ALTERED APOPTOSIS AND BIOTRANSFORMATION SIGNALING IN HCT-116 COLORECTAL CARCINOMA CELLS INDUCED BY Teucrium chamaedrys L. EXTRACT

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(Received March 1st, 2019; Accepted March 31st, 2019)

ABSTRACT. The aim of this study was an investigation of pro-apoptotic activity of methanol extract from T. chamaedrys, a more detailed determination of the signal molecules activated in the process of apoptosis, and effects on mRNA expression of enzymes involved in biotransformation (CYP1A1 and GSTP1) and membrane transporter, MRP-2 in HCT-116 colorectal carcinoma cells. The results show pronounced pro-apoptotic activity of T. chamaedrys extract, due to activation of both extrinsic and intrinsic pathways. The death receptor associated signaling pathway was activated in HCT-116 following treatment by T. chamaedrys, via increased Fas receptor expression and activity of caspase 8. Activation of caspase 9 suggests that mitochondrial signalling also has an impact. The extract reduced mRNA expression of GSTP1 and MRP-2 genes, as one of the causes of multi drug resistance in cancer cells. Observed results offer the possibility for the use of T. chamaedrys extract in the context of cancer prevention and therapy.

Key words: anticancer properties; apoptosis; biotransformation; food supplement, plant extract; Teucrium.

INTRODUCTION

The plants have been used primarily for human nutrition, but also in the treatment of diverse human diseases, and represent a valuable source for novel drug discovery (Crag and Newman, 2013). Bioactive compounds from plants have been applied in different attempts for treatment of cancer, as a leading cause of human mortality in the current century (Choi et al., 2017; Mathur and Hoskins, 2017).

There is a wide number of medicinal plants, plant-derived products or isolated compounds, mainly polyphenols, with significant anticancer activity (Bishayee, 2012; Fridlender et al., 2015). Their anticancer properties are mediated through different mechanisms including interaction with DNA repair systems, stimulation of immune system, antiproliferative activity on cancer cells, induction of apoptosis, alteration to metabolism of
anticancer drug and impact on progressive stages of carcinogenesis by suppression of angiogenesis and metastasis (Milutinović et al., 2015; Manure and Naikwade, 2018). Among these processes, apoptosis has a preventive role as an event in transformed cells during the process of carcinogenesis, as well as a primary mode of action and desirable type of cell death induced in anticancer therapy (Pfeffer and Singh, 2018). A number of crude plant extracts and their constituents induce apoptosis in cancer cells (Gali-Muhtasib et al., 2015; Milutinović et al., 2015). It can be induced through extrinsic and/or intrinsic pathway, wherein the first includes interaction of death receptors and followed activated molecules, while the intrinsic mainly include mitochondria and molecules associated with changes in permeability of mitochondrial membrane, releasing of cytochrome c, which stimulates appropriate apoptotic molecules (Pfeffer and Singh, 2018). Additionally, an important part of drug investigation is defining of its potency to modulate expression and basal activity of phase I and II biotransformation enzymes as a response to therapy (Jain et al., 2007). Cytochrome P450 enzymes (CYP), glutathione-S-transferase (GST), and membrane transporters involved in drug efflux from cells were frequently investigated due to their importance for development of multi drug resistance (Housman et al., 2014). Bulus et al. (2018) reported that there is a high expression of CYP1A1 and GSTP1 isoforms of these enzymes in colon cancer cells.

Based on the above, the investigation of new anticancer drugs from nature is required, especially due to their potential for using as a functional food and food supplements. Teucrium chamaedrys L., from Lamiaceae family, is known as a medicinal plant with traditional use. Previous reports have demonstrated that the T. chamaedrys possessed several biological activities such as antioxidant, antimicrobial, antifungal, etc (Stanković et al., 2010). Recently, it has also been reported that T. chamaedrys induces cytotoxic activity against several cell lines, including HCT-116 colorectal carcinoma cells (Stanković et al., 2011; Stanković et al., 2015). These reports are without data about mechanism of its cytotoxic effects. Thus, the aims of this study were to investigate the pro-apoptotic activity of T. chamaedrys methanol extract in HCT-116 colorectal carcinoma cells, with an accent to its influence on signaling molecules involved in apoptosis by monitoring of Fas receptor protein expression, caspase 8 and 9 activity. Also, mRNA expression of CYP1A1, GSTP1 and MRP-2 genes, related to biotransformation process and development of cancer cell resistance was determined.

