

METHANOLIC EXTRACT OF TEUCRIUM POLIUM EXERTS IMMUNOMODULATORY PROPERTIES IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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ABSTRACT

Teucrium polium has been used in traditional medicine around the world for centuries in treatment of various conditions and diseases. Many studies have confirmed pharmacological effects of its extracts, although the immunomodulatory effect has not been investigated. Therefore, the aim of our study was to examine the immunomodulatory effect of methanolic extract of *T. polium* (TPE) on peripheral blood mononuclear cells (PBMCs) derived from healthy donors and patients with hepatitis C virus HCV infection. We analyzed the effect of the extract on PBMCs viability using the MTT test. The cell death type was determined using Annexin V-FITC/7-AAD staining. Immunophenotyping using anti-CD8 FITC, anti-CD4 PE, anti-CD3 ECD, anti-CD20 PC5, anti-CD14 FITC and anti-CD25 PC7 was performed by flow cytometry. Results of the MTT test indicate that TPE stimulates proliferation of healthy PBMCs, while the HCV PBMCs viability was slightly reduced. The percentage of apoptotic HCV PBMCs was higher after TPE treatment compared to the control. The proportion of CD25-expressing cells was higher among the untreated HCV PBMCs than in the untreated healthy PBMCs. TPE treatment significantly and gradually increased CD25 expression in healthy PBMCs, whereas CD25 expression on HCV PBMCs increased only at the highest TPE concentration. The upregulation of double-positive CD3+CD25+, CD20+CD25+ and CD14+CD25+ cells was significant in TPE treated healthy PBMCs, while only the highest concentration was effective on HCV PBMCs. In summary, TPE exerts a strong immunomodulatory effect on healthy PBMCs and, only at the highest concentration, on HCV PBMCs.

Keywords: *T. polium*, immunomodulation, PBMC, HCV.

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INTRODUCTION

Since ancient times, plants have been used as an alternative source for treatment of different diseases. Inter alia, some plants were used in therapy of some diseases due to their immunomodulatory effect (1). Evaluation of the immunomodulatory activity of plant extract is an interesting and constantly growing area of research.

Excessive or suppressed immune response leads to auto-reactivity, inflammation or increased susceptibility to pathogens. Therefore, immunomodulatory drugs are used in the treatment of many diseases with immunopathological background. Modulation of the immune system represents any change in the immune response regarding induction, expression, amplification, or inhibition of any part or stage of the immune response (2). Immunopharmacology, as a developing branch, aims to manipulate the immune system by modifying endogenous immune responses and many researchers are focused on ethnomedicinal plants and their products as a source of immunomodulatory substances (2, 3). The immune-response-modulation activity of plant extracts and their active compounds has become an acceptable therapeutic measure. Determination of immunomodulatory properties and the mechanism of action of herbal medicine provides affordable, easy access and low side effect agents as a complementary part of therapeutic protocols (4). Secondary metabolites of plants, such as sterols, alkaloids, glycoproteins, polysaccharides and flavonoids are responsible for immunomodulatory effects of plants (3).

Teucrium polium L. (*Labiatae*) is a traditional medicinal plant widely distributed in the Mediterranean countries. It has been traditionally employed for various types of pathophysiological conditions as antidiabetic, anti-inflammatory, anti-ulcer, hypotensive, antispasmodic, anorexic, wound-healing and antipyretic agent. Remedial effects are prescribed to tannins, terpenoids, saponins, sterols and flavonoids content. Numerous *in vitro* and *in vivo* studies confirmed the pharmacological effects of *T. polium* (5). Oral administration of the crude extract during 6 weeks in diabetic rats significantly reduced blood glucose concentration in streptozotocin-induced diabetes in rats (6). Antioxidant testing showed that the aqueous extract of *T. polium* inhibited β -carotene oxidation, AAPH-induced plasma oxidation, Fe^{2+} -induced lipid peroxidation in rat liver homogenates, scavenged free oxygen species, bound iron and increased intracellular GSH levels in HepG2 cells (7). Ethanolic and methanolic extracts exhibited a marked antimicrobial effect on both Gram-negative and Gram-positive bacteria (8). *In vitro* antitumor study underlined the methanolic extract as an effective and safe chemosensitizer in tumor therapy. Namely, the ethanolic extract in combination with vincristine, vinblastine or doxorubicin produced a strong inhibitory effect on tumor cell lines, whereas non-transformed fibroblasts were negligibly affected (9). In a study focused on the anti-inflammatory effect, the ethanolic extract significantly inhibited cotton-pellet granuloma and carrageenan-induced inflammation (10).

