

Polyphenolic contents of *Teucrium polium* L. and *Teucrium scordium* L. associated with their protective effects against MMC-induced chromosomal damage in cultured human peripheral blood lymphocytes

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Abstract: *Teucrium* species have been used in traditional medicine for treatment of different diseases. The aim of this study was to investigate polyphenolic contents by high-performance liquid chromatography (HPLC), and the genotoxic effect of methanolic extracts of *Teucrium polium* and *Teucrium scordium* using the cytokinesis-block micronucleus (CBMN) assay on human peripheral blood lymphocytes (PBLs) from healthy donors. The HPLC analysis showed that extracts consist of phenolic acid (gallic, vanillic, caffeic, chlorogenic, p-coumaric, sinapic) and flavonoids (catechin, rutin, myricetin, luteolin, quercetin and apigenin). Cultures were treated with extracts of both plants separately and in combinations with mitomycin C (MMC). In separate treatments, both herbal extracts significantly induced micronucleus (MN) frequency only at the highest concentrations. All concentrations of *T. scordium*, except the lowest, and all concentrations of *T. polium* extracts in combined treatment with MMC significantly reduced the frequency of MN. The extract of *T. polium* did not significantly affect the nuclear division index (NDI), whereas *T. scordium* in higher concentrations, separately and in combined treatment with MMC, significantly decreased the NDI value. Our results suggest that both herbal extracts in combination with MMC have antimutagenic (*T. polium*) and proapoptotic effects (*T. scordium*), which indicates their protective effects in PBLs.

Key words: Genotoxic potential, HPLC analysis, *Teucrium polium*, *Teucrium scordium*, micronuclei, human peripheral blood lymphocytes

1. Introduction

Plants have been used for centuries in the treatment of many human diseases. According to data from the World Health Organization (WHO), about 70%–80% of the world's population in developing countries relies on plants for their primary health care (Chan, 2003). Medicinal plants can be considered the main source of new chemical substances with potential therapeutic effects (Blumenthal, 2000).

Teucrium L. (Lamiaceae) is a large, polymorphic, and cosmopolitan genus of perennial plants, the largest member of the family Lamiaceae distributed in Europe, North Africa, and the temperate parts of Asia, but mainly in the Mediterranean area, including more than 300 species (Tutin et al., 1972).

Teucrium polium L. (felty germander) is a wild-growing, flowering species; it is a perennial, aromatic

plant, 20–50 cm high, with green-grayish leaves and white to light pink flowers, that occurs from June to August and is found abundantly in Southwestern Asia, Europe (Mediterranean region), and North Africa (Diklić, 1974; Djabou et al., 2012). Traditionally, *T. polium* has been used for the treatment of different diseases in humans such as gastrointestinal disorders (kidney and liver diseases, abdominal and intestinal pain), inflammation, eczema, urinary tract inflammation, diabetes, and rheumatic diseases (Said et al., 2002; Abu-Irmaileh and Afifi, 2003; Everest and Ozturk, 2005). This plant is mainly used in folk medicine to improve mental performance (Perry et al., 1996).

Numerous in vivo and in vitro studies have confirmed different biological activities of *T. polium* such as antiinflammatory and antirheumatoid (Tariq et al., 1989), antimicrobial (Balmekki et al., 2013), antihypertensive

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(Suleiman et al., 1988), hypolipidemic (Rasekh et al., 2001), and hypoglycemic properties (Kasabri et al., 2011). Moreover, many studies reported that different extracts of *T. polium* exhibit significant free radical scavenging activity, hydroxyl radical scavenging, and antioxidant activity in vitro (Kadifkova-Panovska et al., 2005). The biological activity of this plant, including its antioxidant activity as well, depends on its polyphenolic compositions.

Teucrium scordium L. (garlic germander) is a perennial plant that grows in South and Southeast Europe, the Middle East, and North Africa. In traditional medicine, the flowering branches of *T. polium* and *T. scordium* are used for tea and tonic-based medicinal preparation for the treatment of some gastrointestinal ailments, wound healing, and as a spice plant.

Recently, the cytotoxic, anticancer, and antimutagenic effects of the ethanolic and aqueous extracts of *T. polium* on various cell lines have been investigated (Nematollahi-Mahani et al., 2007; Khader et al., 2007; 2011; Rahmouni et al., 2017). There are no literature data on the genotoxic potential of methanolic extracts of *T. polium* or *T. scordium*.

The latest research showed that the micronucleus (MN) assay is most frequently used in the evaluation of genotoxic risks of medicinal plants (Sponchiado et al., 2016). MN rates reflect chromosome damage because they arise from chromosome/chromatid fragments or whole chromosomes, which are lost during cell division (Fenech, 2007). In the cytokinesis-block micronucleus (CBMN) assay, cells that have completed one nuclear division are blocked from performing cytokinesis by cytochalasin-B and are consequently readily identified by their binucleated appearance. MN are scored in binucleated (BN) cells only, which enables a reliable comparison of chromosome damage between cell populations that may differ in their cell division (Fenech, 2000).

