. 2011). Recent findings elucidated that mechanism of mercury toxicity included production of reactive oxygen species (ROS) capable of damaging lipids in membrane, proteins or enzymes in tissues, and DNA to induce oxidative stress (Jan et al. 2011). Therefore, many efforts are being made to minimize the mercury toxicity by antioxidant featured agents such as fish oil (Grotto et al. 2010), selenium (Glaser et al. 2010), boron compounds (Turkez et al. 2011), melatonin (Rao et al. 2010), curcumin (Agarwal et al. 2010) and *Allium sativum* L. extract (Abdalla et al. 2010).

Thymbra spicata L. from Lamiaceae family is a perennial plant known as "Kekik, Zahter or Sater" in Turkey. The plant is naturally grown in Southeast Anatolia (especially in the Gaziantep, Kahramanmaraş and Şanlıurfa Cities) (Daneshvar-Royandezagh et al. 2009). Dried herbs of *Thymbra* are used as herbal tea, condiment and folk medicine for treating asthma, colic, bronchitis, coughs. In addition, it is also used in food industry for flavouring, as preservation agent and aroma in Turkey. Several previous studies reported that the essential oils of *T. spicata*, and their main phenolic constituents such as carvacrol and thymol that show remarkable antimicrobial, antibacterial and antifungal activities (Baytop 1984; Shelef 1983).

Many efforts are being made to investigate therapeutic substances capable of reducing the genotoxicity of man-made or natural mutagens in human life. These include frequently vitamins, minerals and plant products (Turkez and Geyikoglu 2010; Turkez et al. 2010; Haleagrahara et al. 2010; Aboul-Soud et al. 2011; Fujiwara et al. 2011; Turkez and Dirican, 2011). Concomitant treatment with the antioxidants and mutagens provided protection against tissue damages in experimental models (Takaishi et al. 2009; Turkez et al. 2011). To our best knowledge, there is no report on the effects of *T. spicata* extracts against mercury toxicity on human blood cells. Therefore, in this investigation it was aimed to explore the role of *T. spicata* water extracts, methanol extract and ethanol extract on $HgCl_2$-induced genotoxicity in human lymphocyte cells by using SCE and MN tests. Hence, SCE and MN tests covering a wide range of induced genetic damage as primary DNA damage were performed on peripheral lymphocytes.

Materials and methods

Experimental design

Blood samples were obtained by veinpuncture from five healthy non-smoking donors. Human peripheral blood lymphocyte cultures were set up according to a slight modification of the protocol described by Evans and O'Riordan (1975). The heparinized blood (0.5 ml) was cultured in 6 ml culture medium (Chromosome Medium B, Biochrom®, Leonorenstr. 2-6, D-12247, Berlin) with 5 μg/l of phytohemagglutinin (Biochrom®). $HgCl_2$ were purchased from Sigma® (St. Louis, MO, USA; CAS No. 7487-94-7). *T. spicata* was purchased in ready package. For water extraction of *T. spicata*, 20 g leaves was mixed with 400 ml distilled and boiling water using magnetic stirrer for 15 min. Then extract was filtered over Whatman No. 1 paper. Then, $HgCl_2$ (9 mg/L) and *T. spicata* extracts (0, 25, 50, 100 and 200 mg/L) were added into culture tubes separately and together. After supplementation of $HgCl_2$ and plant extracts, the blood samples were incubated for 72 h at 37 °C to adjust body conditions. Each individual whole blood culture without $HgCl_2$ or *T. spicata* extract was studied as a control group.

Preparation of methanol extract

Air-dried and powdered leaves (10 g) were extracted with 250 mL of methanol using a Soxhlet.

extractor (Isopad, Heidelberg, Germany) for 72 h at a temperature not exceeding the boiling point of the solvent. The extract was filtered using Whatman filter paper No. 1 and then concentrated in a vacuum at 40 °C using a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland), yielding a waxy material. The extract was then lyophilized and kept in the dark at 4 °C until tested.