To the best of our knowledge, there is no study investigating the immunomodulatory effect of *T. polium* extract. Therefore, this research is focused on examination of the immunomodulatory potential of *T. polium* methanolic extract on peripheral blood mononuclear cells derived from healthy blood donors and patients with chronic hepatitis C infection (HCV).

MATERIALS AND METHODS

Subjects

Five patients with chronic HCV infection were recruited in the Clinical Center of Kragujevac, Serbia. An inclusion criterion was the presence of liver cirrhosis. The liver biopsies were performed and patients were graded and staged according to Knodell et al. (11). The patients coinfecting with other hepatotropic viruses or with any possible causes of liver injury (alcohol, autoimmune diseases) were excluded from the study. None of the patients had been previously treated with immunomodulatory agents. Five healthy controls were selected from the hospital staff of similar age and with no history of liver diseases. The study was approved by the local Ethics Committee (01-6427/5), and written informed consent was obtained from all subjects in accordance with the Declaration of Helsinki.

Isolation of peripheral blood mononuclear cells

Peripheral blood of healthy volunteers and HCV patients was collected in heparin-coated tubes and mononuclear leukocytes were isolated by density gradient centrifugation (Histopaque 1077, Sigma, Germany). Peripheral blood mononuclear cells (PBMNC) were then washed three times and finally suspended in the supplemented culture medium RPMI 1640 (10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin G and 100 $\mu\text{g}/\text{ml}$ streptomycin, all from Sigma, Germany). The cell number and viability were determined using Acridine orange/Ethidium bromide staining (all from Sigma, Germany).

Plant material and preparation of extract

Aerial parts of *Teucrium polium* were collected from the natural population in the territory of Kragujevac – central Serbia, during June 2015. The voucher specimen was confirmed and deposited at the Herbarium of the Faculty of Science, the University of Kragujevac. The sampled material was dried at ambient temperature in a dark place. The air-dried material was milled in a grinder. The prepared plant material (10 g) was transferred into a dark-colored flask, filled with 200 ml of methanol and stored at room temperature. After 24 h, the infusion was filtered using Whatman No. 1 filter paper and residue was re-extracted with an equal volume of solvent. After 48 h, the process was repeated. Combined supernatants were evaporated to dryness under vacuum at 40 °C. The obtained extract was kept in a sterile sample tube and stored at + 4 °C until the analysis. The stock solution

was prepared as 100mg/ml DMSO and kept at +4°C. The content in *T. polium* methanol extract was defined by Stankovic et al. (12).

Cell viability assay

The effect of *Teucrium polium* extract (TPE) on peripheral blood mononuclear cells was determined by MTT assay that is widely used for assessing cell proliferation, cell viability, and/or cytotoxicity (13, 14). Isolated PBMCs were grown in 96-well plates at a starting density of 0.2×10^6 cells/well in presence of TPE (5, 10, 50, 100 and 500 µg/ml) or in medium alone (control). The cells were cultured for 24h at 37°C in 5% CO₂. The cultured cells viability was determined by assaying the reduction of MTT to formazan. In short, after the incubation, media was removed and MTT (0.5mg/1ml of PBS) was added to each well. The cells were then incubated at 37 °C for 4h, and DMSO (150µl/well) was added to dissolve the formazan crystals. Absorbance was measured at 550 nm with a multiplate reader (Zenith 3100, Anthos Labtec Instruments GmbH, Austria). The results were presented as relative to the control value (untreated cells).