The preservation of the genetic integrity of normal cells is important. The aim of the present work was to analyze the polyphenolic composition of the methanolic extracts of *T. polium* and *T. scordium*, as well as to investigate the genotoxic and antimutagenic effects of these extracts separately and in combination with known mutagen mitomycin C (MMC) in cultured peripheral blood lymphocytes (PBLs) from healthy donors, by CBMN assay.

2. Materials and methods

2.1. Selection of chemicals

All polyphenolic compounds were HPLC- or reagent-grade purity, unless stated otherwise, and were supplied by Fluka (Sigma-Aldrich, Steinheim, Germany). Acetonitrile was obtained from J.T. Baker (Deventer, the Netherlands), while ortho-phosphoric acid was obtained from Merck (Darmstadt, Germany). HPLC-grade water was purified with a Milli-Q system (Millipore, Milford, MA, USA) and

was used for the preparation of all solutions and reagents. PB-MAX karyotyping medium was purchased from Invitrogen (Carlsbad, CA, USA). Cytochalasin-B, dimethyl sulfoxide (DMSO), and MMC were purchased from Sigma (St. Louis, MO, USA). Giemsa stain was purchased from Alfapanon (Novi Sad, Serbia).

2.2. Plant material collections

In August 2014, aerial parts of the selected species of the flowering plant *Teucrium* were collected from the natural population in the territory of Serbia (*Teucrium polium* L. – Suva Planina Mt. in Southeast Serbia, position: 43°16'51.04"N, 22°08'25.26"E, altitude: 589 m, habitat: limestone rocks; *Teucrium scordium* L. – Zasavica in Northwest Serbia, position: 44°52'55.81"N, 19°24'09.23"E, altitude: 80 m, habitat: wet meadows).

The taxonomic authenticity of the samples was conducted at the Faculty of Science, University of Kragujevac. They were dried under ambient laboratory conditions, sealed, and stored until needed for the experiment.

2.3. Preparation of plant extract

The dried herbal parts (10 g) were powdered and mixed with 200 mL of methanol for extraction. After 48 h, the extract thus obtained was filtered through a paper filter (Whatman No. 1) and rotary evaporated at 40 °C under reduced pressure. The extracts were stored in sterile sample bottles for further processing.

2.4. Phytochemical analysis

2.4.1. High-performance liquid chromatography (HPLC) analysis of polyphenols

The extracts were analyzed by HPLC system (Shimadzu, Kyoto, Japan) consisting of a degasser DGU-20A3, analytical pumps LC-20AT, 7125 injector and SPD-M20A diode array detector, and CBM-20A system controller. Separation was achieved on a Luna C18 (250 × 4.6 mm I.D., 5 µm) (Phenomenex, Torrance, CA, USA) column at 30 °C, with 1.0 mL/min flow rate. The chromatography data were processed using LC Solution computer software (Shimadzu).

The modified method described by Stefkov et al. (2012) was used for separation of flavonoids. The gradient elution profile was as follows: 10% B 0–5 min, gradient 10%–60% B during 5–60 min, 60% B held for 5 min, then ramped from 60% to 90% B in 2–3 min, and equilibrated for further 5 min; mobile phases – A: water acidified with ortho-phosphoric acid, pH 3, B: acetonitrile. Compounds were identified by their retention times and UV-absorption peak at 280 nm. Quantification was based on the external calibration of purified flavonoid standards and phenolic acids over the concentration range 0.05–10.0 µg/mL (n = 7).

2.4.2. Determination of flavonoids and phenolic acids

The method to improve the accuracy of quantitation and eliminate interferences should be considered in the

quantitation of bioflavonoids in plant matrices. Complete removal of co-eluting substances could not be achieved in the samples because the matrices are complex and different in terms of composition from one sample to another.

2.5. In vitro CBMN assay

Peripheral blood was collected by venipuncture from three healthy donors aged 20–25 years, who were nonsmokers, and who had not been exposed to known mutagens. The experiments conformed to the guidelines of the World Medical Association (Declaration of Helsinki).

The CBMN assay was performed as described by Fenech (2000). Heparinized whole blood (0.5 mL) was added to 5 mL of PBMax Karyotyping medium. Cultures were incubated for 72 h at 37 °C. Cytochalasin-B dissolved in DMSO was added to the cultures 44 h after the beginning of incubation, at a concentration of 4.0 µg/mL. The cultures were re-incubated and harvested 28 h later. First, the cells were collected by centrifugation and then treated with cold (4 °C) hypotonic solution (0.56% KCl). Then the cells were fixed three times in 3:1 methanol/glacial acetic acid. The suspended cells were dropped onto air-dried slides and stained with 2% Giemsa.

