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Teucrium polium induces apoptosis in peripheral blood lymphocytes isolated from human chronic lymphocytic leukemia

Teucrium polium indukuje apoptozu limfocita iz periferne krvi kod obolelih od hronične limfocitne leukemije

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Abstract

Background/Aim. Chronic lymhocytic leukemia (CLL) is considered more as a disease of cells accumulation due to the defect in apoptosis rather than deregulated cell's proliferation. The activation of apoptosis is one of the main molecular mechanisms responsible for anti-cancer activities of most of the currently studied potential anti-cancer agents, including natural compounds. *Teucrium polium* (TP) extracts exhibited strong cytotoxic effects in murine leukemia cell line, RAW 264.7 and human melanoma cell line, C32, but their cytotoxic effects against human leukemia cells were unknown. **Methods.** The viability of human leukemia cell lines (MOLT 4 and JVM 13), lymphocytes isolated from 28 patients with CLL (CLL cells), and peripheral blood mononuclear cells (PBMCs) isolated from 16 healthy subjects treated with TP leaves methanolic extract, was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Apoptosis of TP treated CLL cells was measured by flow cytometry applying Annexin V/7AAD staining. The expressions of active proapoptotic protein Bax, antiapoptotic protein Bcl-2, cytochrome c and the percentage of cells containing cleaved caspase-3 in treated CLL cells was determined by flow cytometry and immunocytochemistry. **Results.** The TP methanolic extract decreased the viability of all tested human leukemia cells but it had no effect on the viability of PBMCs isolated from healthy subjects. The cytotoxic effect of TP was caused by its induction of CLL cells' apoptosis. TP disarranged the ratio of the expressions of proapoptotic Bax and antiapoptotic Bcl-2 protein in favor of Bax, consequently inducing apoptosis by cytochrome c mitochondrial release and activation of caspase-3 in treated CLL cells. **Conclusion.** The TP leaves methanolic induced selective apoptosis in CLL cells and it affected the expressions of key proteins involved in the regulation of programmed cell death.

Key words:
teucrium extract; apoptosis; leukemia, lymphocytic, chronic, b cell; cell line; mice; humans.

Apstrakt

Uvod/Cilj. Hronična limfocitna leukemija (HLL) se pre smatra bolešću akumulacije ćelija usled defekta u njihovoj apoptozi, nego bolešću ćelijske proliferacije. Aktivacija apoptoze je jedan od glavnih molekulskih mehanizama odgovornih za antitumorsku aktivnost većine agenasa koji se sada ispituju, uključujući i agense prirodnog porekla. Ekstrakti biljke *Teucrium polium* (TP) su ispoljili snažne citotoksične efekte na ćelije mišje leukemije, RAW 264.7, i ćelije humanog melanoma, C32, ali su njihovi citotoksični efekti na ćelije humane leukemije nepoznati. **Metode.** Vijabilnost ćelija humane leukemije (MOLT 4 i JVM 13), limfocita izolovanih iz krvi 28 bolesnika sa HLL (HLL ćelije) i mononukleara periferne krvi (PBMCs) izolovanih iz krvi 16 zdravih ispitanika je određena 3-(4,5-dimetiltazol-2-il)-2,5-difeniltetrazolium bromid (MTT) testom nakon tretmana ispitivanih ćelija metanolskim ekstraktom lista TP. Apoptoza HLL ćelija tretiranih ovim ekstraktom merena je protočnom citometrijom korišćenjem bojenja Annexin V/7AAD kita. Ekspresija aktivnog proapoptotičnog proteina Bax, antiapoptotičnog proteina Bcl-2, citohroma c i procenat ćelija koje sadrže aktivnu kaspazu-3 u tretiranim HLL ćelijama, određivana je pomoću protočne citometrije i imunocitohemijskim metodama. **Rezultati.** Metanolni ekstrakt lišća TP je smanjivao vijabilnost svih leukemijskih ćelija, ali nije uticao na vijabilnost PBMCs. Pokazano je da ovaj ekstrakt deluje citotoksično indukujući apoptozu HLL ćelija, kao i da utiče na odnos ekspresije Bax i Bcl 2 proteina u korist Bax, posledično indukujući apoptozu preko citohroma-c i aktivacije kaspaze-3 u HLL ćelijama.

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Zaključak. Metanolni ekstrakt lista TP selektivno indukuje apoptozu HLL ćelija menjajući ekspresiju ključnih proteina uključenih u proces programirane ćelijske smrti.

