

New Values of *Teucrium* species: *in Vitro* Study of Cytotoxic Activities of Secondary Metabolites

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Abstract

The cytotoxicity of seven *Teucrium* species, a long time ago used as a food spices, for beverages and teas preparing, as well as therapeutics for digestive and respiratory diseases, were examined against human cervix adenocarcinoma HeLa, human melanoma Fem-x, human chronic myelogenous leukemia K562 and human breast adenocarcinoma MDA-MB-361 cells. MTT assay was used for determination of target cell survival. The most prominent cytotoxic effect was observed against K562 cells, especially by *T. scordioides*, *T. montanum* and *T. botrys*. All *Teucrium* extracts showed good cytotoxic activity on HeLa cells, but very low cytotoxic effect on MDA-MB-361 cells. In addition, the cytotoxic activities of *T. scordioides* and *T. montanum* extract were tested on healthy resting and phytohaemagglutinin-stimulated peripheral blood mononuclear cells (PHA-stimulated PBMC). *T. scordioides* and *T. montanum* extracts at concentration of 200 µg/ml reduced the resting PBMC and PHA-stimulated PBMC survival up to 10% and 20%, while the reduction of K562 cell survival at the same concentration of extracts was 94% and 97%, respectively. These results point to selectivity in their antitumor actions. *Teucrium* species can be regarded as promising candidates for natural plant sources of effective biological compounds as a supplements in the food industry, as well as for therapeutic use.

Keywords: anticancer properties, germanders of Serbian flora, malignant cell lines, MTT test

Introduction

Cancer continues to be a leading cause of death worldwide due to a limited success of available treatments for metastatic cancers (Jemal, 2012). There were an estimated 3.45 million new cases of cancer (excluding non-melanoma skin cancer) and 1.75 million deaths from cancer in Europe in 2012 (Ferlay *et al.*, 2013). These facts inspired an increasing interest in plants as source of novel therapeutic agents. Only 1-10% plant species out of the 250,000-500,000 species estimated on this planet, have been studied chemically and pharmacologically (Verpoorte, 2000).

The genus *Teucrium* L. (Lamiaceae) includes over 300 species commonly known as germanders and widespread all over the continents (Amirghofran *et al.*, 2010). Some of these species are endemic to the Mediterranean and Middle East area (Kästner, 1989). Nine species of *Teucrium* are native in central and west Balkan. They are mostly perennial herbs, shrubs or subshrubs, while *T. botrys* is a herbaceous annual herb. The *Teucrium*-based formulations have been used since ancient times as remedial

measures against various human ailments. Traditional medicinal use of *T. polium* for treatment of abdominal pain, indigestion, common cold, diabetes and urogenital diseases dates back over two thousand years (Bahramikia and Yazdanparast, 2012; Rajabalian, 2008; Said *et al.*, 2002). *T. chamaedrys*, *T. montanum* and *T. scordium* are the most popular traditional remedies in the Balkan area (Kundaković *et al.*, 2011). They are used as tonic, bitter, antianemic, cholagogue, antimotility, febrifuge and vulnerary agents (Jarić *et al.*, 2007; Kundaković *et al.*, 2011; Redžić, 2007).

The *Teucrium* species are considered as a potential source of diterpenoids, flavonoids, phenols, iridoids, sterols and terpenoids (Bahramikia and Yazdanparast, 2012; Eskandary *et al.*, 2007; Rajabalian, 2008). Diterpenoids and flavonoids are usually responsible for anti-cancer properties of *Teucrium* extracts. For instance, anti-cancer chemosensitizer effects of diterpenoids and methanolic extracts of *T. polium* and vincristine, vinblastine and doxorubicin against Skmel-3 (melanoma), Saos-2 (osteosarcoma), SW480 (colon carcinoma), MCF-7 (breast

carcinoma), KB (oral cavity epidermal cell line), EL (bladder carcinoma) and A431 (epidermoid carcinoma) cells are demonstrated (Rajabalian, 2008). Also, flavonoids are considered as strong inducers of apoptosis (Haidara, 2011).