Preparation of ethanol extract

About 50 g of air dried leaves were dissolved in 250 ml of ethanol and kept in an orbital shaker for overnight. The extracts obtained were filtered through Whatman No. 1 filter paper and the filtrate was collected. Fifty grams of powdered plant material was extracted in 250 ml of boiling water for 2 h and concentrated to half of the volume by boiling in a

water bath. The extract was cooled and filtered using Whatmann No. 1 filter paper.

Genotoxicity testing

SCE assay

With the aim of providing successive visualization of SCEs, 5-bromo-2′-deoxyuridine (Sigma®) was added at culture initiation. The cultures were incubated in complete darkness for 72 h at 37 °C. Exactly 70 h and 30 min after beginning the incubations, demecolcine (N-Deacetyl-N-methylcolchicine, Sigma®) was added to the cultures. After hypotonic treatment (0.075 M KCl), followed by three repetitive cycles of fixation in methanol/acetic acid solution (3:1, v/v), centrifugation, and resuspension, the cell suspension was dropped onto chilled, grease-free microscopic slides, air-dried, aged for 3 days, and then differentially stained for the inspection of the SCE rate according to fluorescence plus Giemsa (FPG) procedure. For each treatment condition, well-spread 25 s division metaphases containing 42–46 chromosomes in each cell were scored by one observer (by E. Dirican), and the values obtained were calculated as SCEs per cell.

MN assay

The MN test was performed by adding cytochalasin B (Sigma®) after 44 h of culture. At the end of the 72 h incubation period, the lymphocytes were fixed with ice-cold methanol/acetic acid (1:1, v/v). The fixed cells were put directly on slides using a cytospin, and stained with Giemsa solution. All slides were coded before scoring. The criteria for scoring MN were as described by Fenech (1993). At least 1,000 binucleated lymphocytes were examined per concentration for the presence of one, two or more MN by one observer (by E. Dirican).

Statistics

Statistical analysis was performed using SPSS Software (version 18.0, SPSS, Chicago, IL, USA). For statistical analysis of obtained data Duncan's test was used. Statistical decisions were made with a significance level of 0.05.

Results

The effects of $HgCl_2$ and aqueous, ethanol and methanol *T. spicata* extracts on the number of SCEs and MNs in human whole blood cultures are shown in Figs. 1, 2, 3, 4, 5 and 6 respectively. 9 mg/L $HgCl_2$ caused significant increases of SCE and MN frequencies in human peripheral lymphocytes as compared with the controls. But, the plant extracts at four applied concentrations (25, 50, 100 and 200 mg/L) did not indicate statistically significant differences ($p > 0.05$) in the number of SCEs or MN rates. Moreover, the positive effect of *T. spicata* extracts was established on $HgCl_2$-induced SCE and MN formations. The incidences for the SCE and MN values were decreased in comparison with $HgCl_2$-treated group. In addition, the magnitude of the positive effect depended on the concentrations of *T. spicata* different extracts. Moreover, methanol extracts gave an higher effect than ethanol extract (shown in Figs 3, 4, 5 and 6).

Discussion

Our results indicated that one of the targets of $HgCl_2$ in human cells was DNA. Similar to our finding, a dose-related increase in DNA damage was observed in human cultured human peripheral blood lymphocytes

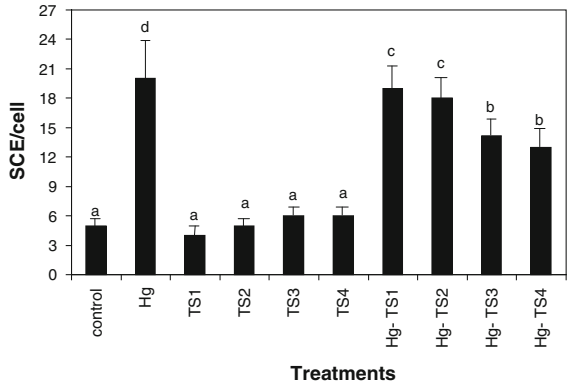

Fig. 1 Effect of *T. spicata* water extracts on mercury induced SCE formations in human peripheral lymphocytes. Values are expressed as mean for five cultures in each group; means in the figure followed by the different letters present significant differences at the $p < 0.05$ level; Hg: 9 mg/L of $HgCl_2$; TS1: 25 mg/L *T. spicata* extract; TS2: 50 mg/L *T. spicata* extract; TS3: 100 mg/L *T. spicata* extract; TS4: 200 mg/L *T. spicata* extract