2.5.1. Teucrium methanol extracts

Methanolic extracts of both *T. polium* and *T. scordium* in the same four concentrations (125, 250, 500, and 1000 µg/mL) were added separately to lymphocyte cultures 24 h after the beginning of incubation. For antimutagenicity testing, the cultures were simultaneously exposed to the extracts and to MMC (Sigma, St. Louis, MO, USA). MMC was added to the cell cultures in the final concentration of 0.5 µg/mL, concomitantly with the plant extract and alone (positive control). Negative controls were untreated cultures.

2.5.2. MN scoring

The analysis of MN was performed based on the criteria for scoring binucleated cells (BN) and MN (Fenech, 2007) using a light microscope (Nikon E50i) at 400× magnification. The frequency of micronucleated BN cells found in 3000 BN cells per extract concentration tested (1000 per donor) was scored, as well as the proportion of mononucleated, binucleated, trinucleated, and tetranucleated cells per 500 cells. The nuclear division index (NDI) was calculated according to the formula $NDI = ((1 \times M1) + (2 \times M2) + (3 \times M3) + (4 \times M4))/N$, where M1–M4 represents the number of cells with 1–4 nuclei, and N is the number of the scored cells (Eastmond and Tucker, 1989).

2.6. Statistical methods

The data were expressed as the mean ± standard deviation (SD). Statistical analysis of genotoxic activities was performed using SPSS (IBM SPSS Statistics 20). Statistically significant difference between MN frequencies and NDI

values was determined by Student's t-test. The levels of significance were $P < 0.05$ and $P < 0.01$. The relationship among the tested concentrations of extracts and MN and NDI was determined by Pearson's correlation coefficient. For the statistical analysis of the difference in polyphenolic compounds between the extracts of *T. polium* and *T. scordium*, Microsoft Office Excel 2010 software was used (t-test: paired two sample for means).

3. Results

The results of the study are presented in Figure 1 and Tables 1–4.

A chromatogram of a synthetic mixture containing polyphenolic acids and flavonoids is shown in Figure 1A. Reproducible peak shapes were obtained under optimum conditions. The chromatograms of extracts of *T. scordium* and *T. polium* are presented in Figures 1B and 1C. The polyphenolic compounds in the extracts of *Teucrium* were identified by retention time and characteristic UV-absorption spectra.

The calibration curve values of polyphenolic compounds are summarized in Table 1. The analytes elicited a linear response in the concentration range 0.05–10.0 µg/mL. The values of the limit of detection (LOD) and limit of quantification (LOQ) indicated the reliable identification and quantification of phenolic acids and flavonoids in the plant extracts. The % relative standard deviation (RSD) based on the peak area at LOQ was found to be less than 5%, indicating the satisfactory sensitivity of quantification.

Table 2 shows the content of the polyphenolic compounds in the plant extracts examined ($\times 10^{-4}$ µg/mL extract). In the methanolic extract of *T. polium*, five phenolic acids and six flavonoids were found, whereas in the extracts of *T. scordium* three phenolic acids and two flavonoids were found. The extract of *T. polium* contained the highest amount of chlorogenic acid (90.0 ± 0.2), while the extract of *T. scordium* included vanillic acid (501.1 ± 0.6). The lowest phenolic content was determined for caffeic acid (1.7 ± 0.2) in the extract of *T. polium* and for sinapinic acid (26.9 ± 0.2) in the extract of *T. scordium*.

Analyzing the content of flavonoids, our results showed that both plants (*T. polium* and *T. scordium*) contained the highest content of catechin (235.0 ± 0.5 ; 32.5 ± 0.6 respectively), while the lowest contents were measured for luteolin (22.0 ± 0.6) in the extract of *T. polium* and for rutin (24.7 ± 0.01) in the extract of *T. scordium*. The second most abundant flavonoid in the extract of *T. polium* was apigenin (157.0 ± 0.3).

Table 3 shows the effects of four different concentrations (125, 250, 500, and 1000 µg/mL) of methanolic extracts of *T. polium* and *T. scordium* on MN frequency per 1000 BN cells (±SD) and distribution of both MN and NDI