Flow cytometric analysis

Freshly isolated PBMCs were incubated with TPE (5µg/ml, 50µg/ml and 500µg/ml) or with media alone (control) for 24 h at 37 °C in an atmosphere of 5% CO₂ and absolute humidity. Then, the cells were harvested, washed in PBS and used for determination of apoptosis/necrosis and immunophenotyping.

RESULTS

T. polium extract exerts different effects on viability of healthy and HCV patients PBMCs

Firstly, the effect of TPE on viability of isolated PBMCs was investigated by the MTT assay and flow cytometry. The MTT test showed not only that TPE is not cytotoxic to PBMCs from healthy individuals, but also it stimulates their proliferation, and this effect was dose-dependent: 10µg/ml induced 15,29%, 50µg/ml 32,04%, 100µg/ml 40,44% and 500µg/ml 66,79% increase in a cell number in relation to control, untreated cells.

Since we established that the TPE treatment stimulates proliferation of healthy PBMCs, further, we included the TPE testing on PBMCs derived from HCV patients. Contrarily to healthy donors PBMCs, the MTT test showed that 24h-treatment with TPE slightly decreased viability of PBMCs from HCV patients (Figure 1A.). Flow cytometry using Annexin V/7-AAD staining demonstrated a high incidence of apoptosis in untreated HCV cells (18,2%) and an increase in percentage of apoptotic cells after the treatment with 50 and 500µg/ml TPE (24,19% and 26,63%, respectively). There

Determination of apoptosis/necrosis

For apoptosis/necrosis detection, an Annexin V-FITC/7-AAD Kit was used according to the manufacturer's instructions (Beckman Coulter, USA). In short, PBMCs (2×10^5 cells) were suspended in 100µL of ice-cold binding buffer. The cells were stained with 10µl of Annexin V-FITC and 20µl of 7-AAD and incubated for 15 minutes at +4°C in the dark. Then, 400µl of binding buffer was added to each tube and the samples were immediately analyzed by flow cytometer Cytomics FC500 (Beckman Coulter). The data were analyzed using Flowing Software (<http://www.flowingsoftware.com/>).

Immunophenotyping

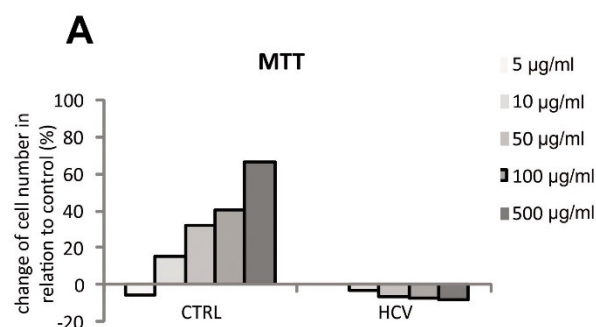
PBMCs (2×10^5 cells) were suspended in 100µl of PBS and stained with anti-CD8 FITC, anti-CD4 PE, anti-CD3 ECD, anti-CD20 PC5, anti-CD14 FITC and anti-CD25 PC7 (Beckman Coulter) and isotype controls (Beckman Coulter) for 20 minutes in the dark. Then, 400µl of PBS was added to each tube and the samples were acquired on a Cytomics FC500. The data were analyzed with Flowing Software.

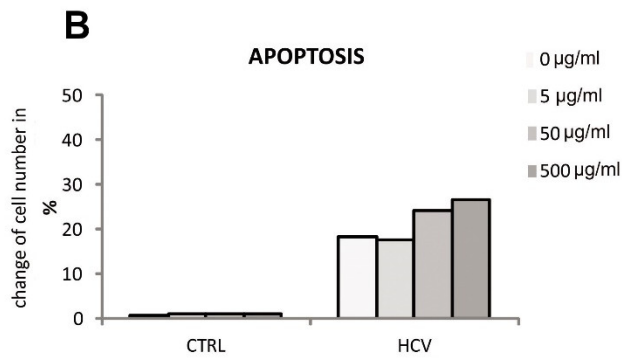
Statistics

Statistical analysis was performed in SPSS program (version 19.0, SPSS Inc., Chicago, IL). Normality of the data distribution was determined by Kolmogorov-Smirnov test. Comparing the data between the patient and control group was performed using Mann-Whitney *U* test. P-values <0.05 and <0.001 were considered statistically significant. The data are presented as column charts.

was less than 1% apoptosis in healthy PBMCs in any concentration used (Figure 1B.). In both healthy and HCV PBMCs, the percent of necrotic cells was negligible.