Ključne reči:
teucrium ekstrakt; ćelija, smrt; leukemija, b ćelije, hronična; ćelijska linija; miševi, ljudi.

Introduction

Chronic lymhocytic leukemia (CLL) originates from the antigen-stimulated mature B lymphocytes that either avoid death through the intercession of external signals or die by apoptosis, only to be replenished by proliferating precursor cells [1]. For that reason, CLL is considered more as a disease of cells accumulation due to the defect in apoptosis rather than deregulated cells proliferation [2]. The activation of apoptosis is one of the main molecular mechanisms responsible for the anti-cancer activities of most of the currently studied potential anti-cancer agents, including natural compounds [3, 4]. Several, novel drugs designed to interfere with the proteins regulating the cell cycle, the apoptotic machinery or leukemic microenvironment, are currently being tested in *in vitro* or *in vivo* studies, as well as in clinical trials [5–9].

There are two main cellular death pathways leading to caspase activation and apoptosis: the extrinsic pathway, initiated by "death" receptors and the intrinsic pathway, initiated after cytosolic discharge of mitochondrial derived cytochrome c and the other apoptotic proteins, caused by the mitochondrial outer membrane permeabilization induced by the formation of the proapoptotic proteins oligomerisation pores, such as Bax. Both pathways merge into the activation of caspase-3, the executioner caspase, that ultimately finalizes the apoptosis. The mitochondrial outer membrane permeabilization by pro-apoptotic protein Bax that by following the death signal, translocates from the cytosol to the mitochondrial outer membrane, is suppressed through the actions of cytosolic antiapoptotic proteins such as Bcl-2. Therefore, the changes in cytosolic expressions of Bcl-2 and Bax, play a significant role in the execution of apoptosis [10, 11].

In the present study, for the first time, we investigated the antitumor activity of *Teucrium polium* (TP) leaves methanolic extract against two human leukemic cell lines, MOLT 4 and JVM 13, and peripheral blood lymphocytes isolated from human chronic lymphocytic leukemia patients (CLL lymphocytes). We also determined the cellular pathway responsible for the activation of apoptosis induced by the TP methanolic extract in CLL lymphocytes.

Methods

Chemicals

Unless stated otherwise, all reagents were from Sigma-Aldrich (St. Louis, MO, USA), and all dishes for culturing cells were from Sarstedt (Numbrecht, Germany).

Preparation of drug solution

Leaves of TP were collected in the late summer period of 2015, from the region of Šumarice, Kragujevac, Serbia. The voucher specimen of TP was confirmed and deposited in Herbarium at the Department of Biology and Ecology, Faculty of Science, University of Kragujevac. The sampled leaves were air-dried in darkness at room temperature (20ºC).

Air-dried leaves (10 g) were transferred to the dark-coloured flasks. They were mixed with 200 mL of methanol and stored at room temperature. After 24 h, the extracts were filtered through Whatman No.1 filter paper and residues were again mixed with equal volumes of the solvent. After 48 h, the process was repeated. Combined supernatants were evaporated to dryness under vacuum at 40ºC using rotary evaporator. The obtained extracts were kept in sterile sample tubes and stored in a refrigerator at 4ºC.

Stock solution of the TP extract was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Germany) at a concentration of 20 mM, filtered through a 0.22 mm Millipore filter before use, and diluted by the nutrient medium Roswell Park Memorial Institute (RPMI 1640) to various working concentrations, so the final concentration of DMSO in the culture medium never exceeded 0.5% (v/v).

Patients

The local Ethics Committee accepted the study and prior to the initiation of the study, the written informed consent was obtained from all individual participants included in the study according to the Declaration of Helsinki. CLL was diagnosed by establishing the clinical criteria and it was confirmed by immunophenotypic analysis for the expression of CD5, CD19 and monoclonal immunoglobulin in accordance with updated the National Cancer Institute (NCI) Working Group Guidelines [12]. The control group was composed of healthy volunteers without known acute and chronic diseases. Peripheral blood samples from 28 CLL patients and 16 healthy control subjects were included in study.