The anticancer properties of *Teucrium* extracts and compounds are tested on various types of human cancer cell lines, such as bladder carcinoma (EL, Fen), breast adenocarcinoma (MCF-7, estrogen-dependant MDA-MB-361, estrogen-nondependant MDA-MB-453), Burkitt's lymphoma (Raji), cervix epitheloid carcinoma (HeLa), chronic myelogenous leukemia (K562), colon carcinoma (CACO-2, HCT-116, LoVo, SW480), epidermoid carcinoma (A431), glioblastoma multiforme (REYF-1), hepatoblastoma (HepG2), larynx carcinoma (Hep-2), lung carcinoma (COR-123), non-small cell lung cancer (H322, A549), melanoma (C32, Skmel-3), osteosarcoma (Saos-2), prostate carcinoma (DU145, PC3), T cell leukemia (Jurkat), etc. (Abu-Dahab and Affifi, 2007; Amirghofran et al., 2010; Eskandary et al., 2007; Haidara, 2011; Kandouz et al., 2010; Kundaković et al., 2011; Menichini et al., 2009; Pacifico et al., 2012; Rajabalian, 2008; Sghaier et al., 2012; Stanković et al., 2011; Talib and Mahasneh, 2010; Yin et al., 2009).

Previously, chemical content of methanolic extracts of seven *Teucrium* species – *T. scordioides*, *T. scordium*, *T. chamaedrys*, *T. polium*, *T. montanum*, *T. arduini* and *T. botrys* was compared with their antiproliferative, proapoptotic and antioxidant properties (Stanković et al., 2011). The highest content of phenolic compounds and the best cytotoxicity activity on human colon carcinoma cells HCT-116 were observed with methanolic extract of *T. chamaedrys* and *T. arduini* (Stanković et al., 2011). Kundaković and colleagues (2011) explored cytotoxicity and antimicrobial activity of cyclohexane, dichloromethane and methanolic extracts of *T. scordioides*. They observed high cytotoxicity of cyclohexane and dichloromethane and lack of cytotoxicity of the methanolic extracts of *T. scordioides* on breast cancer cell lines (MDA-MB-361 and MDA-MB-453).

The aim of this study was further evaluation of anticancer potential of methanolic extracts of seven *Teucrium* species – *T. scordioides*, *T. scordium*, *T. chamaedrys*, *T. polium*, *T. montanum*, *T. arduini* and *T. botrys* against novel set of cancer cells (HeLa, Fem-x, K562, MDA-MB-361). Also, the extracts which showed the most notable cytotoxicity (*T. scordioides* and *T. montanum*) were examined on normal human immunocompetent peripheral blood mononuclear cells (PBMC) – on resting and stimulated to proliferate by the mitogen phytohemagglutinin (PHA).

Materials and methods

Chemicals

Methanol, potassium hydroxide (KOH) and sodium nitrite (NaNO_3) were purchased from - Zorka pharma, Serbia. *N*-(1-naphthyl)ethylenediamine were purchased from Fluka Chemic AG, Buchs, Switzerland. Fetal bovine serum (FBS) and trypsin-EDTA were from PAA (The cell culture company), Austria. Dimethyl sulfoxide (DMSO), nitro blue tetrazolium (NBT), ethidium bromide and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from SERVÁ, Germany and sulfanilic acid from MP Hemija, Serbia.

Plant material

From June to September 2009 aerial flowering parts of *Teucrium* species were collected from natural populations in the regions of Serbia and Montenegro. The voucher specimens of *T. scordioides*, *T. scordium* L., *T. chamaedrys* L., *T. polium* L., *T. montanum* L., *T. arduini* L. and *T. botrys* L., were confirmed and deposited in Herbarium at 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 extracts

Prepared plant material (10 g) was transferred to dark-colored flasks and was soaked in 200 ml of methanol and stored at room temperature. After 24 h, the infusions were filtered through Whatman No. 1 filter paper and 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 Rotary evaporator. The obtained extracts were kept in sterile sample tubes and stored in a refrigerator at 4 °C.