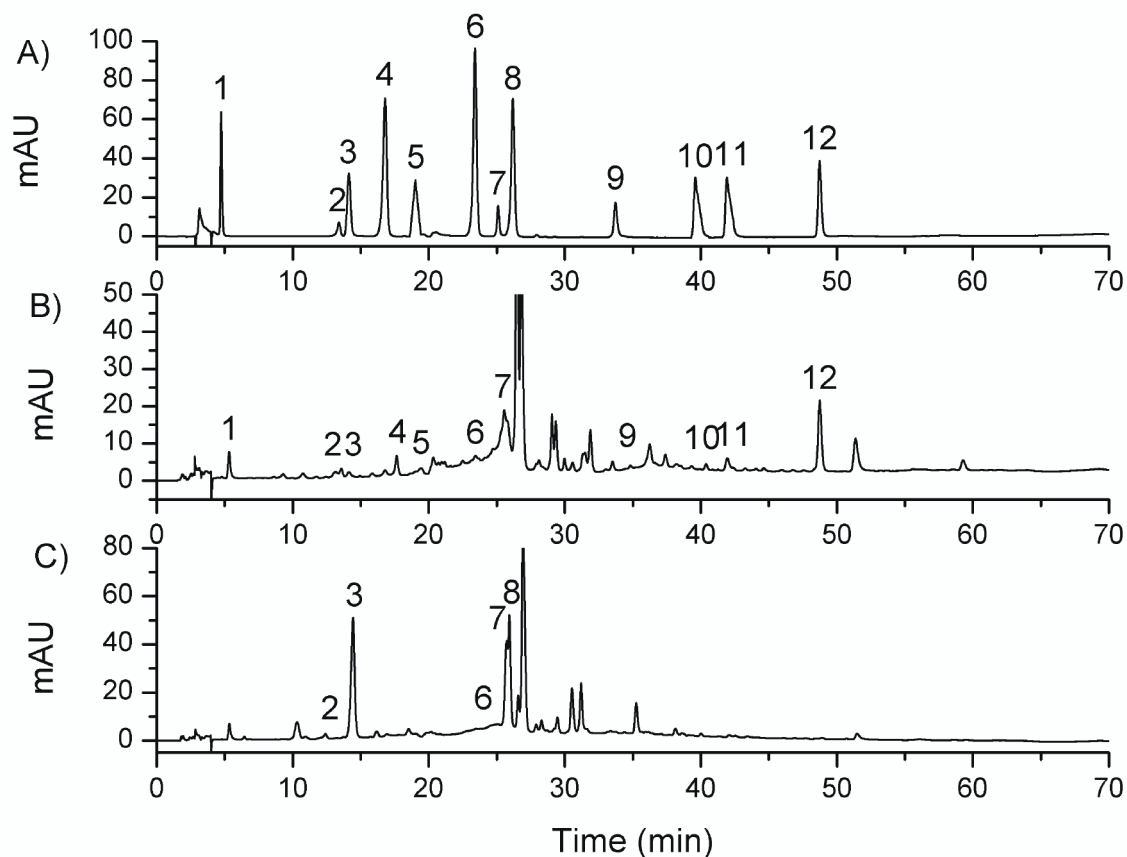


Figure 1. HPLC chromatogram of polyphenolic standards (A); polyphenolic compounds in methanolic extracts obtained from *T. polium* (B) and *T. scordium* (C) (Compounds: 1 gallic acid, 2 catechin, 3 vanillic acid, 4 caffeic acid, 5 chlorogenic acid, 6 rutin, 7 p-coumaric acid, 8 sinapinic acid, 9 myricetin, 10 luteolin, 11 quercetin, 12 apigenin).

Table 1. Linear regression analysis – polyphenolic acids and flavonoids.

Polyphenolic compounds	$Y = a \times 10^6 X + b \times 10^4$	R^2	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)
Polyphenolic acid				
Gallic acid	$Y = 1.6 X + 1.3$	1.000	0.11	0.33
Vanillic acid	$Y = 1.8 X + 3.7$	0.999	0.16	0.48
Caffeic acid	$Y = 2.2 X + 0.7$	0.988	0.09	0.27
Chlorogenic acid	$Y = 1.9 X + 1.9$	0.999	0.07	0.23
p-Coumaric acid	$Y = 0.5 X + 2.9$	0.996	0.35	1.05
Sinapinic acid	$Y = 2.1 X + 3.8$	0.999	0.17	0.52
Flavonoids				
Catechin	$Y = 1.6 X + 0.1$	0.997	0.31	0.96
Rutin	$Y = 2.4 X + 0.2$	1.000	0.03	0.11
Myricetin	$Y = 1.0 X + 1.7$	0.999	0.17	0.54
Luteolin	$Y = 1.8 X + 0.6$	0.993	0.29	0.89
Quercetin	$Y = 1.7 X + 0.7$	0.999	0.15	0.46
Apigenin	$Y = 1.7 X + 16.1$	0.995	0.34	1.02

R^2 - coefficient correlation; LOD- limit of detection; LOQ- limit of quantification

Table 2. Polyphenolic compounds in methanolic extracts of *T. polium* and *T. scordium* ($\times 10^{-4}$ $\mu\text{g/mL}$ extract).