Cell preparation

All blood samples were obtained in the morning and collected in potassium-ethylendiaminetetracetic acid (EDTA) coated blood collection tubes (Terumo). Peripheral blood samples (9 mL) were centrifuged at 400 x g for 10 minutes to separate plasma and cells. Peripheral blood mononuclear and polymorphonuclear cells were

separated by single step continuous density-gradient centrifugation with Histopaque 1077. The separated mononuclear cells were washed three times with culture medium RPMI 1640 and resuspended in RPMI 1640 supplemented with 10% autologous serum. The monocytes were removed by adhesion on plastic Petri dishes [13].

Cell lines

Human leukemia cell lines, MOLT- 4 (ATCC® CRL-1582™) and JVM 13 (ATCC® CRL-3003™), were acquired as a gift from professor Sonja Denčić, Department of Biochemistry, Belgrade University School of Medicine, Serbia. Both cell lines were maintained in culture medium consisting of RPMI-1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS).

MTT assay

The viability of cultured cells was determined by assaying the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan [14]. In brief, cells were treated with different concentrations of the TP methanolic extract (10 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, 250 µg/mL and 500 µg/mL) or cultivated in the cell culture medium containing the appropriate amount of DMSO (control). After 24 and 48 hours of cells incubation at 37°C in the atmosphere containing 5% CO_2, the 96 well plates were centrifuged for five minutes at 400 x g, the culture medium was removed, and MTT solution (5 mg/mL) was added to the cells. After additional 4h of incubation, the microtiter plates were centrifuged again for five minutes at 400 x g, the culture medium with MTT solution was removed and DMSO (150 µL/well) was added to dissolve the formazan crystals. Absorbance was measured at 595 nm with a multiplate reader (Zenyth 3100, Anthos Labtec Instruments, Austria). The results were presented as relative to the control value (untreated cells).

Detection of apoptosis

Apoptosis of CLL lymphocytes was measured using annexin V–fluorescein isothiocyanate (FITC)/7-amino-actinomycin D (7-AAD) Apoptosis Kit (BD Biosciences) according to manufacturer's instructions. CLL cells were treated with the TP methanolic extract at earlier specified concentrations and the percentage of apoptotic cells were determined by flow-cytometer FC 500 (Beckman Coulter). Data were presented as density plots of Annexin V-FITC and 7AAD stainings.

Assessment of apoptosis mechanism

In order to understand the mechanism of apoptosis induced by the TP methanolic extract, we analyzed the expressions of the active proapoptotic protein Bax, antiapoptotic protein Bcl-2, cytochrome c and the percentage of cells containing cleaved caspase-3. Lymphocytes from 28 CLL patients were incubated for 24 hours with 250 µg/mL and 500 µg/mL of the TP extract investigated or with the culture medium alone (control), washed three times with ice cold (PBS), and then resuspended, fixed and permeabilized (Fixation and Permeabilization Kit, eBioscience). Four types of stainings were separately performed afterward. Intended for Bcl-2 staining, the permeabilized cells were then incubated with Bcl-2 fluorescein isothiocyate primary antibody (mhbcl01, Life technologies) for 15 minutes at room temperature. Other three types of staining included incubation of permeabilzed lymfocytes for 30 minutes with primary antibodies against Bax (N20, sc-493; Santa Cruz Biotech. Inc), cytochrome-c (G7421, Promega) and caspase-3 (#9661, Cell signaling Technology). These cells were washed and then incubated with the appropriate secondary antibodies for 30 minutes. We used Alexa488 goat anti-mouse IgG (H+L) antibody (A-11001, Life Technologies) for cytochrome c, and goat anti-rabbit IgG FITC (Ab6717-1, Abcam) for Bax and caspase-3 staining. All cells were then washed with phosphate buffer saline (PBS) and analysed by flow cytometry and/or immunocytochemistry.

Immunocytochemistry

Observation of cells by fluorescent microscope was performed to localize the presence of Bax, Bcl-2, cytochrome c and cleaved caspase-3 in CLL lymphocytes. The images were aquired with a Olympus BX51 at 1000x magnification.

Flowcytometric evaluation

Fluorescence of at least 10,000 events per sample was measured using flow-cytometer FC500 (Beckman Coulter). Fluorescence intensity was standardized using isotype-matched negative control antibodies. The mean fluorescence intensities (MFIs) of Bax and Bcl-2 were calculated as the ratio of raw mean channel fluorescences to isotype control levels, respectively. Cytochrome c and cleaved caspase-3 levels were evaluated as percentage of cells displaying the fluorescence.