Cell culture

Human cervix adenocarcinoma HeLa, human melanoma Fem-x and human breast adenocarcinoma MDA-MB-361 cells were cultured as monolayers. Human chronic myelogenous leukemia K562 cells were grown in a suspension in nutrient medium. Cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The complete nutrient medium was RPMI 1640 supplemented with 3 mM L-glutamine, 100 µg/ml streptomycin, 100 IU/ml penicillin, 10% heat-inactivated (56 °C) fetal bovine serum and 25 mM Hepes adjusted to pH 7.2 with a bicarbonate solution. The cells were grown at 37 °C in an atmosphere of 5% CO₂ and humidified air. RPMI 1640, L-glutamine and Hepes were obtained from PAA (Pasching Austria).

Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were separated from whole heparinized blood of two healthy volunteers by Lymphoprep (Oslo, Norway) gradient centrifugation. Interface cells were washed three times with Haemacel (aqueous solution supplemented with 145 mM Na⁺, 5.1 mM K⁺, 6.2 mM Ca⁺, 145 mM Cl⁻ and 35 g/l gelatin polymers, pH 7.4), counted and resuspended in nutrient medium.

Treatment of cancer cell lines

HeLa (2,000 cells per well), Fem-x (5,000 cells per well), MDA-MB-361 (10,000 cells per well) were seeded into 96-well microtiter plates and 20 h later, after cell adherence, five different concentrations of the plant extracts were added to the wells. Nutrient medium was only added to the cells in the control wells. K562 cells (5,000 cells per well) were seeded 2 h before addition of the extracts. Stock solutions of the extracts were made in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/ml. They were diluted with complete nutrient medium and applied to cells at five different final

concentrations that ranged from 12.5 µg/ml to 200 µg/ml. All experiments were done in triplicate. Cisplatin was used as a positive control.

Treatment of PBMC

PBMC (150,000 cells per well) were seeded into nutrient medium or in nutrient medium enriched with PHA (5 µg/ml) in 96-well microtiter plates. After 2 h, five different concentrations of the plant extracts were added to the wells, in triplicate, except to the control wells where a nutrient medium only was added to the cells. The final concentrations of the tested extracts ranged from 12.5 µg/ml to 200 µg/ml. PHA was obtained from INEP (Belgrade, Serbia). Cisplatin was used as a positive control.

Determination of target cell survival

Cell survival was determined by the MTT test according to the method of Mosmann (1983) modified by Ohno and Abe (1991). Briefly, after the treatment with plant extracts for 72 h, 10 µl of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was added to each well. Samples were incubated for a further 4 h, followed by the addition of 100 µl of 10% SDS. Absorbance at 570 nm was measured the next day.

To quantify cell survival (S%), the absorbance of a sample with cells grown in the presence of different concentrations of the investigated agents was divided by the absorbance of the control cells grown only in the nutrient medium, and multiplied by 100. It is implied that the absorbance of the blank was always subtracted from the absorbance of the corresponding sample with target cells. The IC₅₀ was defined as the concentration of the agent that inhibited cell survival by 50%, compared to the vehicle-treated control.

Results and discussions

Cytotoxic effects of methanolic extracts of seven different plant species from the genus *Teucrium* were examined on the selected malignant cell lines: human cervix adenocarcinoma