MATERIALS AND METHODS

Plant material

The voucher specimen of T. chamaedrys has been deposited with the number 16695 at the Herbarium of the Faculty of Biology, University of Belgrade, Serbia. Methanol extract was prepared according to standard procedure, formerly described (Milutinović et al., 2015). Stock solution of crude plant extract was prepared in DMSO (Dimethyl sulfoxide), where the highest concentration of DMSO used for final dilution of extracts did not exceed 0.5%.

Maintenance of cells used for assays and treatment

Human colorectal carcinoma cell line (HCT-116) was obtained from American Type Culture Collection. The cells were maintained in optimum conditions and standard protocols (Milutinović et al., 2015). All assays were performed 24 h after treatment with T. chamaedrys extract, in concentration of 50 µg/ml, and nontreated cells were used as control.
**Determination of T. chamaedrys pro-apoptotic activity**

Dual fluorescent acridine orange/ethidium bromide (AO/EB) staining assay was performed in order to investigate the type of cell death induced by *T. chamaedrys* extract (BASKIĆ et al., 2006). Method was used for investigation of pro-apoptotic activity, by detection of normal, early and late apoptotic cells, as well as necrosis. Assay protocol used in our experiments was described earlier in detail (ČURČIĆ et al., 2012). The images were taken using fluorescence microscope at 400 x magnification.

**Monitoring of signaling molecules involved in apoptosis**

Protein expression of Fas receptors was marked by immunofluorescence method, previously described in detail (ČURČIĆ et al., 2014). Micrographs were taken on Nikon inverted fluorescent microscope (Ti-Eclipse) at 600 x magnification. Nuclei were stained blue (DAPI color), Fas was stained red (second antibody Cy3). The quantification of cellular fluorescence in control and treated cells was measured on fluorescence micrographs using ImageJ software (Wayne Rasband, ImageJ, http://rsb.info.nih.gov/ij/).

Measuring of activity of caspase 8 and 9 was performed by colorimetric assay kits (RD Systems), according to the manufacturer’s protocols, as formerly described in detail (MILUTINOVIĆ et al., 2015).

**Monitoring of signaling molecules involved in biotransformation**

For determination of mRNA expression for *CYP1A1*, *GSTP1* and *MRP-2* genes, extraction of total RNA was carried out according to the phenol-chloroform method by CHOMCZYNSKI and SACCHI (1987). RNA concentration was measured using Biophotometer (Eppendorf BioPhotometer plus).

Conversion of single-stranded RNA molecules into their complementary DNA (cDNA) was performed using a method by BUSTIN (2000) with a commercially available Qiagen Sensiscript RT Kit in the Eppendorf Master-cycler PCR, programmed according to the manufacturer's protocol. Simplex PCR method was used for amplification of sequences defined by specific primers for investigated genes β-actin - F: 5’-AAGCAGGAGTATGACGAGTCCG-3’ and R: 5’-GCCTTCATACATCTCAAGTTGG-3’; *CYP1A1* - F: 5’-TAGACACTGATCTGGCTGCAG-3’ and R: 5’-GGTCTGGCCAGGTC TAGGCA-3’; *GSTP1* - F: 5’-TCAAGGCCTCCCTGCTATAC -3’ and R: 5’-AGGTGACGCA GGATGGTATT -3; *MRP-2* - F: 5’-ATACCAATCCAGCTCCTAC-3’ and R: 5’-GAATTGG TCACCCCTGTAAGAG-3’ (ZHAI et al., 2005). Method was performed according to manufactured instructions from commercially available Qiagen PCR Kit.

The amplificated samples obtained for each gene in control and treated HCT-116 cells were separated by electrophoresis at 1.5% agarose gel and photographed on the Transilluminator. The quantification of the bands from gels was performed densitometrically in the ImageJ program. The expression of investigated sequences was compared to the expression of β-actin, as a housekeeping-reference gene. The results are shown as a relative expression of investigated gene in relation to β-actin.

**Statistical analysis**

All the assays were performed in two individual experiments, in triplicate for each dose, where the results were expressed as mean ± standard error (SE) from both independent experiments. Statistical significance (p <0.05) was determined using the Student’s t-test or the
one-way ANOVA test in SPSS statistical software package (SPSS for Windows, ver. 17, 2008).