Statistical analysis

All values were expressed as mean ± standard deviation (SD). Each experiment was performed in triplicate and conducted on every sample. Commercial SPSS version 20.0 for Windows was used for statistical analysis. The distributions of data were evaluated for normality using the Shapiro-Wilk test. Statistical evaluation was performed by Student's *t*-test for paired observations, or one-way ANOVA depending on data distribution. *P* values less than 0.05 were considered significant.

Results

Teucrium polium methanol extract decreased viability of human leukemic cells

After 24 hours of incubation of MOLT-4 leukemia cells with 10 μg/mL, 25 μg/mL and 50 μg/mL of the TP methanolic extract, there was no statistically significant decrease in the viability of the examined cells compared to the viability of untreated MOLT-4 leukemia cells ($p > 0.05$). However, at the TP extract concentrations of 100 μg/mL, 250 μg/mL and 500 μg/mL, there was a statistically significant reduction in the viability of MOLT-4 leukemic cells compared to the viability of untreated cells ($p < 0.05$). Specifically, after 24 h of incubation of MOLT-44 cells with 100 μg/mL, 250 μg/mL and 500 μg/mL of the TP extract, the viability of MOLT-4 cells was reduced to 63.56 ± 1.27%, 30.32 ± 2.39% and 41.83 ± 1.42%, respectively.

After 48 hours of incubation with 100 μg/mL of the TP extract, the viability of the MOLT-4 cells was 71.41 ± 2.39% ($p < 0.05$). Also, at concentrations of 250 μg/mL and 500 μg/mL of the extract, after 48 h of the treatment, the extract significantly decreased viability of MOLT-4 cells to 14.41 ± 1.14% and 13.92 ± 0.94%, respectively ($p < 0.05$) (Figure 1).

After 24 hours incubation of JVM-13 cells with 10 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL and 250 μg/mL of the TP extract, there was no statistically significant decrease in JVM-13 cells viability compared to the viability of untreated JVM-13 cells ($p > 0.05$). However, 24 h of incubation of JVM-13 cells with 500 μg/mL of the TP extract, reduced viability of these cells to 36.24 ± 1.52% compared to the untreated JVM-13 cells, was observed.

Nevertheless, a statistically significant decrease ($p < 0.05$) in JVM-13 cells viability to 84.96 ± 3.53%, 59.85 ± 8.98% and 11.31 ± 1.35% compared to untreated cells was noticed after 48 h of cells incubation with 100 μg/mL, 250 μg/mL and 500 μg/mL of the TP extract, respectively (Figure 2).

Afterwards, we analyzed effects of the TP extract on viability of CLL cells and peripheral blood mononuclear cells (PBMCs) isolated from healthy subjects. After 24 hours, 250 μg/mL and 500 μg/mL of the TP extract statistically significantly decreased viability of treated CLL cells to 76.85 ± 7.25% and 50.27 ± 9.25%, respectively compared to the control group of untreated CLL lymphocytes ($p < 0.05$).

Also, after 48 hours, 250 μg/mL and 500 μg/mL of the TP extract statistically significantly decreased viability of treated CLL cells to 65.63 ± 8.13% and 40.39 ± 6.67%,

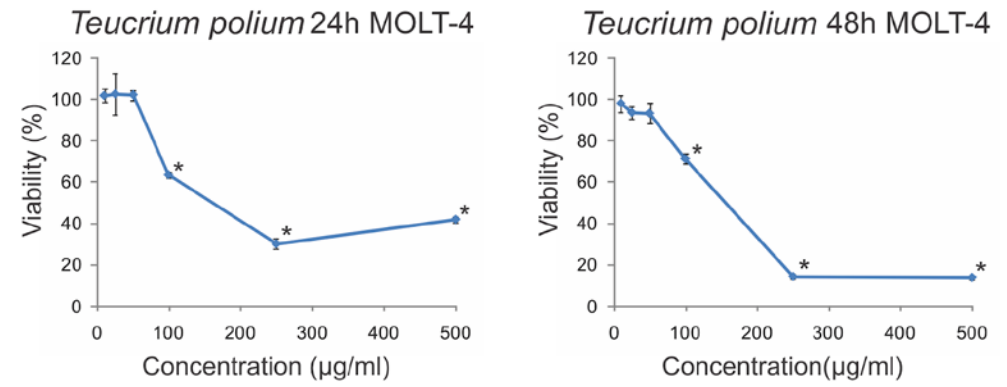

Fig. 1 – Effects of the *Teucrium polium* leaves methanolic extract on viability of MOLT-4 cells (*$p < 0.05$).

