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Cytotoxic activity of *Nepeta rtanjensis* Diklić & Milojević essential oil and its mode of action

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ABSTRACT

Nepeta rtanjensis Diklić & Milojević (fam. Lamiaceae) is an endemic, critically endangered plant, protected by the law in Serbia. Various biological activities have been ascribed to its major constituents, nepetalactones. In this study we describe for the first time cytotoxic activity of *N. rtanjensis* essential oil (EO), obtained from field cultivated plants, which was found to be especially rich in *trans,cis*-nepetalactone. MTT assays indicated that after 72 h of treatment the EO exhibited cytotoxic activity on investigated cancer cell lines: HeLa, K562, A549, LS-174 and MDA-MB-231. Normal cell line (MRC-5) was the least sensitive to the treatment and IC_{50} value for this cell line was not reached within the tested range of EO concentrations (up to 0.1 μL/mL). Analysis of morphological changes of treated cells confirmed the higher sensitivity of tumor cells than normal cells to the tested EO. Application of *N. rtanjensis* EO resulted in the appearance of morphological changes in tested cancer cell lines characteristic for apoptotic cell death, and induced perturbations of the cell cycle of HeLa cells. In addition, upregulation of *Bax* and *p53*, and downregulation of *Bcl-2*, and *Skp2* genes, involved in apoptotic signalling cascades, confirmed an apoptosis-inducing effect of *N. rtanjensis* EO on HeLa cells. Presented results highlighted the potential of *N. rtanjensis* EO in anticancer therapy.

1. Introduction

Nepeta rtanjensis Diklić & Milojević (fam. Lamiaceae) belongs to the genus *Nepeta* (catmints), which comprises species that contain a variety of secondary metabolites belonging to terpenoids (monoterpenoids, diterpenoids, triterpenoids, sesquiterpenoids) and phenolics (Formisano et al., 2011; Sharma and Cannoo, 2013; Mišić et al., 2015). Numerous *Nepeta* species are traditionally used for the treatment of different gastrointestinal and respiratory disorders such as colic, diarrhea, cough and asthma due to spasmolytic and myorelaxant activities (Gilani et al., 2009; Formisano et al., 2011). These plants exhibit effects as cats and aphids attractants (Sakurai et al., 1988; Formisano et al., 2011), or act as repellents against various insects such as mosquitoes, ixodid ticks and red poultry mites (Birkett et al., 2011). Antimicrobial activity against a variety of bacteria and fungi, antioxidant and phytotoxic activities of *Nepeta* plants have been well documented (Stojanović et al., 2005; Tepe et al., 2007; Ljaljević Grbić et al., 2008; Nestorović et al., 2010; Formisano et al., 2011; Kumar et al., 2014). Furthermore, these plants have shown antiviral, phytotoxic and allelopathic effects (Kobaisy et al., 2005; Formisano et al., 2011; Dmitrović et al., 2015; Nestorović Živković et al., 2016). Several studies investigated the cytostatic and cytotoxic activities of various *Nepeta* species (Rigano et al., 2011; Tsuruoka et al., 2012; Kahkeshani et al., 2014; Shakeri et al., 2014, 2016). The majority of aforementioned biological activities of *Nepeta* species are ascribed to nepetalactones, cyclopentanoid monoterpenes which are usually the main constituents of the essential oils (Birkett et al., 2011; Formisano et al., 2011; Stojanović et al., 2005; Nestorović et al., 2010; Kumar et al., 2014; Mišić et al., 2015). Certain *Nepeta* species (Nestorović et al., 2010; Sharma and Cannoo, 2013) do not produce nepetalactones (or produce them in small amounts), thus their biological activity could be attributed to other terpenoid or phenolic compounds

Abbreviations: EO, essential oil; GC, gas chromatography; HeLa, human cervix carcinoma cells; MDA-MB-231, human breast cancer cells; LS-174, human colon carcinoma cells; A549, lung adenocarcinoma cells; K562, human myelogenous leukemia cells; MRC-5, human fetal lung fibroblast cells; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide.

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(Nestorović et al., 2010; Formisano et al., 2011; Mišić et al., 2015). The biological activity of nepetalactones depends on their stereochemistry (Gkinis et al., 2003; Nestorović et al., 2010). There are eight stereoisomers of nepetalactone, four diastereoisomers and their corresponding enantiomers, and with some exceptions, only the (7S)-diastereomers exist in natural sources (Formisano et al., 2011).

