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ORIGINAL RESEARCH

Comparative study of genotoxicity and antimutagenicity of methanolic extracts from *Teucrium chamaedrys* and *Teucrium montanum* in human lymphocytes using micronucleus assay

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Abstract Since *Teucrium chamaedrys* and *Teucrium montanum* are the most popular plants used in the treatment of many diseases, we evaluated genotoxic potential of their methanolic extracts on cultured human peripheral blood lymphocytes (PBLs) using cytokinesis-block micronucleus (MN) assay. Cultures were treated with four concentrations of both plants (125, 250, 500 and 1,000 μg/ml), both separately and in combination with mitomycin C (MMC). The results revealed that extract of *T. chamaedrys* administered at the tested concentrations did not significantly affect the mean MN frequency in comparison to untreated cells. Methanolic extract of *T. montanum* increased the mean MN frequency in PBL at the tested concentrations, but significantly only at the concentration of 1,000 μg/ml. In all tested concentrations, the extract of *T. chamaedrys* significantly reduced the MMC-induced MN frequency, in a dose dependent manner ($r = -0.687$, $p < 0.01$). The extract of *T. montanum* decreased the MMC-induced MN frequency at the tested concentrations, but statistically only at 125 μg/ml. Both extracts administered alone did not significantly affect the nuclear division index (NDI) at the tested concentrations. In the combined treatments with MMC, the extract obtained from *T. chamaedrys* in the concentrations of 500 and 1,000 μg/ml significantly decreased NDI values in comparison to MMC-treated cells alone, while the extract of *T. montanum* significantly decreased NDI at all tested concentrations. Both extracts nonsignificantly decreased NDI at all tested concentrations in comparison to untreated cells. Our results suggest the important function of *T. chamaedrys* extract in cancer therapy, this methanolic extract may prevent genotoxic effects of chemotherapy in PBLs.

Keywords Micronucleus test · Human lymphocytes · Genotoxicity · Antimutagenicity · *Teucrium chamaedrys* · *Teucrium montanum*

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Introduction

The species of genus *Teucrium* have been used as medicinal plants for more than 2,000 years. These species are known for their medicinal and biological properties such as hypoglycemic, hypolipidemic, hepatoprotective, antipyretic, anti-inflammatory, antiulcerogenic, antitumor and antimicrobial activities (Rasekh et al. 2001; Baluchnejadmojarad et al. 2005; Sundaresan et al. 2006; Vuković et al. 2007; Shtukmaster et al. 2010; Sghaier et al. 2011a; Sghaier et al. 2011b). Many species of this genus show antimicrobial, antioxidant and

antifungal activities and are used in food industry (Salah et al. 2006; Ozkan et al. 2007; Ahmad et al. 2008). The most investigated species are *Teucrium chamaedrys* (wall germander), which was used in herbal medicine for anti-inflammatory, anti- rheumatic, digestive and diuretic effects, and *Teucrium montanum* (mountain germander), widely used as diuretic, stomachic, analgesic and antispasmodic agent with antibacterial, antifungal, anti-inflammatory and antioxidative activity.

In the flora of Europe, the genus *Teucrium* has been divided into seven sections with 49 species, including 7 on the territory of Serbia. Wall germander, *T. chamaedrys* L. belongs to the section *Chamaedrys*. It is a perennial herbaceous plant with half-ligneous and shrub-like low stem being up to 30 cm high. It has a poorly-branched stem with oval serrated leaves and tiny blooms on the branch-tops. The plant inhabits rocky limestone areas, dry mountain meadows and pastures, edges of the sparse oak and pine forests up to 1,000 m above the sea level in Central Europe, the Mediterranean region and Western Asia. Mountain germander, *T. montanum* L. belongs to the section *Polium*. This is a perennial, shrub-like plant with half-ligneous branches, is up to 25 cm high and inhabits thermophilic limestone and serpentine rocks, dry mountain meadows and edges of forests in Europe and Anatoly (Tutin and Wood 1972; Diklić 1974).

For screening chromosome damages in human peripheral blood lymphocytes (PBL), micronucleus assay is the most frequently used assay (Fenech 2000). Micronuclei (MN) are defined as small, round nuclei, clearly separated from the main cell nucleus. They are formed from acentric fragments/chromatids or whole chromosomes that lag behind in anaphase, and are left in telophase outside the daughter nuclei. This test is very attractive and MN frequency in peripheral blood lymphocytes is a well established method for monitoring chromosome damages in human population (Fenech 1998).