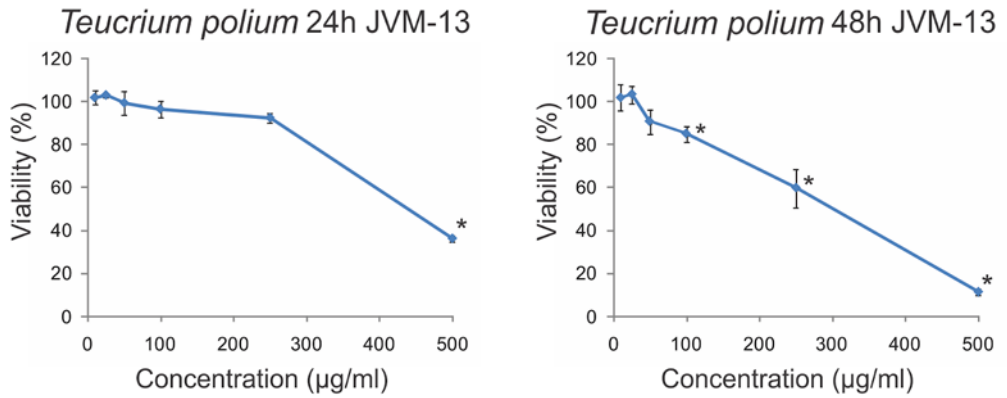

Fig. 2 – Effects of the *Teucrium polium* leaves methanolic extract on viability of JVM-13 cells (*$p < 0.05$).

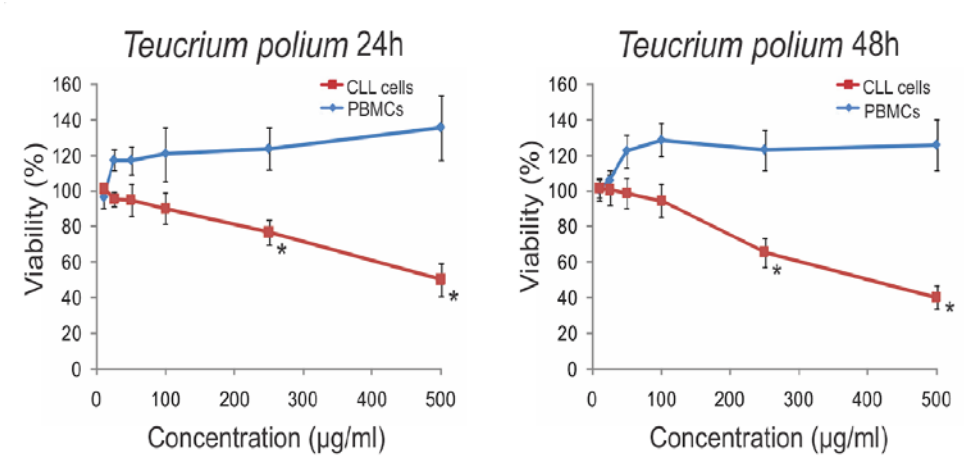

Fig. 3 – Effects of the *Teucrium polium* leaves methanolic extract on viability of chronic lymphocytic leukemia (CLL) cells and peripheral blood mononuclear cells (PBMCs) (*$p < 0.05$).