No.	Polyphenolic compounds	t_r	<i>T. polium</i>	<i>T. scordium</i>	t-test t_{crit} 4.302
Phenolic acids					
1	Gallic acid	4.736	8.1 \pm 0.2		
3	Vanillic acid	14.129	2.1 \pm 0.3	501.1 \pm 0.6	+121.9*
4	Caffeic acid	16.793	1.7 \pm 0.2		
5	Chlorogenic acid	19.057	90.00 \pm 0.2		
7	p-Coumaric acid	25.095	30.0 \pm 0.6	111.5 \pm 0.1	+166.6*
8	Sinapinic acid	26.178		26.9 \pm 0.2	
Flavonoids					
2	Catechin	13.401	235.0 \pm 0.5	32.5 \pm 0.6	+59.4*
6	Rutin	23.392	77.0 \pm 0.1	24.7 \pm 0.1	+68.7*
9	Myricetin	33.713	55.0 \pm 0.1		
10	Luteolin	40.125	22.0 \pm 0.6		
11	Quercetin	41.912	24.0 \pm 0.4		
12	Apigenin	48.722	157.0 \pm 0.3		

*Statistically significant difference in polyphenolic compounds between plant extracts (t-test: two paired sample means)

Table 3. Micronuclei (MN) frequency and nuclear division index (NDI) in cultured peripheral blood lymphocytes (PBLs) of healthy donors after treatments with different concentrations of methanolic extracts of *T. polium* and *T. scordium*.

Treatments	Concentrations ($\mu\text{g/mL}$)	AnalyzedBN cells	MN/1000BN cells (X \pm S.D.)	BN with MN (%)	Distribution of MN			NDI(X \pm S.D.)
					1MN (%)	2MN (%)	3MN (%)	
Control untreated cells	0	3000	5.67 \pm 2.52	16 (0.50)	15 (0.47)	1 (0.03)	-	1.73 \pm 0.02
<i>T. polium</i>	125 $\mu\text{g/mL}$	3000	6.33 \pm 2.89	17 (0.57)	15 (0.50)	2 (0.07)	-	1.75 \pm 0.08
<i>T. polium</i>	250 $\mu\text{g/mL}$	3000	6.67 \pm 2.31	19 (0.63)	18 (0.60)	1 (0.03)	-	1.75 \pm 0.11
<i>T. polium</i>	500 $\mu\text{g/mL}$	3000	8.67 \pm 2.08	24 (0.80)	22 (0.73)	2 (0.07)	-	1.64 \pm 0.09
<i>T. polium</i>	1000 $\mu\text{g/mL}$	3000	12.33 \pm 1.53*	35 (1.17)	33 (1.10)	2 (0.07)	-	1.58 \pm 0.10
<i>T. scordium</i>	125 $\mu\text{g/mL}$	3000	7.33 \pm 1.53	21 (0.70)	20 (0.67)	1 (0.03)	-	1.69 \pm 0.07
<i>T. scordium</i>	250 $\mu\text{g/mL}$	3000	10.67 \pm 2.52	28 (0.93)	25 (0.83)	2 (0.07)	1 (0.03)	1.66 \pm 0.11
<i>T. scordium</i>	500 $\mu\text{g/mL}$	3000	12.33 \pm 2.08*	36 (1.20)	35 (1.17)	1 (0.03)	-	1.55 \pm 0.09*
<i>T. scordium</i>	1000 $\mu\text{g/mL}$	3000	17.33 \pm 4.62*	47 (1.57)	44 (1.47)	3 (0.10)	-	1.39 \pm 0.03**

% of cells with 1–3 MN in relation to total number of analyzed cells. *P < 0.05; **P < 0.01, statistically significant difference in comparison to untreated control cells (Student's t-test)

values (\pm SD). The results show that the extract of *T. polium* only at the highest concentration tested (1000 $\mu\text{g/mL}$) significantly induced chromosome damage with probability P < 0.05. Pearson's correlation coefficient demonstrated a dose-dependent increase in MN frequency ($r = 0.789$, P < 0.01) in the extract of *T. polium*. There

was no significant difference between the NDI values of all treatments and untreated PBLs and the correlation between the concentrations of the extract and NDI values was negative ($r = -0.661$; P < 0.05).

On the other hand, *T. scordium* showed genotoxic effects in the two highest concentrations tested (500 and

1000 µg/mL), and the correlation between the tested concentrations and MN frequencies in PBLs ($r = 0.817$; $P < 0.01$) appeared to be positive and statistically significant. The correlation between the concentration of the extract and the NDI was negative ($r = -0.870$; $P < 0.01$).

The combined effects of methanolic extracts of *T. polium* and *T. scordium* and MMC on MN frequency and the distribution of MN and NDI in cultured human PBLs are shown in Table 4. MMC itself significantly increased the MN frequency in PBLs in comparison to untreated PBLs ($P < 0.05$). All the tested concentrations of methanolic extracts of *T. polium* in combined treatments with MMC significantly reduced the MN frequency in a dose-dependent manner ($r = -0.813$; $P < 0.01$), and insignificantly reduced NDI values ($r = -0.434$; $P > 0.05$).