HeLa, human melanoma Fem-x, human chronic myelogenous leukemia K562 and human breast adenocarcinoma MDA-MB-361 cells. The investigated *Teucrium* extracts exhibited selective dose-dependent cytotoxic activities against target malignant cells (Table 1 and Fig. 1). Each of the *Teucrium* extracts exerted the most pronounced cytotoxic action against myelogenous leukemia K562 cells. The highest cytotoxic activity against K562 cells was demonstrated for methanolic extract of *T. scordioides* known previously for the highest content of phenolic compounds and the best cytotoxicity activity on human colon carcinoma cells HCT-116 (Stanković et al., 2011). Besides, high performance liquid chromatography (HPLC) analysis of this extract revealed presence of flavonoid aglycones (luteolin, apigenin and diosmetin) and their glycosides (luteolin-7-O-glucoside, luteolin-7-O-rutinoside and diosmetin-7-O-glucoside) (Kundaković et al., 2011). Extracts of *T. montanum* and *T. botrys* showed similar intensities of notable cytotoxic action on K562 cells as the one above reported for the extract of *T. scordioides*. All seven *Teucrium* extracts demonstrated pronounced cytotoxic effects against cervix adenocarcinoma HeLa cells. Additionally, these plant extracts exhibited very low cytotoxic effects against melanoma Fem-x and breast adenocarcinoma MDA-MB-361 cells as noticed previously (Kundaković et al., 2011). The intensities of cytotoxic activity of seven methanolic *Teucrium* extracts against particular malignant cell line did not differ significantly (Table 1). Photomicrographs of HeLa and K562 cells obtained after the treatment with plant extracts for 72 h applied at a concentration of 200 µg/ml demonstrate that *Teucrium* plant extracts induced significant decrease in the number of survived cells in comparison to cells in the control sample (Fig. 2).

To evaluate the sensitivity of the healthy immunocompetent cells, involved in the antitumor immune response, the cytotoxic activities of *T. scordioides* extract and *T. montanum* extract were tested against resting and PHA-stimulated PBMC. These data are presented in Table 2 and Fig. 3. At concentrations up to 200 µg/ml these plant extracts did not exert notable cytotoxic action against unstimulated PBMC. *T. scordioides* extract and *T. montanum* extract at a concentration of 200 µg/ml reduced the

Table 1. Concentrations of *Teucrium* extracts which induced 50% decrease in cancer cell survival, determined by the MTT test

	HeLa	Fem-x	K562	MDA-MB-361
<i>T. scordioides</i> IC ₅₀ [µg/ml]	139.96 ± 12.11	>200	96.63 ± 28.61	196.5 *
<i>T. scordium</i> IC ₅₀ [µg/ml]	144.82 ± 10.24	≈ 200	102.48 ± 22.04	≈ 200
<i>T. chamaedrys</i> IC ₅₀ [µg/ml]	146.47 ± 22.04	190.16 ± 13.92	102.71 ± 24.46	188.28 ± 2.94
<i>T. polium</i> IC ₅₀ [µg/ml]	148.02 ± 4.99	199.79 ± 0.30	116.75 ± 24.40	≈ 200
<i>T. montanum</i> IC ₅₀ [µg/ml]	152.34 ± 15.88	196.44 ± 5.03	99.15 ± 11.92	>200
<i>T. arduini</i> IC ₅₀ [µg/ml]	137.51 ± 2.22	>200	113.38 ± 14.94	≈ 200
<i>T. botrys</i> IC ₅₀ [µg/ml]	164.23 ± 18.92	≈ 200	98.78 ± 7.84	≈ 200
Cisplatin IC ₅₀ [µM]	5.39 ± 1.21	6.16 ± 0.31	5.34 ± 1.60	37.42 ± 7.32

*n=1

Time of continuous agent's action was 72 h

IC₅₀ data are mean ± SD values of three independent experiments

Table 2. Concentrations of *Teucrium scordioides* and *T. montanum* extracts which induced 50% decrease in PBMC survival, determined by the MTT test

	PBMC	PBMC + PHA
<i>T. scordioides</i> IC ₅₀ [µg/ml]	>200	>200
<i>T. montanum</i> IC ₅₀ [µg/ml]	>200	>200
Cisplatin IC ₅₀ [µM]	>66.67	>66.67