Figure 1. The effect of TPE on healthy (CTRL) and HCV PBMCs after 24h-treatment determined by the MTT assay (A) and flow cytometry (Annexin V/7-AAD) analysis (B).





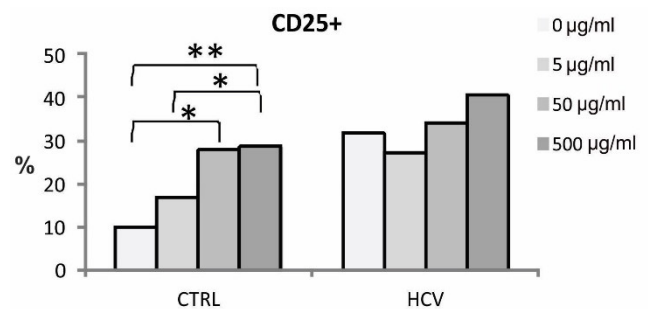
The results are median from five independent experiments.

TPE-treated healthy and HCV patients PBMCs have different immunophenotypic characteristics

Further, we analyzed the expression of CD25 as an activation marker on peripheral blood mononuclear cells. In healthy untreated PBMCs, about 10% of population expressed CD25 and after the treatment with TPE, the number of activated cells significantly increased: 5 µg/ml – 16,75%, 50 µg/ml – 27,84%, $p < 0,05$ and 500 µg/ml – 28,56% ($p < 0,001$). In the population of untreated PBMCs isolated from HCV patients, there was a higher percent of CD25+ cells (31,76%) than in untreated CTRL PBMCs. The TPE treatment resulted in a slight increase of CD25 expression (50 µg/ml – 33,96 and 500 µg/ml – 40,63%), but without statistical significance (Figure 2.).

In order to demonstrate better the effect of TPE on CD25 expression, we calculated the percentage ratios of CD25+ cells in PBMCs populations. As shown in Figure 3, the TPE treatment in healthy controls engendered a gradual increase of CD25 expression in CD3+, CD20+ and CD14+ cells, while in HCV PBMCs only the highest tested concentration affected CD25 expression.

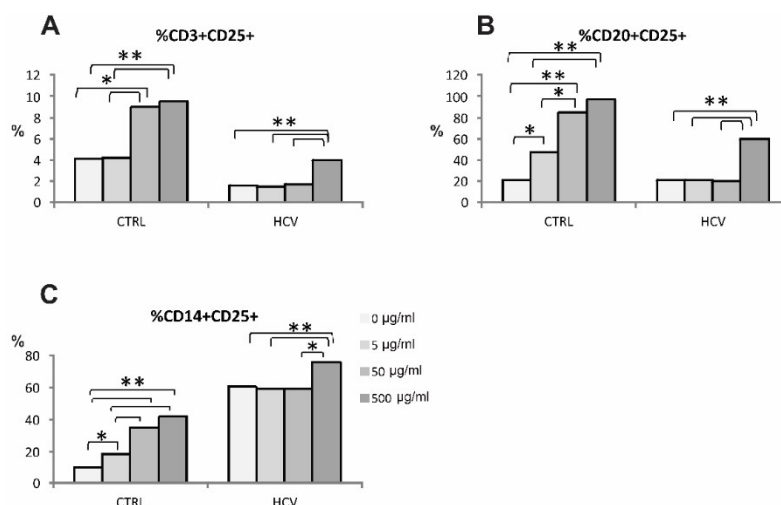
Figure 2. Expression of CD25 in untreated and TPE treated PBMCs of healthy individuals (CTRL) and HCV patients (HCV).