All concentration levels of *T. scordium*, except the lowest one (125 µg/mL), significantly reduced MMC-induced MN frequency ($P < 0.05$). Pearson's correlation coefficient between the extract concentrations and MN frequency was not significantly negative ($r = -0.549$; $P > 0.05$). All *T. scordium* extract concentrations dose-dependently decreased the MMC-induced NDI values ($r = -0.709$, $P < 0.01$), but the effect was significant only at the highest concentrations tested (1000 µg/mL) with probability $P < 0.05$. There were no differences in the NDI values between negative and positive (MMC alone) controls. MMC alone insignificantly decreased NDI if compared to the untreated cells ($P > 0.05$).

The analysis of the distribution of MN in PBLs separately and in combination with methanolic extracts of *T. polium* and *T. scordium* revealed that the BN cells with 1MN were mostly present, BN cells with 2MN were fewer in number, whereas the cells with 3 and 5MN were found only in the cultures treated with *T. scordium* 250 µg/mL extract and MMC and in positive controls (Tables 3 and 4).

4. Discussion

Intensive research over the last few decades has shown the beneficial protective effects of medicinal plant extracts on different cell cultures both under in vitro (Ljubuncic et al., 2006; Leskovac et al., 2007; Milošević-Djordjević et al., 2013) and in vivo (Damasceno et al., 2016; Rahmouni et al., 2017) conditions. In the present study, we investigated the polyphenolic contents and genotoxic and antimutagenic effects of methanolic extracts of *T. polium* and *T. scordium* on PBLs from healthy donors.

The most important polyphenolic compounds in plants are phenolic acid and flavonoids (Petti and Scully, 2009). They play an important role in reducing the risk of cardiovascular disease, as antioxidants, and exhibited a wide variety of other biological activities, including hepatoprotective and antibacterial activities (Tiwari, 2001). Due to their importance in plants and human health, it would be useful to know the concentration of the polyphenolic compounds and biological activities that could indicate their potential as therapeutic agents (Benedec et al., 2013).

Table 4. Micronuclei (MN) frequency and nuclear division index (NDI) in cultured peripheral blood lymphocytes (PBLs) of healthy donors after combined treatments with mitomycin C (MMC = 0.5 µg/mL) and different concentrations of methanolic extracts of *T. polium* and *T. scordium*.

Treatments	Concentrations (µg/mL)	Analyzed BN cells	MN/1000BN cells ($X \pm S.D.$)	BN with MN (%)	Distribution of MN				NDI ($X \pm S.D.$)
					1MN (%)	2MN (%)	3MN (%)	5MN (%)	
Control untreated cells	0	3000	5.67 ± 2.52	16 (0.50)	15 (0.47)	1 (0.03)	-	-	1.73 ± 0.02
Positive control cells	0 + MMC	3000	41.33 ± 5.13	110 (3.67)	99 (3.30)	10 (0.33)	-	1 (0.03)	1.67 ± 0.16
<i>T. polium</i>	125 µg/mL + MMC	3000	27.33 ± 2.89*	81 (2.70)	80 (2.67)	1 (0.03)	-	-	1.65 ± 0.11
<i>T. polium</i>	250 µg/mL + MMC	3000	20.33 ± 1.53*	59 (1.97)	57 (1.90)	2 (0.07)	-	-	1.65 ± 0.10
<i>T. polium</i>	500 µg/mL + MMC	3000	20.00 ± 2.65*	58 (1.93)	56 (1.86)	2 (0.07)	-	-	1.62 ± 0.10
<i>T. polium</i>	1000 µg/mL + MMC	3000	13.67 ± 4.62*	38 (1.27)	35 (1.17)	3 (0.10)	-	-	1.55 ± 0.10
<i>T. scordium</i>	125 µg/mL + MMC	3000	29.33 ± 7.23	81 (2.70)	74 (2.47)	7 (0.23)	-	-	1.54 ± 0.04
<i>T. scordium</i>	250 µg/mL + MMC	3000	22.67 ± 7.37*	60 (2.00)	53 (1.77)	6 (0.20)	1 (0.03)	-	1.46 ± 0.12
<i>T. scordium</i>	500 µg/mL + MMC	3000	20.67 ± 5.69*	61 (2.03)	60 (2.00)	1 (0.03)	-	-	1.46 ± 0.07
<i>T. scordium</i>	1000 µg/mL + MMC	3000	17.33 ± 6.66*	52 (1.73)	52 (1.73)	-	-	-	1.34 ± 0.08*

% of cells with 1–3 and 5 MN in relation to total number of analyzed cells. *Statistically significant difference in comparison to positive control cells ($P < 0.05$, Student's t-test)

By HPLC analysis, we showed that the extracts of the two analyzed plants contained phenolic acids (gallic, vanillic, caffeic, chlorogenic, p-coumaric, and sinapinic) and flavonoids (catechin, rutin, myricetin, luteolin, quercetin, and apigenin). There were different qualitative and quantitative contents of flavonoids and phenolic acids in the plant extracts. In the extract of *T. polium*, there were five phenolic acids and six flavonoids, while in the extracts of *T. scordium* three phenolic acids and two flavonoids were found. In the extract of *T. polium*, the most abundant phenolic acid was chlorogenic acid. On the other hand, the most abundant phenolic acid in the extract of *T. scordium* was vanillic acid. The most abundant flavonoid was catechin in both herbal extracts. Apigenin was found in higher amounts in *T. polium* than in *T. scordium*.