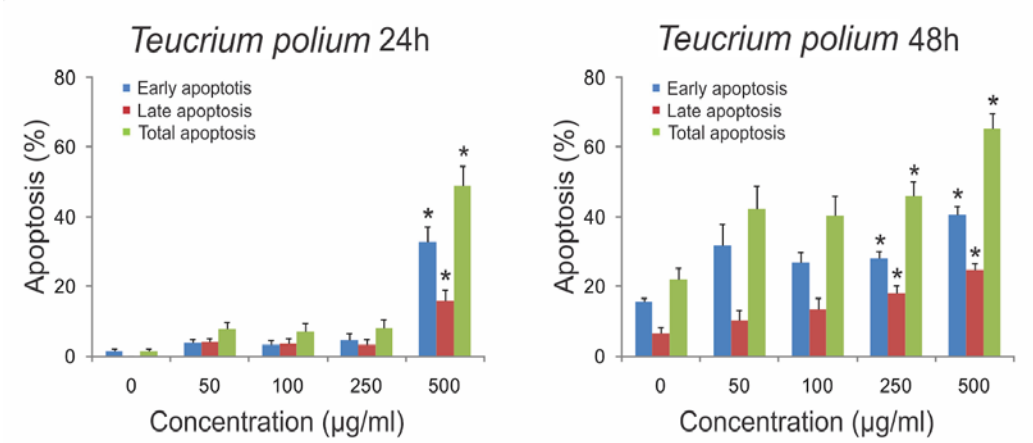

Fig. 4 – The *Teucrium polium* leaves methanolic extract induces apoptosis of chronic lymphocytic leukemia (CLL) cells. The extract induces apoptosis in peripheral blood lymphocytes isolated from human CLL patients via mitochondrial apoptotic pathway (*$p < 0.05$).

respectively compared to the control group of untreated CLL lymphocytes ($p < 0.05$).

After cultivation of PBMCs for 24 and 48 hours with the TP extract at concentrations ranging from 10 µg/mL to 500 µg/mL, there were no statistically significant changes in the viability of PBMCs relative to the control group of PBMCs that were not exposed to the TP extract ($p > 0.05$) (Figure 3).

Teucrium polium methanol extract induced apoptosis of CLL cells

Considering that our previous results showed that the TP extract produced the cytotoxic effect on human leukemia cells, especially on CLL cells, our next goal was to examine the type of cell death induced by the TP extract in CLL cells. The type of cell death was determined by Annexin V/7AAD staining.

Results obtained by Annexin V/7AAD staining after 24 hours of incubation of CLL cells with 500 µg/mL of the TP extract displayed significant increase of percentage of total apoptotic cells of about 50% compared to the untreated cells ($p < 0.05$, Figure 4). Nevertheless, results obtained after 48 hours of incubation of CLL cells with 250 µg/mL and 500 µg/mL of *TP*, also showed significant increase of percentage of total apoptotic cells of 50% and 70%, respectively compared to the untreated cells ($p < 0.05$, Figure 4).

To investigate whether the TP extract activated the mitochondrial apoptotic pathway, CLL cells were either treated with 500 µg/mL of the TP extract or cultivated in complete medium (control) for 24 hours and the localisation of cytochrome c was analyzed by fluorescent microscopy.

In parallel, we examined the localisation of active Bax proapoptotic protein and the fluorescence intensity representing the total expression of Bcl-2 antiapoptotic protein, along with presence of cleaved (activated) caspase-3 in treated and control cells. Our results showed that the TP extract induced the activation and translocation of Bax from cytosol to mitochondria, decreased the expression of cellular Bcl-2 protein, and induced the release of cytochrome c from mitochondria to cytosol and caspase-3 cleavage (Figure 5).

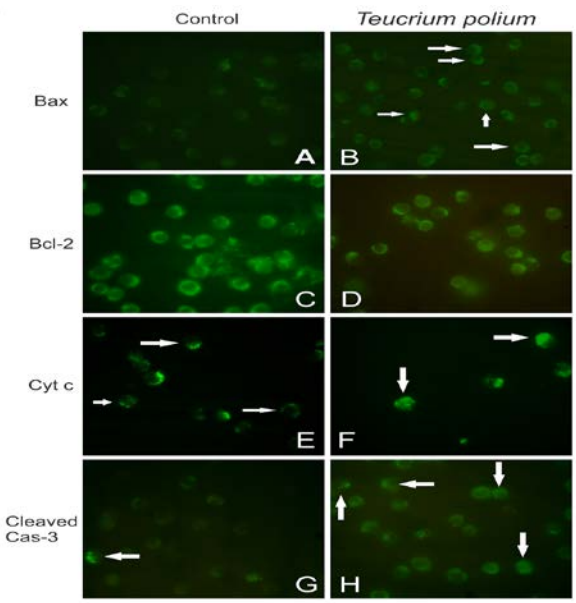

Fig. 5 – *Teucrium polium* (TP) leaves methanolic extract induces Bax translocation, decreases of cellular Bcl-2 protein level, release of cytochrome-c to cytosol and caspase-3 activation.