Time of continuous agent's action was 72 h

IC₅₀ data are mean ± SD values of two independent experiments

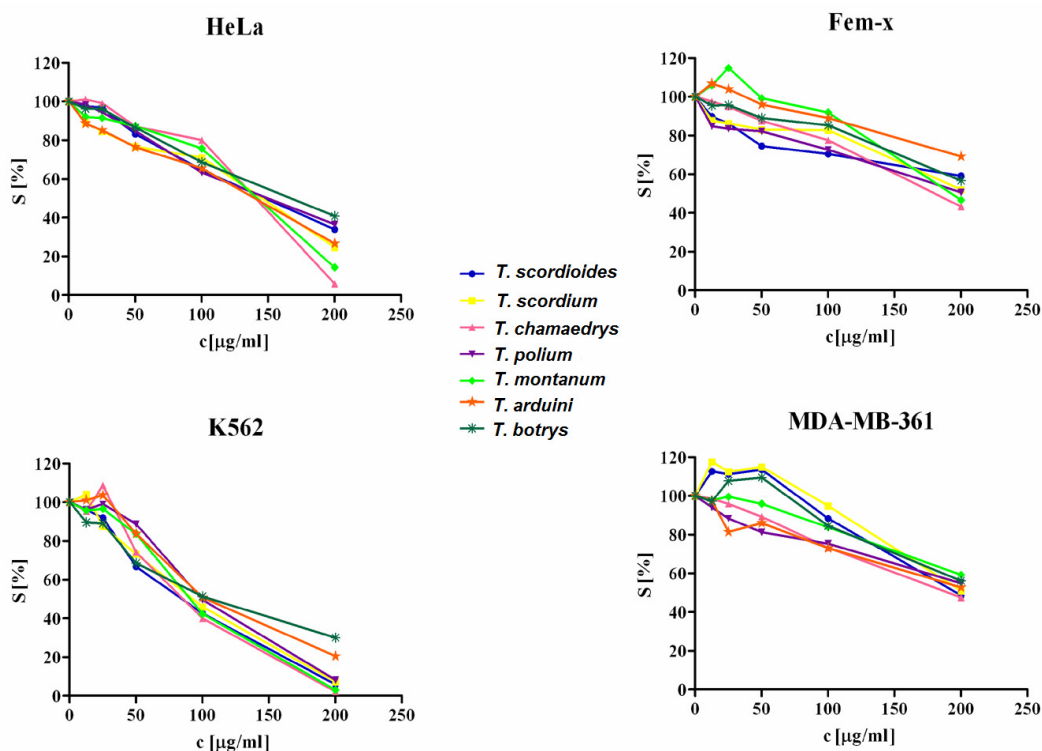


Fig. 1. Survival of HeLa, Fem-x, K562 and MDA-MB-361 cells grown for 72 h in the presence of increasing concentrations of *Teucrium* extracts determined by the MTT test. Representative graphs are shown