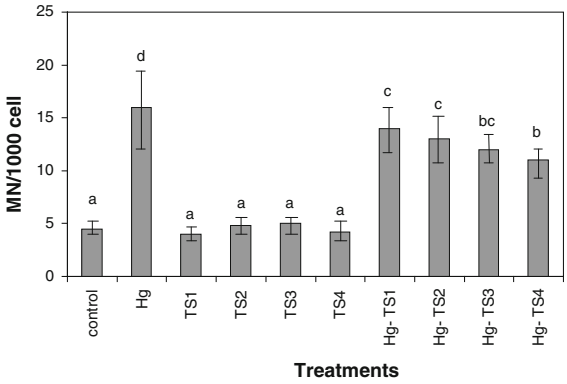

Fig. 2 Effect of *T. spicata* water extracts on mercury induced MN formations in human peripheral lymphocytes. Abbreviations are as in Fig. 1

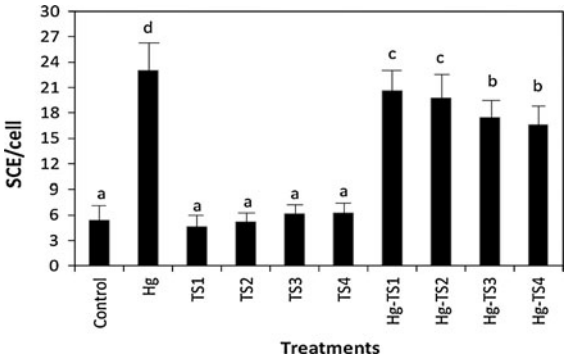

Fig. 3 Effect of *T. spicata* ethanol extracts on mercury induced SCE formations in human peripheral lymphocytes. Abbreviations are as in Fig. 1

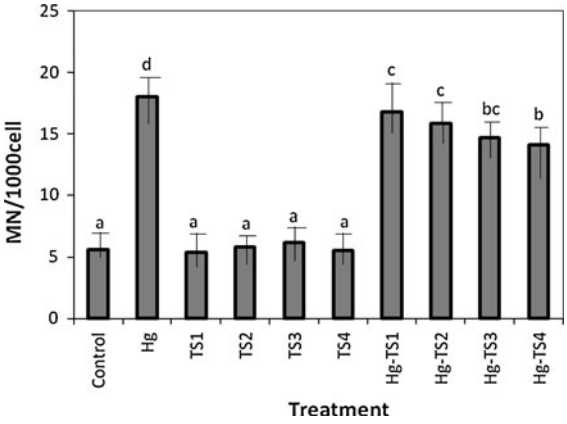

Fig. 4 Effect of *T. spicata* ethanol extracts on mercury induced MN formations in human peripheral lymphocytes. Abbreviations are as in Fig. 1

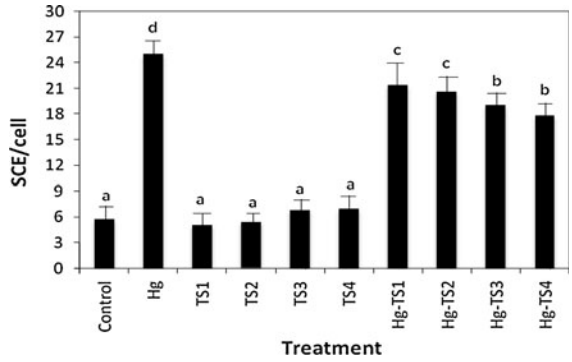

Fig. 5 Effect of *T. spicata* methanol extracts on mercury induced SCE formations in human peripheral lymphocytes. Abbreviations are as in Fig. 1