Chronic lymphocytic leukemia (CLL) lymphocytes were incubated for 24 h with Roswell Park Memorial Institute (RPMI) (control) or 500 μg/mL the TP methanolic extract. In a group of control cells, (A) Bax was localized in the cytosol, while in treated lymphocytes (B), Bax becomes organelle membrane-associated, and especially, mitochondrial membrane associated. Treated lymphocytes (D) displays a reduced amount of fluorescence intensity compared to the control group (C) suggesting decreased amount of antiapoptotic protein Bcl-2. The TP methanolic extract also stimulates cytochrome-c release to cytosol (F) compared to the untreated cells (E). Besides, the number of cleaved caspase-3 positive cells shows a trend of increase in a group of CLL lymphocytes treated with 500 μg/L of the TP methanolic extract (H) compared to the control group of lymphocytes (G).

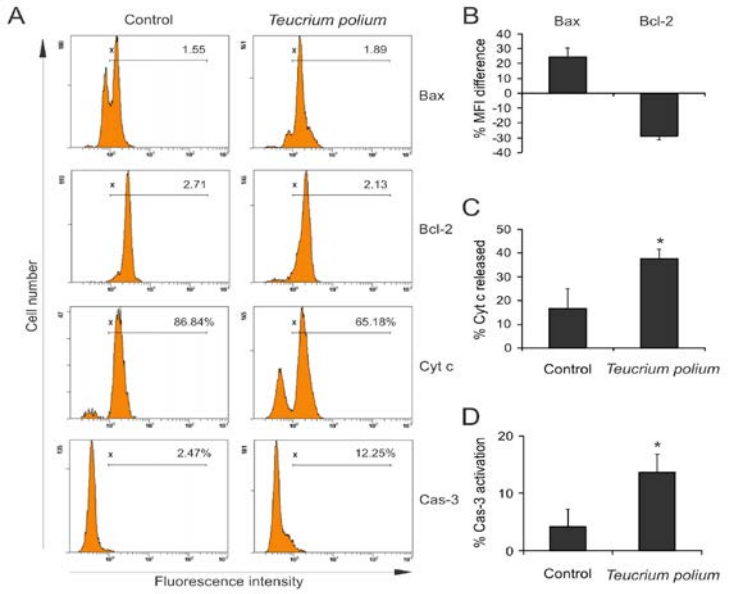

Fig. 6 – *Teucrium polium* (TP) leaves methanolic extract increases active Bax concentration and decreases cytosolic Bcl-2 concentration in treated CLL lymphocytes, consequently inducing apoptosis by cytochrome-c (Cyt c) mitochondrial release and activation of caspase-3 (Cas-3).

Lymphocytes of CLL patients were incubated in RPMI (Control) or 500 μg/mL of the TP methanolic extract for 24 h and stained with antibodies specific to Bax, Bcl-2, Cyt cand cleaved Cas-3. Cells were analyzed using single-colour flow cytometry. (A) Representative histograms that show Bax and Bcl-2 mean fluorescence intensities (MFIs) and percentage of cells displaying fluorescence for Cyt c and cleaved Cas-3 are presented. (B) Percentage of MFIs suppression or increase compared to untreated cells was calculated by formula (TP-C)*100/C where TP and C are MFIs of cells treated with TP methanolic extract or control cells, respectively. (C) Cyt c translocation was determined by selective permeabilisation of plasma membrane followed by flow cytometry. The percentage of cells with low fluorescence (100% of cells displaying fluorescence), where Cyt was translocated during apoptosis, is displayed. (D) The percentage of cells displaying fluorescence for cleaved Cas-3 (*$p < 0.05$ compared to the untreated cells).

Additionally, in order to quantify these apoptotic changes induced by the TP extract in CLL cells, we analysed the expression levels of Bax, Bcl-2 and cytochrome c by flowcytometry. Furthermore, we identified the amount of cells displaying cleaved caspase-3, to verify that the apoptotic pathway induced by the TP extract in CLL cells was caspase-dependent. The expression of active-Bax and cytosolic concentrations of cytochrome c were significantly increased, while the cytosolic expression of Bcl-2 was significantly decreased in treated CLL lymphocytes compared to the untreated cells. Furthermore, the percentage of cells containing cleaved caspase-3 was significantly increased (Figure 6). These finding showed that the TP extract disarranged the ratio of the expressions of proapoptotic Bax and antiapoptotic Bcl-2 protein in favor of Bax, consequently inducing apoptosis by cytochrome c mitochondrial release and activation of caspase-3.