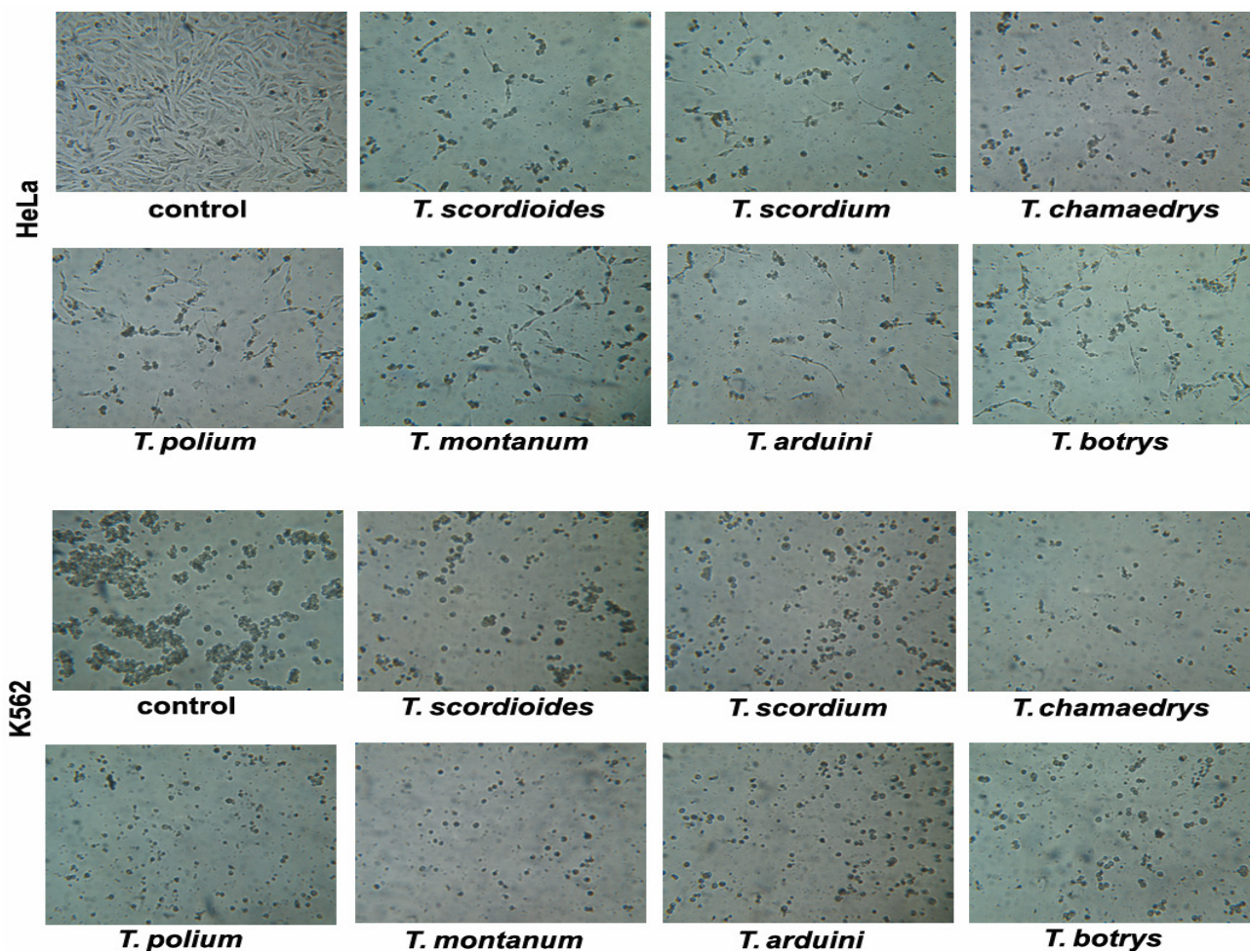


Fig. 2. Photomicrographs of HeLa and K562 cells obtained after 72 h treatment with *Teucrium* extracts