Since medicinal plants and their derivatives such as extracts and syrups have been traditionally used in the treatment of different human disorders and many of them have been successfully screened for biological activities with often unknown effects on human health and genetic material; thus the aim of this study was to evaluate the genotoxic potential of methanolic extracts obtained from *T. chamaedrys* and *T. montanum*, which were tested by analyzing the MN frequency and nuclear division index (NDI) in cultured human lymphocytes of healthy donors. This study is the first report of a series of investigations performed to evaluate the genotoxic potential of this herbal medicine.

Materials and methods

Plant material

In June 2011, aerial flowering parts of *T. chamaedrys* were collected from natural populations in the region of Trgovište in south Serbia (position: 42°21′53.93″N, 22°05′11.53″E, altitude: 697.68 m, exposition: SW, substratum: limestone). In July 2011, aerial flowering parts of *T. montanum* were collected from natural populations in the region of Goč Mt. in Central Serbia (position: 43°36′43.56″N, 20°41′52.25″E, altitude: 381.91 m, exposition: W, substratum: serpentinite). The voucher specimens of *T. chamaedrys* L. and *T. montanum* L. were confirmed and deposited at the Herbarium of the Department of Biology and Ecology, Faculty of Science, University of Kragujevac. The collected plant material was air-dried in darkness at ambient temperature (20 °C). The dried plant material was cut up and stored in tightly sealed dark containers until needed.

Preparation of plant extract

Prepared plant material (10 g) was transferred to dark-colored flasks, soaked in 200 ml of methanol and stored at laboratory conditions. After 24 h, the infusions were filtered through Whatman® No. 1 filter paper and the residue was re-extracted with equal volume of solvents. After 48 h, the process was repeated. Combined supernatants were evaporated to dryness under vacuum at 40 °C using a rotary evaporator.

In vitro cytokinesis-block micronucleus (MN) test

Cell cultures of three healthy donors aged 27 years, nonsmokers, who had not been exposed to known mutagenic agents, were used in the investigation of in vitro effects of methanolic extracts obtained from *T. chamaedrys* L. and *T. montanum* L. by cytokinesis-block micronucleus (CBMN) test. Informed consent was obtained from all donors and experiments were conformed to the guidelines of the World Medical Assembly (Declaration of Helsinki). The study was approved by the Ethics Committee of the Clinical Center of Kragujevac.

Micronuclei were prepared using the Fenech method (2000). Whole heparinized blood (0.5 ml) was added to 5 ml of PBMax Karyotyping (Invitrogen, Carlsbad, CA, USA), the complete medium for lymphocyte culture. All cultures were carried out in duplicate and incubated at 37 °C for 72 h.

Forty-four hours after the beginning of incubation, cytochalasin B (Sigma, St. Louis, MO, USA) was added at a final concentration of 4 μg/ml. Cultures were harvested 28 h later. The cells were collected by centrifugation and treated with cold (+4 °C) hypotonic solution (0.56 % KCl). Then the cells were fixed with fixative methanol: glacial acetic acid = 3:1 and the procedure was repeated three times. The cell suspensions were dropped onto clean slides, air-dried and stained with 2 % Giemsa (Alfapanon, Novi Sad, Serbia).

Plant methanolic extracts

Methanolic extracts of the *Teucrium* species at four concentrations (125, 250, 500 and 1,000 μg/ml) and small volume (0.1 ml) were added to lymphocyte cultures 24 h after the beginning of incubation. To determine the co-mutagen/antimutagenic effect, mitomycin C (MMC, Sigma, St. Louis, MO, USA) at the concentration of 0.5 μg/ml and plant methanolic extract were concomitantly added to cell cultures.

MN scoring

The scoring was performed using a light microscope (Nikon E50i) at 400× magnification and following the criteria for MN scoring in binucleated (BN) cells only, as described by Fenech (2007).

The MN frequencies were scored in one thousand binucleated cells (BN) from each donor (3,000 BN cells per concentration). Five hundred cells from each donor were scored to determine the frequency of cells with 1, 2, 3 or 4 nuclei and to calculate the nuclear division index (NDI) using the formula $NDI = ((1 \times M1 + (2 \times M2) + (3 \times M3) + (4 \times M4))/N$, where M1–M4 represent the number of cells with 1–4 nuclei, and N is the total number of the cells scored (Fenech 2000).

Statistical analysis

The results are shown as mean ± standard deviation (SD). Statistically significant difference between mean baseline and induced MN frequencies was determined using the Student's *t* test. Levels of significance were $p < 0.05$, $p < 0.01$ and $p < 0.001$. The relationship between the tested concentrations of extracts and MN and NDI was determined by Pearson correlation coefficients.

Results

The results of the genotoxic potential of *Teucrium* species *T. chamaedrys* and *T. montanum* on genomic damage in PBL of healthy donors are shown in Tables 1 and 2.

Table 1 shows the effects of different concentrations of *T. chamaedrys* extract on MN frequency per 1,000 BN cells (±SD) and NDI values (±SD). The presence of the extract at different concentrations did not significantly affect both mean MN frequency and NDI in comparison to untreated control PBLs ($p > 0.05$).

The results obtained for PBL treated with different concentrations of methanolic extract of *T. chamaedrys* with concomitantly added are MMC shown in Table 2. MMC alone significantly increased the MN frequency in PBL ($p < 0.001$). However, for all tested concentrations, the methanolic extract significantly reduced the MMC-induced MN with the increase of concentration ($r = -0.687$, $p < 0.01$). The MMC-reduced NDI values non- significantly decreased for all tested concentrations of the methanolic extract in comparison to the untreated control cells ($p > 0.05$), but significantly decreased NDI in the treatment with the extract of 500 and 1,000 μg/ml in comparison to the MMC-treated cells alone ($p < 0.05$). A negative correlation was observed between NDI in the combined treatments with MMC and at different concentrations of *T. chamaedrys* extract ($r = -0.717$, $p < 0.01$).

The analysis of the distribution of MN (Tables 1, 2) revealed that the BN cells with 1 MN were mostly present in both treatments (the methanolic extract of *T. chamaedrys* alone and in combination with MMC). This extract in MMC-treated PBL decreased not only the number of BN cells that contained MN, but also the number of MN in BN cells.

The results obtained for PBL treated with different concentrations of methanolic extract of *T. montanum* alone are shown in Table 1. A significant increase of MN frequency was observed only in PBL treated with a concentration of 1,000 μg/ml of the extract compared to the untreated control cells ($p < 0.01$). No

Table 1 The frequency of micronuclei (MN) and nuclear division index values (NDI) in peripheral blood lymphocytes (PBL) of healthy donors after the treatments with four concentrations of methanolic extracts from *T. chamaedrys* and *T. montanum* in vitro

Treatments	Concentration (μg/ml)	No. of analyzed BN cells	MN/1,000 BN cells (X ± SD)	BN with MN (%)	Distribution of MN		NDI
					1 MN (%)	2 MN (%)	
Controls	0	3,000	5.00 ± 1.73	15 (0.50)	15 (0.50)		1.80 ± 0.37
T. chamaedrys	125	3,000	6.33 ± 1.53	18 (0.60)	17 (0.57)	1 (0.03)	1.48 ± 0.09
T. chamaedrys	250	3,000	8.00 ± 1.00	23 (0.77)	22 (0.73)	1 (0.03)	1.55 ± 0.07
T. chamaedrys	500	3,000	11.00 ± 3.61	30 (1.00)	27 (0.90)	3 (0.10)	1.37 ± 0.10
T. chamaedrys	1,000	3,000	12.67 ± 4.93	37 (1.23)	36 (1.20)	1 (0.03)	1.43 ± 0.08
T. montanum	125	3,000	5.67 ± 1.15	17 (0.57)	17 (0.57)		1.58 ± 0.04
T. montanum	250	3,000	7.00 ± 2.00	21 (0.70)	21 (0.70)		1.56 ± 0.04
T. montanum	500	3,000	7.67 ± 1.53	22 (0.73)	21 (0.70)	1 (0.03)	1.64 ± 0.03
T. montanum	1,000	3,000	9.33 ± 1.53[a]	27 (0.90)	26 (0.87)	1 (0.03)	1.60 ± 0.08

% of cells with 1 and 2 MN in relation to total number of analyzed cells

[a] Statistically significant difference in the MN frequency between untreated control and PBL treated with 1,000 μg/ml *T. montanum* methanolic extract

significant decrease of NDI values was detected in PBL for all tested concentrations of the extract ($p > 0.05$).

The results of combined treatments with different concentrations of *T. montanum* extract and MMC on both MN and NDI are shown in Table 2. The reduction of MMC-induced MN frequency was significant at the 125 μg/ml concentration of extract compared to MMC alone ($p < 0.05$), but non-significant for combined 250, 500 or 1,000 μg/ml concentrations of the extract and MMC. On the contrary, significant decrease of MMC-reduced NDI was observed for all tested concentrations of the extract (125–1,000 μg/ml) and MMC in comparison to MMC- treated cells alone ($p < 0.05$). There were no significant differences in NDI in comparison to untreated control cells ($p > 0.05$).

The correlation was negative between MMC-decreased NDI and the tested concentrations of *T. montanum* extract ($r = -0.591$, $p < 0.01$).

The analysis of the distribution of MN revealed that 250–1,000 μg/ml concentrations of *T. montanum* extract separately administered in PBL at 250–1,000 μg/ml increased both the number of BN cells with MN and the number of MN in BN cells (Table 1). In combined treatments with four different concentrations and MMC, only the lowest tested concentration (125 μg/ml) significantly decreased both the number of MMC-induced BN cells with MN and the number of induced MN in BN cells. As the concentration of extract increased, the number of MN increased in BN cells as well as the number of BN cells with MN (Table 2).

Discussion

Although plants are very often used in traditional medicine, 70–95 % of the populations in a majority of developing countries rely on traditional medicine for their primary health care and for the management of health (Robinson and Zhang 2011), but their effect on the genetic material is poorly known in most cases.

Teucrium chamedrys and *T. montanum* are very rich in phenolic compounds with very strong biological activity and antioxidative effects (Čanadanović-Brunet et al. 2006; Ozgen et al. 2006; Gursoy and Tepe 2009; Stanković et al. 2010; Stanković et al. 2011b). Stankovic et al. (2011a) reported that extracts from different *Teucrium* species caused cytotoxicity and induced apoptosis in human HCT-116 cells (human colon cancer cell line), hence they could be considered suitable natural anticancer products. For these purposes, it is important to evaluate the genotoxic and anti-mutagenic effects of these plants on PBL in vitro.

Our results showed that all tested concentrations of methanolic extract from *T. chamaedrys* had no genotoxic effect on the PBL in vitro. Additionally, all tested concentrations of *T. chamaedrys* lowered the mutagenic effects of MMC by decreasing MN frequencies,

Table 2 The frequency of micronuclei (MN) and nuclear division index values (NDI) in peripheral blood lymphocytes (PBL) of healthy donors after the combined treatments with mitomicin C (MMC = 0.5 μg/ml) and different concentrations of methanolic extracts from *T. chamaedrys* and *T. montanum* in vitro

Treatments	Extract + MMC	MN/1,000 BN cells (X ± SD)	BN with MN (%)	Distribution of MN				NDI
				1 MN (%)	2 MN (%)	3 MN (%)	>4 MN (%)	
Negative control cells	0	5.00 ± 1.73	15 (0.50)	15 (0.50)				1.80 ± 0.37
Positive control cells	0 + MMC	169.00 ± 60.61	415 (13.83)	346 (11.53)	48 (1.60)	19 (0.63)	2 (0.07)	1.60 ± 0.11
T. chamaedrys	125 μg/ml + MMC	81.67 ± 63.01[a]	207 (6.90)	173 (5.77)	30 (1.00)	4 (0.13)		1.42 ± 0.10
T. chamaedrys	250 μg/ml + MMC	64.00 ± 46.51[b]	159 (5.30)	128 (4.27)	29 (0.97)	2 (0.07)		1.37 ± 0.03
T. chamaedrys	500 μg/ml + MMC	61.00 ± 30.35[c]	168 (5.60)	154 (5.13)	13 (0.43)	1 (0.03)		1.33 ± 0.07[f]
T. chamaedrys	1,000 μg/ml + MMC	33.67 ± 14.22[d]	94 (3.13)	87 (2.90)	7 (0.23)			1.28 ± 0.01[g]
T. montanum	125 μg/ml + MMC	37.67 ± 18.18[e]	101 (3.37)	90 (3.00)	10 (0.33)	1 (0.03)		1.35 ± 0.06[h]
T. montanum	250 μg/ml + MMC	57.33 ± 17.01	150 (5.00)	132 (4.40)	15 (0.50)	2 (0.07)	1 (0.03)	1.35 ± 0.08[i]
T. montanum	500 μg/ml + MMC	71.33 ± 19.86	192 (6.40)	174 (5.80)	15 (0.50)	2 (0.07)	1 (0.03)	1.42 ± 0.09[j]
T. montanum	1,000 μg/ml + MMC	82.67 ± 35.23	214 (7.13)	189 (6.3)	18 (0.60)	5 (0.17)	2 (0.07)	1.39 ± 0.09[k]

[a,b,c,d,e] Statistically significant difference in MN frequencies between the cells treated with MMC alone (positive control) and cells treated with MMC and methanolic extracts of the plants in co-treatments

[f,g,h,i,j,k] Statistically significant difference in NDI values between the cells treated with MMC alone (positive control cells) and cells treated with MMC and methanolic extracts of the plants in co-treatments

with the strongest antimutagenic activity obtained for the highest concentration (1,000 μg/ml). The presented data show that the applied treatment decreased both the number of BN cells containing MN and the number of MN in binucleated cells. The obtained results clearly indicate the protective properties of *T. chamaedrys* against the genotoxic effect of MMC in a dose depending manner, hence it may be considered a chemoprotective drug in cancer therapy. Chemical analyses of *T. chamedrys* showed that the extracts were rich in phenolic compounds (Stanković et al. 2010) and could be considered responsible for antimutagenic properties of the plant.

Considering methanol extract of *T. montanum*, our results showed that the treatment with the highest concentration (1,000 μg/ml) only, significantly induced MN frequency in PBL in comparison to the untreated control cells, indicating careful usage of higher dose. In addition, only the lowest concentration of *T. montanum* extract (125 μg/ml) decreased the mutagenic effects of MMC, and the protective properties decreased with the increase of the plant extract concentration.

According to our knowledge, there are no available literature data about the genotoxic and antimutagenic effect of the examined plant extracts, but on the other hand there is a study that considered the genotoxic and mutagenic potential of species from the *Teucrium* genus. Sghaier et al. (2011c) reported no mutagenic and no genotoxic effects of *Teucrium ramosissimum* extracts by using different tests. Moreover, extracts of *T. ramosissimum* possess antimutagenic effects against sodium azide (SA), aflatoxin B1 (AFB1), benzo[*a*]pyrene (B[*a*]P), and 4-nitro-*o*-phenylenediamine (NOPD), and it also decreased the genotoxicity induced by aflatoxin B1 and nitrofurantoin.

The NDI value provides a measure of the proliferative status of the viable cells and therefore, it can be used as an indicator of cytostatic effects of the examined agent (Fenech 2007). In our study, the extracts from both *T. chamaedrys* and *T. montanum* have no impact of proliferative status of cultured human lymphocytes, suggesting no cytotoxic effect of the plants on the PBL in vitro. Considering cultures with co-treatment, our results revealed that the extracts of *T. chamaedrys* at concentrations of 500 and 1,000 μg/ml and *T. montanum* at all tested concentrations (125–1,000 μg/ml) significantly decreased the NDI compared to the MMC-treated cells alone; this decrease in cell proliferation may be explained by permitting the repair of MMC induced DNA damages. Eroglu et al. (2009) considered that by permitting repair of genotoxic lesions, mitotic delay may have changed the frequency of cells with two or more nuclei at the time of harvest by changing the number of cells that underwent mitosis.

To the best of our knowledge, this is the first report of the genotoxic and antimutagenic potentials of *T. chamaedrys* and *T. montanum*. The data presented here broaden the knowledge about the properties of *T. chamaedrys*, which has antimutagenic properties as well; further studies are needed to identify bioactive compounds with the protective effect. Moreover, except the highest examined concentrations of *T. montanum*, both plant extracts had no genotoxic effects. Accordingly, the protective properties of *T. montanum* extract decreased with the increase of the extract concentration.

It is known that natural bioactive substances can modify redox status and interfere with basic cellular functions such as cell cycle and apoptosis (Ramos 2008). Many studies of different cell lines (normal and malignant) suggest that polyphenols, separately and in combination with chemotherapeutic agents induced apoptosis (Ferguson 2001; Rajabalian 2008; Ćurčić et al. 2012), which is an important mechanism of chemoprevention and chemotherapy of cancer. In accordance with these conclusions, preventive effect of methanolic extract of *T. chamaedrys* was reflected in the induction of apoptosis in the cells which collected a large quantity of genetic damage induced with MMC. This conclusion is confirmed by the fact that in the combined treatments of the extract with MMC, NDI values gradually decrease until a statistically significant decrease occurs in the treatments with the highest concentrations of the tested extract.

In conclusion, our results confirmed the safe use of *T. chamaedrys* extract in the tested concentrations and its important function in prevention from induced genetic damage. In the cancer therapy, this methanolic extract may prevent genotoxic effects of chemotherapy in PBLs.

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Conflict of interest The authors have no conflicts.

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