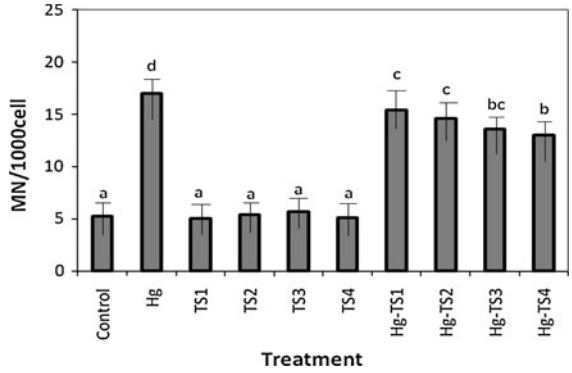

Fig. 6 Effect of *T. spicata* methanol extracts on mercury induced MN formations in human peripheral lymphocytes. Abbreviations are as in Fig. 1

exposed to $HgCl_2$ for 72 h in SCE and MN assays (Turkez et al. 2011). Likewise, significant increases in the frequencies of chromosome aberrations (CAs) and SCEs were observed for $HgCl_2$ in cultured human lymphocytes (Rao et al. 2001; Silva-Pereira et al. 2005; Halder et al. 2005). $HgCl_2$ was found to be genotoxic in bacterial mutagenicity assays (Schurz et al. 2000). Again, Bonacker et al. (2004) reported that $HgCl_2$ induced concentration-dependently MN formations in V79 cells. $HgCl_2$ treatment was shown to produce CAs, MNs and sperm head anomaly in mice (Datta et al. 2004). $HgCl_2$ genotoxicity in rats following oral exposure was also established by comet assay (Rozgaj et al. 2005). On the contrary, $HgCl_2$ exhibited a lack of genotoxic activity in the wing spot assay of *Drosophila melanogaster* (Carmona et al. 2008).

It was reported that the genotoxicity of mercuric compounds including $HgCl_2$ indicated by the CA and MN tests in peripheral blood lymphocytes could be partly due either to the disturbance of the spindle mechanism, or to the elevated level of 8-OH-dG (a product of oxidatively damaged DNA) brought by the generation of ROS. In addition, some authors have suggested that ROS may be implicated in the production of high basal SCE frequencies in chromosome instability syndromes (Lee et al. 1990; Therman and Susman 1993; Cinkilic et al. 2009). Similarly, oxidative stress could lead to MN formations (Tsangaris et al. 2010). Thus, the increases of SCE and MN rates after $HgCl_2$ exposure could be explained by possible prooxidant effect of this compound.

The results of the current study also revealed that treatment with *T. spicata* extracts (water extract, methanol extract and ethanol extract) at different concentrations provided antigenotoxic effects against $HgCl_2$. Also, there is considerable evidence that Thyme presents positive effects with increasing concentrations without leading to any genetic damage in human blood cells. Avci et al. (2006) have reported that *T. spicata* had antihypercholesterolaemic and antioxidant activities. Likewise Dorman et al. (2004) found antioxidant properties of aqueous *T. spicata* extracts (Dorman et al. 2004). Especially, proteins and nucleic acids in mammalian cells defend themselves with antioxidants (Kedziora et al. 2004). In this study, *T. spicata* could support the antioxidant defense mechanism against $HgCl_2$. In fact, the previous studies revealed that many Lamiaceae family species such as *Teucrium polium* L., *Thymbra spicata* L., *Ocimum basilicum* L. and *Foeniculum vulgar* included pharmacologically and biologically active essential oils (Toroğlu et al. 2005). A recent finding indicated that the essential oils from *T. spicata* had antibacterial or antifungal activity on tested bacteria and fungi (Toroğlu et al. 2005). Thus, *T. spicata* essential oil was considered as a natural antimicrobial source (Unlü et al. 2009). The main components of this plant were determined as carvacrol, p-cymene, beta-myrcene, gamma-terpinene, alpha-terpinene and trans-caryophyllene (Kiliç 2006). Recent findings revealed that these components exhibited more or less antioxidant properties (El Babili et al. 2011; Asbaghian et al. 2011).

In conclusion, the findings of this research clearly indicated that *T. spicata* modulated $HgCl_2$-induced genetic damage in human blood cultures due to its antioxidant and detoxifying nature. So, *T. spicata* can be a new resource of therapeutics against oxidative DNA damages as recognized in this study. Further investigation will be done for developing commercial formulation based on field trail and toxicological experiment. It is an important observation that all the biomolecules are polar in nature with a higher solubility in water, methanol, and ethanol.

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