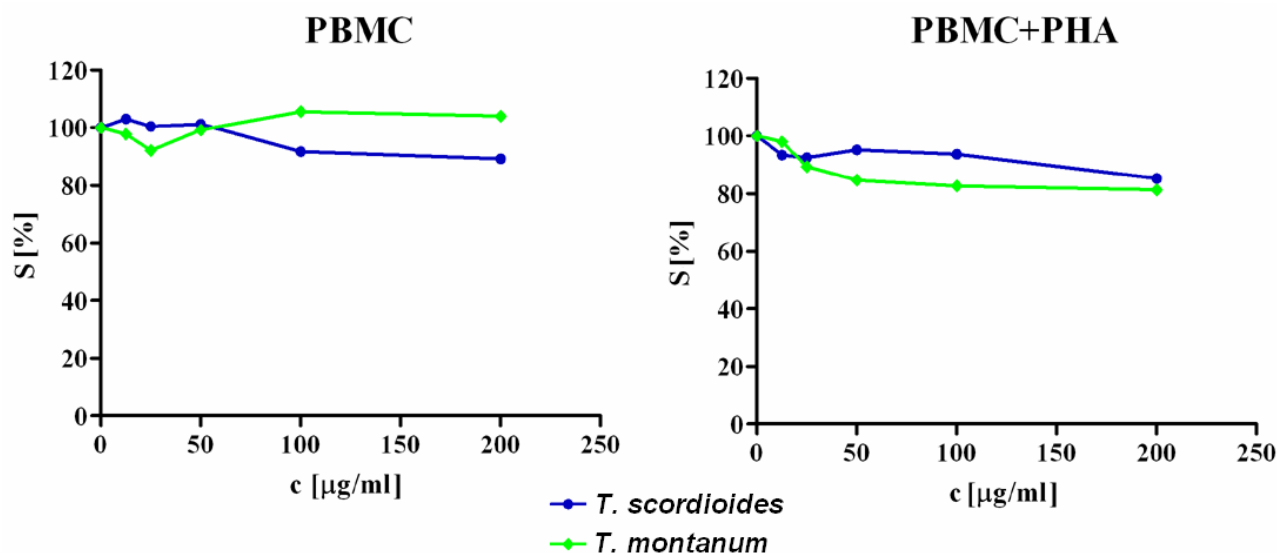


Fig. 3. Survival of resting PBMC and PHA-stimulated PBMC grown for 72 h in the presence of increasing concentrations of *Teucrium scordioides* and *T. montanum* extracts, determined by the MTT test. Representative graphs are shown

resting PBMC survival up to 10%. On the other hand, tested extracts at the concentration of 200 µg/ml induced decrease in the PHA-stimulated PBMC survival up to 20%. It is noteworthy that when applied at the same concentrations these two extracts exhibited pronounced cytotoxic actions against malignant cell lines, especially against K562 cells (by reducing the K562 cell survival for 94% and 97% respectively), pointing to selectivity in their antitumor action. In view of obtained results, other *Teucrium* plant extracts whose effects against PBMC were not tested, are expected to display the same selectivity in the cytotoxic action against malignant cells in comparison to healthy immunocompetent cells.

Generally, the cell-type specific sensitivity to the tested extracts are considered to reflect the difference in the presence of various classes of compounds in the extracts (such as polyphenols and flavonoids) and their modes of action. Phenolic compounds as the most abundant polar fraction of methanolic extract and the most responsible for its antioxidative activities modulate carcinogenesis through two main mechanisms: modification of redox status and interference of basic cellular functions (cell cycle, apoptosis, inflammation, angiogenesis, invasion and metastasis) (Amiri 2010; Kampa, 2007; Stanković et al., 2011). On the other hand, flavonoid compounds in *Teucrium* extracts are believed to be apoptosis-inducers through p53 and other regulators of apoptosis (Haidara et al., 2011; Lin et al., 2008). Also, flavonoids are able to influence a variety of cell functions by modulating cell signaling and inhibiting cancer cell proliferation and migration. For instance, flavonoids as major bioactive compounds of *T. polium* extract showed inhibition of human prostate cancer cells (DU145 and PC3) proliferation, decrease of the cancer cell invasion and metastasis, induction of differentiation to an epithelial phenotype "mesenchymal-epithelial transition" and re-localization of the expression patterns of E-cadherin and catenins (Al Bahtiti, 2012; Haidara et al., 2011; Kandouz et al., 2010). *T. polium* extract inhibited the phosphorylation of beta-catenin, via Src dephosphorylation, and consequently converted its role from a transcriptional regulator to a cell-cell adhesion molecule

(Kandouz et al., 2010). Kandouz et al. (2010) concluded that *T. polium* extract inhibited signaling pathways involved in regulating the E-cadherin/catenin complex and possibly other cell-cell adhesion genes via beta-catenin alteration.

Conclusions

Seven *Teucrium* species out of nine widespread in the Balkan (*T. scordioides*, *T. scordium*, *T. chamaedrys*, *T. polium*, *T. montanum*, *T. arduini* and *T. botrys*) were evaluated against four different malignant cell lines (HeLa, Fem-x, K562 and MDA-MB-361). Examined *Teucrium* extracts showed selective dose-dependent cytotoxic activities against target malignant cells. The strongest cytotoxic effects of the tested extracts were observed against leukemia K562 cells, especially of the *T. scordioides*, *T. montanum* and *T. botrys* methanolic extracts. It should be noted that *T. scordioides* and *T. montanum* extracts exerted notable selectivity in their cytotoxic actions against K562 cells in comparison to normal human immunocompetent PBMC. These species should be studied thoroughly for effective anti-cancer components. The *Teucrium* species represent potent natural source of future anti-cancer drugs or chemosensitizers of available cytostatics.

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