**RESULTS**

**Pro-apoptotic activity of *T. chamaedrys* extract**

In the present study, the pro-apoptotic activity of *T. chamaedrys* methanol extract on HCT-116 cells was determined. After 24 h of the treatment, the characteristics of apoptotic cells were occurred, including the increase of bright green color observed by AO/EB and showed on micrograph, as well as evident chromatin condensation and DNA fragmentations (Figure 1).

For the quantitative values of *T. chamaedrys* pro-apoptotic activity, the percentages of early and late apoptosis were evaluated, related to total cell number. The result showed pronounced increasing of early apoptosis, as a dominant stage of induced cell death. Necrosis induced by *T. chamaedrys* extract appeared in low percentage. Observed morphological changes and quantitative values clearly showed pronounced pro-apoptotic in treated HCT-116 compared to untreated control cells.

![Figure 1. Morphological changes of HCT-116 cells (A) and percentages of VC - control cells (viable cells); EA - early apoptosis; LA - late apoptosis; N – necrosis induced by *T. chamaedrys* extract (50 µg/ml), 24 h after treatment. The images were taken using fluorescence microscope at 40 x magnification.](attachment:figure1.png)

**Mechanism of *T. chamaedrys*-induced apoptosis**

To determine the mechanism of the *T. chamaedrys*-induced apoptosis, the activation and protein expression of some signaling molecules of apoptosis were monitored. The protein expression of Fas receptors was pronouncedly increased in treated cells compared to control HCT-116 cells. It is shown on fluorescent micrographs (Figure 2A and B), as well as on the graph that expressed the measured intensity of fluorescence (Figure 2C). The relative fluorescence of cell was calculated by ImageJ computer program, where the data are means ± SE of more than 30 cells per control/treatment.
The activity of caspases -8 and -9, as an initial caspase in extrinsic and intrinsic apoptotic pathways, was evaluated after 24 h of incubation HCT-116 cells with *T. chamaedrys* extract. The activity of both caspases was elevated significantly compared to control (Figure 3).

*Effects on gene expression of biotransformation enzymes and membrane transporter*

Expression of *β-actin* was examined in control and treated HCT-116 cells. It was equally expressed in both samples and used as a *housekeeping gene*. Relative mRNA expression of *CYP1A1, GSTP1* and *MRP-2* genes was calculated in relation to the *β-actin*. Figure 4 shows that mRNA of *GSTP1* and *MRP-2* genes was significantly reduced in HCT-116 cells treated by methanol extract of *T. chamaedrys* compared to control, while treatment has not changed the expression of *CYP1A1*. 
Figure 4. Relative mRNA expression of CYP1A1, GSTP1 and MRP-2 genes in control and HCT-116 cells treated by T. chamaedrys (50 µg/ml), calculated in relation to the β-actin. The data are means ± SE. *P < 0.05 compared to untreated controls.

DISCUSSION

Studies of natural compounds from plants and their activities are still of interest, as well as discovering of novel drugs as a currently important filed in oncology (THOMFORD et al., 2018). The induction of apoptosis in cancer cells is considered as a valuable way for treatment of cancer (WONG, 2011; HASSAN et al., 2014). A variety of natural substances have the ability to induce apoptosis in different cancer cell lines (MILUTINOVIĆ et al., 2015; LICHOTA and GWOZDINSKI, 2018). It is important to investigate the new plant extracts and constituents isolated from them with pro-apoptotic activity, especially since the current chemotherapeutics show numerous side effects (NURGALI et al., 2018). In relation to them, bioactive substances originated from nature, achieve the valuable properties for using them in many attempts and strategies for cancer therapy.