According to the literature, Proestos et al. (2006) analyzed the polyphenolic compounds in *T. polium* and found caffeic acid, ferulic acid, and luteolin. On the other hand, Mitreski et al. (2014) in dry extract of *T. polium* did not detect caffeic acid, and they found only luteolin and apigenin. In contrast, in dry extract of *T. scordium*, caffeic acid and apigenin were found, but luteolin was not. The results of our study suggested that both plants, especially *T. polium*, may be considered a potential source of polyphenols.

One of the most commonly used cytogenetic assays in the genotoxic assessment of different agents is the CBMN assay in cultivated human PBLs and it is simultaneously used as a method in human biomonitoring studies (Decordier et al., 2009; Speit et al., 2011). Our results showed that only the highest concentrations of methanolic extract of both plants have genotoxic effects. High levels of flavonoids contained in higher concentrations of the extract can possibly contribute to the promutagenic effect. Stopper et al. (2005), Silva et al. (2000), and Boos and Stopper (2000) showed that flavonoids may cause genetic damage in a variety of prokaryotic and eukaryotic systems.

Furthermore, numerous studies provide evidence for dual biological activities of flavonoids. The antimutagenic/promutagenic and antioxidant/prooxidant activities largely depend on the concentration used (Labieniec et al., 2003; Labieniec and Gabryelak, 2003; Stopper et al., 2005), as well as the physiological parameters (Skibola and Smith, 2000; Noel et al., 2006). Several studies confirmed some phenolic acids as well-known antioxidants and possible prooxidants, depending on concentration, thus being a significant cause of DNA damage. Accordingly, Erdem et al. (2012) demonstrated that a high dose of the vanillic acid itself had genotoxic effects on DNA in PBLs in vitro, and when used at an appropriately low dose this acid could prevent oxidative DNA damage.

To determine the protective effects of both herbal extracts against MMC-induced chromosomal damages

in PBLs, the cell cultures were treated with the tested concentrations of extracts in combination with MMC. MMC is a natural antitumor antibiotic drug used in clinical chemotherapy regimens in various carcinomas (Verweij and Pinedo, 1990). However, MMC induces chromosomal aberrations as a noncell-cycle-specific agent, therefore showing strong genotoxic effects in different cell lines (Ho and Schärer, 2010; Vasquez, 2010). During metabolism, the MMC can generate reactive oxygen species (ROS) such as superoxide radical anions, hydrogen peroxide, and hydroxyl radicals (Albertini et al., 2003; Ortega-Gutierrez et al., 2009). ROS induce numerous lesions in DNA including deletions, base modifications, and single and double-strand breakages (Cadet et al., 1994). It is known that free radicals involve micronucleus formation (Vijayalaxmi et al., 1999) and MMC generates micronuclei-induced genotoxic damage in animal models (Hayashi et al., 1992; Grisolia, 2002). Thus, for example, MMC induced genotoxic effect that can be detected by different methods such as chromosomal aberrations and sister chromatid exchanges (Krishnaja and Sharma, 2008) and MN frequency (Fauth et al., 2000) in the cultures of PBLs. In this study, we observed that MMC induced micronuclei in PBLs. In our opinion, these chromosomal damages were, to some extent, the result of the activity of free radicals formed during the metabolism of MMC.

The results of our study clearly indicate the protective properties of methanolic extracts of *T. polium* and *T. scordium* against the genotoxic effect of MMC in a dose-dependent manner. All the concentrations of *T. polium* and *T. scordium* (except the lowest one) significantly decreased the genotoxic effect of MMC. However, the strongest protective activity was observed in the highest tested concentration for both plants. These results are in accordance with our recently published results (Milošević-Djordjević et al., 2013) confirming the protective effect of methanolic extract of *T. chamaedrys* against MMC-induced genetic damage in healthy PBLs.

Since a plant extract is a mixture of natural compounds, their different biological activities (antioxidant, antimicrobial, antigenotoxic, and anticancer) are not only the result of different activities of individual components and may also be the result of their interactions, possibly producing different effects on the overall activity of extracts. Therefore, numerous authors have suggested that the activity of the extracts may be the result of synergistic effects of several compounds (Romero-Jimenez et al., 2005; Kosanić et al., 2016).

In recent years, a large number of authors have suggested the use of medicinal plants as antimutagenic agents in the prevention of genotoxic effects of different chemotherapeutic agents (Verschaeve et al., 2004; Sibanda and Okoh, 2007; Kumar et al., 2012; Vlastos et al., 2013).

Antimutagenic activity of the tested extracts may be ascribed to flavonoids (Calomme et al., 1996), tannins (Baratto et al., 2003), and total polyphenols (Ben Ammar et al., 2008).

The protective effects of *T. polium* and *T. scordium* reported in this study can be justified by the presence of phytochemical compounds in the methanolic extracts of both species. Phytochemical analysis showed that the extracts were rich in polyphenolics (catechin in extracts of both plants; vanillic acid in the extract of *T. scordium*, and chlorogenic acid in the extract of *T. polium*). Some studies indicate the antigenotoxic potential of catechin hydrate against cadmium toxicity in PBLs (Alshatwi et al., 2014). The second most abundant flavonoid in the extract of *T. polium* is apigenin. Some previous studies have shown that apigenin had protective effects on radiation-induced chromosome damage in human lymphocytes (Rithidech et al., 2005; Sharma, 2013). Similarly, Siddique et al. (2008) concluded that apigenin is potent in decreasing MMC and cyclophosphamide-induced genotoxic damage, thereby reducing the possible development of secondary tumors during therapy. Siddique and Afzal (2009) indicated a protective role of apigenin against the genotoxicity of MMC on mouse bone marrow cells. In addition, Siddique et al. (2010) showed a clear reduction in genotoxic damage in PBLs induced by ethinylestradiol observed with increasing doses of apigenin, suggesting a protective role for apigenin during ethinylestradiol therapy. On the other hand, Cinkilic et al. (2013) showed that chlorogenic and quinic acids had a protective effect on in vitro radiation-induced DNA damage in human lymphocytes isolated from two healthy human donors, using the alkaline comet assay.

Cell proliferation was evaluated by nuclear division index (NDI), which indicates the average number of cell cycles. NDI is a marker of cell proliferation in cultures and is considered a measure of general cytotoxicity. The NDI provides a measure of the proliferative status of the viable cells and, therefore, can be used as an indicator of the cytostatic effect of the examined agents (Eastmond and Tucker, 1989; Fenech, 2000).

In our study, the methanolic extract of *T. polium*, separately and in combination with MMC, had no effect on cell proliferation, and this extract had no cytotoxic effect on PBLs in vitro. These results are consistent with previous reports from our laboratory (Milošević-Djordjević et al., 2013) showing that both methanolic extracts of *T. chamaedrys* and *T. montanum* administered alone in PBLs in vitro did not significantly affect the NDI in the concentrations tested.

Numerous studies have shown that extracts of *T. polium* have significant effects on cell proliferation in

different cell types. Khader et al. (2007) demonstrated that aqueous extract of *T. polium* significantly reduced the mitotic indices in primary rat hepatocyte cultures after simultaneous treatment with mutagen *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), indicating that the extract enhances MNNG-mediated cytotoxicity. In a later study, Khader et al. (2010) showed that the ethanolic extract of *T. polium* had an antimutagenic effect on primary rat hepatocyte cultures before, during, and after treatment with MNNG. Moreover, investigations demonstrated that the extract of *T. polium* potentiates the cytotoxic and apoptotic effects of anticancer drugs (vincristine, vinblastine, and doxorubicin) against a panel of cancerous cell lines (Rajabalian, 2008). Some other studies also showed the cytotoxicity of *T. polium* extracts (Lekehal et al., 1996).

MN frequencies and NDI values obtained in our study showed the antimutagenic effect of the methanolic extract of *T. polium*, which can be explained by the antioxidative properties of the bioactive compounds present, as well as the decrease in MMC mutagenicity. However, the extract of *T. scordium* in a separate treatment significantly decreased the NDI values only at higher concentrations (500 and 1000 µg/mL) when compared to the untreated cells. The highest concentration (1000 µg/mL) of the extract in combination with MMC significantly decreased both the NDI values and MN frequency if compared to the cells treated with MMC alone. The decrease may be explained by permitting MMC-induced DNA damage repair. The cells with a substantial amount of genetic damage were brought to apoptosis, indicating the proapoptotic effect of the highest concentration of *T. scordium* extract. Thus, MN is a possible mechanism for introduction into cell death – apoptosis. Similar results were obtained by Hess et al. (1999) and Abou-Eisha et al. (2004), who studied NDI values in cultured PBLs treated with various agents.

Based on our results, we concluded that the methanolic extracts of plants in combination with MMC have antimutagenic (*T. polium*) and proapoptotic effects (*T. scordium*). These effects could be ascribed to polyphenolic compounds. The methanolic extracts of plants analyzed in our study were different in terms of the qualitative and quantitative content of flavonoids and phenolic acid. Future studies should examine the individual components present at high concentrations as HPCL analysis showed.

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