The results are median from five independent experiments. * $p < 0.05$; ** $p < 0.001$

In healthy controls, the treatment with 50 and 500 µg/ml TPE resulted in more than a double increase in the percent of CD3+CD25+ cells, from 4,09% in untreated cells to 9,04% and 9,49%, respectively, while in HCV patients only the highest TPE concentration had the same effect (control: 1,63%; 500 µg/ml: 4,02%). The percent of CTRL CD25+ B lymphocytes gradually increased from 20,87% in untreated cells to 97,65% in cells treated with 500 µg/ml TPE. In HCV B lymphocytes, only the highest concentration of TPE influenced CD25 expression (60,11% CD25+ cells in comparison to 21,30% in untreated cells) (Figure 3B.). Similarly, in CD14+ subpopulation, the percent of CD25+ cells gradually rose from 9,30% to 42,04% in healthy controls, whereas in HCV patients, the expression of CD25 increased only after the treatment with 500 µg/ml TPE (Figure 3C.).

Figure 3. The percentage ratio of CD25+ cells in CTRL and HCV CD3+ lymphocytes (A), CD20+ lymphocytes (B) and CD14+ cells (C).



The results are median from five independent experiments. * $p < 0.05$; ** $p < 0.001$

DISCUSSION

The immune system is a highly regulated host defense mechanism playing the main role in maintaining homeostasis and disease-free state of the body. Modulation of the immune response, in terms of either stimulation or suppression, takes part in mitigating many diseases. Therefore, immunomodulators are used to manage a variety of disease conditions. This study examined the immunomodulatory activity of the methanolic extract of *T. polium* by assessing its effect on viability, apoptosis induction, immunophenotypic characteristics and activation status of PBMCs obtained from healthy donors and HCV patients.

Chronic hepatitis C infection represents one of the leading cause of morbidity and mortality globally. Recent studies have recorded a significant increase of HCV-infected people in the last decade, with prevalence of HCV infection worldwide estimated at 2.5% (15). The WHO strategy for viral hepatitis, which includes the administration of antiviral drugs, vaccinations and various prevention programs, provides an assessment of the elimination of viral hepatitis worldwide by 2030. (16). Acute HCV infections are usually asymptomatic or with mild symptoms and in about 70-80% of cases, the infection goes into the chronic phase (17). The eradication of viruses in the acute phase results from the mechanisms of cell-specific immunity, in particular HCV-specific cytotoxic CD8⁺ T-lymphocytes and antiviral cytokines. The inability of the immune system to eradicate the virus results in persistent infection, which leads to chronic HCV infection. During the chronic infection, liver fibrosis develops in most patients, translating into cirrhosis in 15-25% of patients over a period of 10 to 40 years from the infection (18).

HCV persistence is repercussion of the viral ability to evade the immune surveillance by viral mutation, inhibition of innate immune cells by HCV proteins or by alteration of both arms, innate and adaptive, of the immune response which results in a gradually deterioration of the immune response. A strong and persistent cellular immune response is necessary for elimination of HCV virus. However, the adaptive immune response is often delayed in HCV infection, allowing HCV virus to spread before the host establishes effective T and B cellular response (19). Chronic antigen load and changes within targeted epitopes, resulting from a high viral error prone replication, trigger an excessive and prolonged activation of T cells, but they fail to hold on with viral epitope changes and elimination of viral infected cells (20). Waning of T cell response is depicted with an increased susceptibility to apoptosis (21, 22), disruption of differentiation, proliferation and effector functions (23). All of these are characteristics of the terminally exhausted immune response, particularly viral specific CD8⁺ T cells, that is mediated by the expression of numerous inhibitory molecules (23). Exhausted T cells highly express two or more inhibitory receptors such as, in the first place PD-1, CTLA-4, then lymphocyte activation gen 3 protein (LAG3), T cell immunoglobulin and mucin-domain containing-3 (TIM-3), 2B4 (known as CD244), but

also T-box transcription factor and reduce expression of the IL-7 receptor α chain (CD127) (23, 24). In addition, directed T cell response collapses, resulting in a global immune dysfunction.