The antiproliferative activity of T. chamaedrys has been examined on several cancer cells with positive results (ABU-DAHAB and AFIFI, 2007; STANKOVIĆ et al., 2015). Previously reported results about cytotoxic activity of methanol extract from T. chamaedrys against HCT-116 cells (STANKOVIĆ et al., 2011) showed remarkable cytotoxic activity, according to fact that crude extracts with IC_{50} values ≤ 30 µg/ml are considered as a pharmaceutically active (SUFFNES and PEZUTO, 1990). This study aimed to evaluate the pro-apoptotic potential of T. chamaedrys on HCT-116 colorectal carcinoma cells. Taken together with our obtained results, we suggest that achieved anticancer effects are a consequence of cell apoptosis, because 24 h after treatment cells were dominantly in the stage of early apoptosis (56.29±3.81%). Then the specific morphological and biochemical changes in HCT-116 cells were observed (Figure 1A). Results observed by measuring the protein expression of Fas receptors on membrane and activities of initiator caspases 8 and 9 contribute to this conclusion. The extrinsic apoptosis pathway receives signals through the interaction of extracellular death ligands to death receptors, building the complex that transmits signals to the pro-caspase 8 (PFEFFER and SINGH, 2018). Protein expression of Fas receptors and activity of caspase 8, as crucial proteins and initiators of death receptor-mediated apoptosis, were increased in HCT-116 cells treated by T. chamaedrys extract (Figure 2 and 3). It suggests the role of these proteins in triggering of the process of apoptosis. In the intrinsic pathway, mitochondria have a crucial role, where the mitochondrial membranes were permeabilized, cytochrome c was released from the mitochondria into the cytoplasm (FULDA et al., 2010). It causes a series of events, including the conversion of the pro-caspase 9 into caspase 9 and
other effector caspase activation (PARRISH et al., 2013). In T. chamaedrys-induced apoptosis the activity of caspase 9 was increased (Figure 3). Based on the results, it can be concluded that phytochemicals from T. chamaedrys extract induces apoptosis in HCT-116 cells through mechanisms involving both, the receptor mediated and mitochondria-dependent pathway.

Various phytochemicals, present or isolated from T. chamaedrys, may be responsible for the pro-apoptotic activity. The known compounds observed in the T. chamaedrys were mainly phenolics, essential oil, diterpenoids teucrins A, E, F and G, dihydroteugin, teucroxide, syspirensin, teuchamaedryn D and many other (LIN et al., 2009; ELMASTASA et al., 2015). The terpenes isolated from the T. alopecurus triggered apoptosis in HCT-116, U266, SCC4, Panc28, KBM5 and MCF-7 human cell lines (GUESMI et al., 2018). Pro-apoptotic activity was reported for the other species from the same genus. So, T. sandrasicum induces apoptosis in HeLa and MCF-7 cells (TAHRAN et al., 2016), T. polium and T. montanum in HCT-116 and MDA-MB-231 breast cancer cells (NIKODIJEVIĆ et al., 2016).

Metabolism of xenobiotics, as well as anticancer drugs, occurs in several stages and includes several enzymes and membrane transporters for the drug export from the human cells (PATHANIA et al., 2018). Their crucial and primary role is to protect healthy cells against harmful effects of carcinogens. However, their overexpression and increased activity can lead to the development of resistance to the anticancer drugs in cancer cells (HOUSMAN et al., 2014). Extract of T. chamaedrys has not changed the expression of mRNA for CYP1A1, which indicate that constituents from the extract are not metabolized or may not be a substrate for this enzyme. The significant result is the ability of T. chamaedrys to inhibit the expression of mRNA for GSTP1 and MRP-2. The inhibition of GSTP1 by natural plant products was investigated previously, with the aim to reduce expression or decrease activity of these enzymes (MUKANGANYAMA et al., 2011). MRP-2, from the MRP family of ABC transporters exhibits the highest affinity for the plant constituents. Overexpression of MRP-2 and other ABC transporters in cancer cells is associated with multidrug resistance, thorough ejection and elimination of anticancer drugs from cells, which reduces their intracellular concentration (SODANI et al., 2012). Related to this field of investigation, T. chamaedrys shows high potential to alternate expression of GSTP1 and MRP-2. This is an important result considering the need for investigation of some chemo-modulators, GST and ABC transporters inhibitors, as therapeutic agents in order to reverse drug resistance (ZHAO et al., 2007; PATHANIA et al., 2018).

Observed results show that T. chamaedrys, with pronounced pro-apoptotic activity is the valuable source of bioactive substances, as potent apoptosis inducers and GSTP1 and MRP-2 inhibitors for possible uses in chemo-modulation and cancer therapy. Using this plant as a healthy dietary may be a potentially beneficial approach in the context of cancer prevention and therapy.

Acknowledgments

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (project III41010).

References:


