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

Milena G. Milutinović*, Danijela D. Nikodijević, Milan S. Stanković, Danijela M. Cvetković and Snežana D. Marković

University of Kragujevac, Faculty of Science, Department of Biology and Ecology, Radoja Domanovića 12, 34000 Kragujevac, Serbia
*****Corresponding author; E-mail: milenagen@gmail.com

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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 (ĆURČIĆ *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 (ĆURČIĆ *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'-TCAAAGCCTCCTGCCTATAC -3' and R: 5'-AGGTGACGCA GGATGGTATT -3; *MRP-2* - F: 5'-ATACCAATCCAAGCCTCTAC-3' and R: 5'-GAATTG TCACCCTGTAAGAG-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 (Figure1).

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.

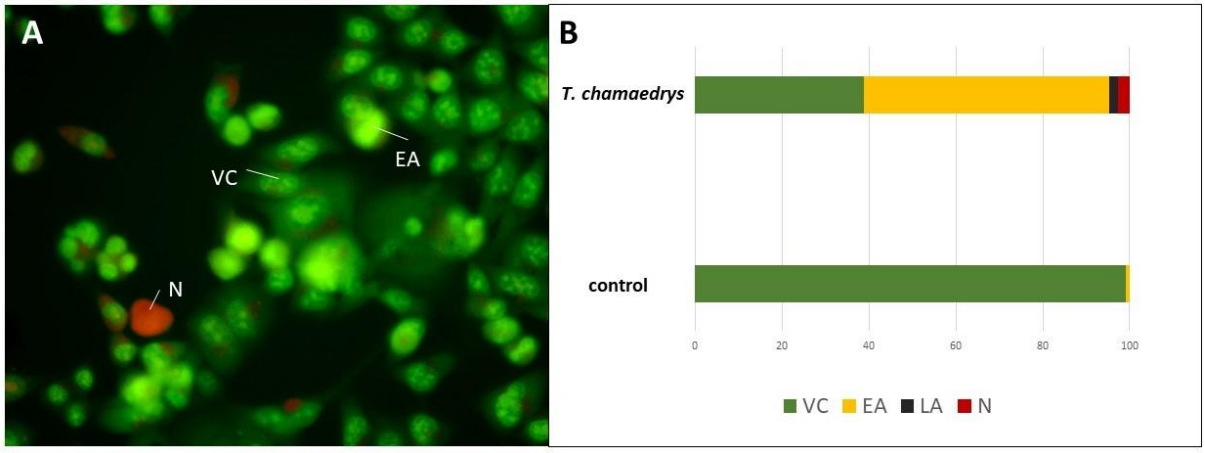

Figure 1. Morphological changes of HCT-116 cells (A) and percentages of VC - control cells (viable cells); EA - early apoptosis; LA - late apoptosis; N – necrosis induced by *T. chamaedrys* extract (50 µg/ml), 24 h after treatment.
The images were taken using fluorescence microscope at 40 x magnification

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.

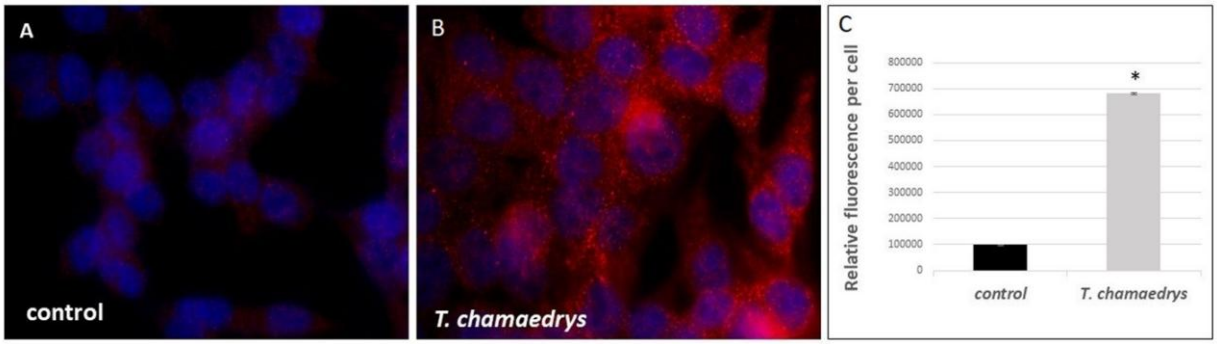

Figure 2. Protein expression of Fas receptors in HCT-116 cells. Fluorescence micrographs: (A) control cells; (B) cells treated by *T. chamaedrys* extract (50 µg/ml); (C) the relative fluorescence of cell calculated by ImageJ computer program. *P < 0.01 compared to control

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).

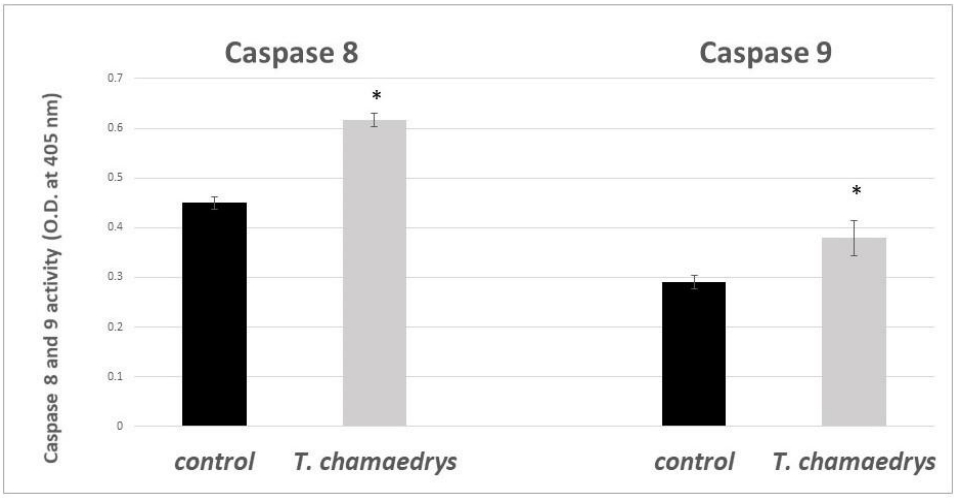

Figure 3. Caspase 8 and 9 activities in HCT-116 control and cells treated by *T. chamaedrys* extract (50 µg/ml), 24 h after treatment. The data are means ± SE. *P < 0.05 compared to untreated controls

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*.

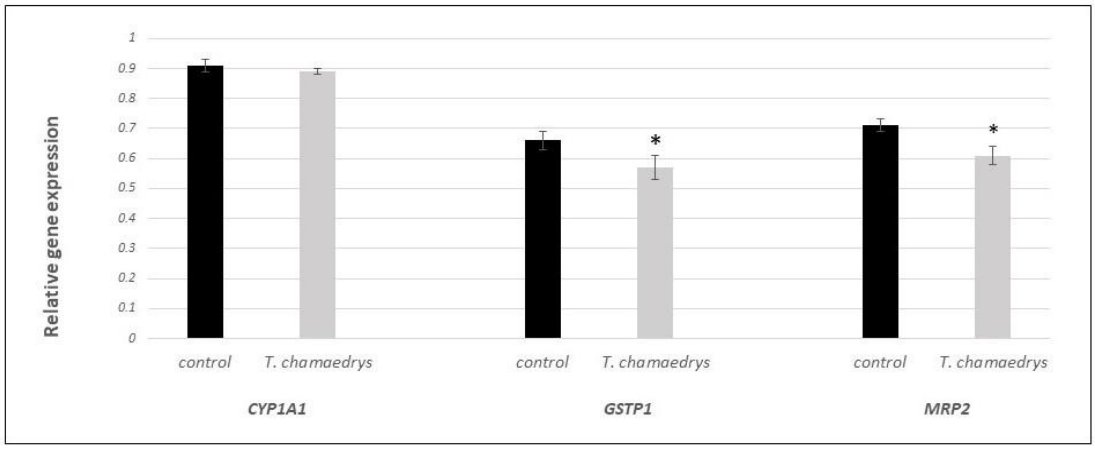

Figure 4. Relative mRNA expression of *CYP1A1*, *GSTP1* and *MRP-2* genes in control and HCT-116 cells treated by *T. chamaedrys* (50 μg/ml), calculated in relation to the *β-actin*. The data are means ± SE. *P < 0.05 compared to untreated controls.

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, trough 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.

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