Discussion

The results of our study showed for the first time that the methanolic extract of *Teucrium polium* reduced the viability of MOLT-4 and JVM-13 cells, and of CLL cells isolated from CLL patients after 24 hours and 48 hours of cells incubation. A very important result of our study was the absence of changes in cells viability of treated PBMCs compared to the untreated PBMCs, after 24 and 48 hours of cultivation of these cells with the TP methanolic extract. Although diverse mechanisms of action might contribute to the anti-cancer effects of TP, we showed that inhibition of Bcl-2 protein expression and activation of Bax were directly involved in TP-induced CLL cells apoptosis.

The results of our research demonstrating antitumor effects of the methanolic extract of *Teucrium polium* in human leukemic cells correlated with the results of previous studies showing cytotoxic effects of TP extracts on cell lines of prostate, colon, lung, and skin tumors [15–20]. In these tumor cell lines, as well as in leukemic cells used in our study, TP decreased the viability of examined cells. Stanković et al. [20] have demonstrated that the half maximal inhibitory concentration (IC_{50}) values of TP extracts after 72 hours of cells incubation were between 100 μg/mL and 200 μg/mL that were in correlation with the results of our research. Particularly, the concentration of the TP extract required to reduce the viability of HeLa cervical adenocarcinoma cells to 50% after 72 hours was 148.02 ± 4.99 μg/mL, for Fem-x human melanoma cells was 199.79 ± 0.30 μg/mL, and for K562 cells chronic myelogenous leukemia's cells, the concentration was 116.75 ± 24.40 μg/mL [21].

Species of plants of the genus *Teucrium*, are very rich in phenols and flavonoids, which are the carriers of the strong biological activity of various extracts of this plant. Extracts of TP have recently been subjected to the increasing number of *in vitro* studies in which their anticancer potential was tested. The results obtained using the HCT-116 cell line clearly indicated that the methanol extract of *Teucrium polium* reduced the viability of these cells by the induction of apoptosis. In a study of Stanković et al. [15], it was shown that after 24 hours of HCT-116 cells incubation with 250 μg/mL of the TP methanolic extract, apoptosis occurred in 85% of the total cell population. Our findings were also consistent with the results of a study of Stanković et al [15], since we showed that the TP extract induced apoptosis in CLL cells. Hence, in our research the percentage of apoptotic CLL cells after 48 hours of incubation in the presence of 250 μg/mL of the TP extract was about 46%. Therefore, reasults from our study suggest that active compunds from the TP methanolic extract reduce the viability of CLL cells by inducing apoptosis.

Permeabilization of outer mitochondrial membrane allows apoptotic molecules such as cytochrome c to be released into the cytoplasm that consequently induce the activation of caspases and subsequently execution of apoptosis. Permeabilization of outer mitochondrial membrane is mainly regulated by Bcl-2 family of proteins, such as proapoptotic protein Bax and antiapoptotic protein Bcl-2 [22]. The results of our study for the first time demonstrated that the TP methanolic extract induced selective mitochondrial apoptosis of CLL cells, by activating proapoptotic protein Bax and reducing the cytosolic expression of antiapoptotic protein Bcl-2, that led to the mitochondrial release of cytochrome c into the cytosol and the activation of caspase-3. The results of various studies that have been published to date were only confirming that the methanol extract of *Teucrium polium* could induce apoptosis in some cell lines, but to date there have not yet been accurately investigated the potential cellular mechanisms involved in this process [12]. The results of some previous studies emphasized the antioxidant capacity of the TP methanolic extract, but these studies have not yet been performed on tumor cell lines [15, 22]. It was shown that methanol extracts of other plants of the genus *Teucrium*, such as *Teucrium chamaedris*, *Teucrium montanum*, *Teucrium arduini* and *Teucrium scordium*, could have an antioxidant activity [15].

Conclusion

The methanolic extract of *Teucrium polium* affected key proteins involved in the regulation of programmed cell death of CLL cells. It selectively induced mitochondrial apoptosis in peripheral blood lymphocytes isolated from human CLL and it had no cytotoxic and apoptotic effects on PBMCs of healthy subjects. In order to precisely define the molecular component, one or more of them, carrying the biological activity of methanolic extract of TP leaves, it is necessary to perform additional research. The results of our experiments, therefore, represent a promising start for the future studies in the investigation of cytotoxic and apoptotic effects of specific components of TP in human leukemia cells.

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Conflict of interest

The authors declare no conflict of interest.

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