Based on the MTT test results and flow cytometry analysis, *T. polium* methanolic extract exerted its non-cytotoxic and proliferative properties on healthy PBMCs. As opposed to healthy PBMCs, untreated HCV PBMCs are prone to spontaneous apoptosis, whereby the TPE treatment further augmented apoptosis in a dose dependent manner. Since spontaneous apoptosis and impaired proliferation are one of the main features of exhausted immune response in the chronically infected patients, HCV PBMCs under pressure of a proliferation stimulus to which they cannot respond spontaneously undergo apoptosis (21).

Many plants and plants-derived compounds have been shown to exert a proliferative effect on PBMCs (25-27). Primarily, we have to mention phytohemagglutinin and concavalin A as well-known mitogens in common proliferation models (27). Several previous studies showed that some flavonoid and phenolic compounds increase a proliferative response of PBMCs from healthy individuals (25, 26), despite their reputation as immunosuppressants. Namely, flavonoid compounds like cyanidanol, derivatives of (+)-3-methoxy-5,7,3',4'-tetrahydroxyflavan, (+)-3-palmitoyl-5,7,3',4'-tetrahydroxyflavan (25) and baicalin and baicalein derived from *Plantago* genus (26) stimulated proliferation of human PBMCs at lower concentration. Jose J et al. reported a similar effect of flavonoid from *Phyllanthus niruri* on un-stimulated human PBMCs (28). Further, the stimulatory effect of cyanidanol on in vitro lymphocyte responses in healthy individuals and in patients with chronic active hepatitis B was recorded (29, 30). Phenolic compounds like p-coumaric acid and vanillic acid have a high proliferative capacity with the stimulation index equal to 4.59 for PBMCs (26). The extracts of *Teucrium* genus plants have a high content of phenolic and flavonoid compounds which are carriers of various biological activities. Stankovic et al. have determined flavonoid and phenolic content of the examined extract, showing that the methanolic extract of *T. polium* leaves had a higher flavonoid concentration compared to the methanolic extract of Green tea as a reference substance and the highest phenolic content compared to the extract from other solvent (12). Flavonoids are known for their antioxidant properties, due to the ability to reduce free radical formation and to scavenge free radicals. The interaction of flavonoids with cell membrane lipids has been demonstrated. Also, some flavonoids inhibited catabolism of cyclic GMP leading to a high level of this nucleotide (31). Nevertheless, the mechanism of proliferative effect on PBMCs of some flavonoids has not been elucidated.

In the final step, we performed immunophenotyping of TPE-treated PBMCs in order to examine changes in the activation status and ratio of activated PBMCs population of both healthy individuals and HCV patient derived PBMCs.

Notable higher percentage of activated (CD25+) PBMCs in healthy controls is in line with previously mentioned prolonged and excessive activation of immune cells due to HCV agility (20). It should be noted that an increase of CD25 expression upon the treatment with TPE was more pronounced in all population of healthy PBMCs than in HCV PBMCs at the highest TPE concentration. HCV PBMCs inability to respond to weak stimuli, but only to stronger ones, such as the highest TPE concentration, is due to the outworn and exhausted immune response (23, 24). At the same time, the percent of CD14+CD25+ was multiple times higher in untreated HCV PBMCs than in healthy PBMCs, as a result of excessive stimulation present in chronic HCV infection (20).

CONCLUSION

In summary, the methanolic extract of *T. polium* exerts a strong immunomodulatory effect on healthy PBMCs. It stimulates the proliferation and activation of healthy T lymphocytes, B lymphocytes and monocytes, while the stimulation of HCV PBMCs activation was present, albeit to a lesser extent, only at the highest concentration. Future research should be directed towards determining the type of immunomodulatory effect and possible complementary administration of TPE in the treatment of a patient with chronic HCV infection.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the local Ethics Committee (01-6427/5), and written informed consent was obtained from all subjects in accordance with the Declaration of Helsinki.

CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

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None.

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