N. rtanjensis Diklić & Milojević is listed in Red Data Book of Flora of Serbia as critically endangered plant species which belongs to the aggr. *N. sibthorpii* (Diklić, 1999). It is a rare and strictly endemic perennial found only in a few localities of Mt Rtanj in North-East Serbia (Diklić, 1999; Mišić et al., 2005). Therefore, *in vitro* plant propagation, field cultivation and reintroduction were performed (Mišić et al., 2005) in order to obtain sufficient plant material for the investigation of biological activities (Ljaljević Grbić et al., 2008; Nestorović et al., 2010; Dmitrović et al., 2015; Nestorović Živković et al., 2016). Until now various biological activities of *N. rtanjensis* are well demonstrated: antibacterial (Stojanović et al., 2005; Nestorović et al., 2010), antifungal (Stojanović et al., 2005; Ljaljević Grbić et al., 2008; Nestorović et al., 2010), allelopathic (Nestorović Živković et al., 2016), and phytotoxic (Dmitrović et al., 2015). The essential oil (EO) of *N. rtanjensis* is characterized by a high content of *trans-cis* nepetalactone which is considered to be the main component principally responsible for the biological activities (Stojanović et al., 2005; Nestorović et al., 2010; Mišić et al., 2015). To our knowledge there are no data about the cytotoxic activities of this EO.

EOs and/or their individual components are widely used for treatment of various human diseases including cancer (Edris, 2007; Bhalla et al., 2013; Saad et al., 2013; Raut and Karuppayil, 2014). EOs exhibit direct anticancer activity on numerous human cancer cell lines and have positive effects in chemoprevention due to their antioxidant, antiproliferative and antimutagenetic activities (Bhalla et al., 2013; Saad et al., 2013; Raut and Karuppayil, 2014). Furthermore EOs could inhibit process of angiogenesis, enhance immune system and induce process of detoxification by induction of different enzymes (Bhalla et al., 2013). It is found that active components of many EOs could alter different genes expression, ion homeostasis, mitochondrial and plasma membrane functions, signaling and/or metabolic pathways leading to cell cycle arrest and/or cell death (Bhalla et al., 2013; Saad et al., 2013; Raut and Karuppayil, 2014; Greay and Hammer, 2015).

There are scarce literature data concerning cytotoxic activities of EOs of some other *Nepeta* species with considerable amounts of diverse nepetalactones (Shakeri et al., 2016; Tsuruoka et al., 2012; Kahkeshani et al., 2014) or germacrene D (Shakeri et al., 2014), and 1,8-cineol (Kahkeshani et al., 2014). However, detailed knowledge about the mode of action of these EOs is still lacking. In this study we describe the cytotoxic effects of *N. rtanjensis* EO, and explore the type of cell death it induces, the cell cycle phase it affects and the genes expression of which it alters. These intricate information are particularly important for evaluating the application possibilities, and facilitating implementation of *N. rtanjensis* EO in anticancer therapies.

2. Materials and methods

2.1. Plant material and essential oil preparation

Plants of *Nepeta rtanjensis* Diklić & Milojević were field cultivated at the Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia. Voucher specimens are deposited at the Herbarium of the Institute of Botany and Botanical Garden 'Jevremovac', Faculty of Biology, Belgrade (16064 BEOU), and at the Department of Plant Physiology, Institute for Biological Research "S. Stanković", University of Belgrade, Serbia. Aerial parts of flowering plants were collected in July 2014, air-dried at room temperature until constant mass, mechanically chopped, and used for the isolation of EO as previously described by Ljaljević Grbić et al. (2008), with some modifications. Hydrodistillation was performed for 2 h, in a Clevenger type apparatus connected to a 5 L Borosilicate Glass Flat Bottom Flask (Isolab, Germany) containing 300 g of plant material and 3 L deionized H_2O (Millipore, Billerica, USA). Essential oil extraction procedure, yielding around 0.5 μL essential oil per g DW, was repeated 5 times; obtained oils were pulled and further subjected to GC analysis. Total yield of essential oils was 750 μL.

2.2. Gas chromatography of N. rtanjensis essential oil

Analysis of *N. rtanjensis* EO was performed by gas chromatography (GC) using two types of detectors, flame ionization (FID) and mass-spectrometric (MSD) as previously described (Dmitrović et al., 2015). GC-FID analysis of the EO was carried out on an Agilent Technologies, model 5890A gas chromatograph on HP-5 column (25 m × 0.32 mm, 0.53 μm film thickness). Flow rate of H_2 (carrier gas) was 1 mL/min, while detector and injector temperatures were 300 °C and 250 °C, respectively. Column was heated linearly from 40 °C to 260 °C and held at 260 °C for 10 min. Quantification was performed using area percent reports as a base. The same analytical conditions as those mentioned for GC-FID were used for GC–MS analysis, which was carried out on HP G 1800C Series II GCD system (Hewlett-Packard, Palo Alto, CA, USA) attached to a HP- 5MS column (30 m × 0.25 mm, 0.25 μm film thickness). Helium was a carrier gas (1 mL/min flow rate), while the transfer line was heated at 260 °C. Mass spectra were acquired in EI mode (70 eV) in *m*/*z* range 40–450. Identification of constituents was performed by comparison of their mass spectra to those from Wiley275 and NIST/NBS libraries, using different search engines. Experimental values for retention indices were determined using calibrated Automated Mass Spectral Deconvolution and Identification System Software (AMDIS ver. 2.1, National Institute of Standards and Technology (NIST), Standard Reference Data Program, Gaithersburg, MD, USA), and by comparison with the available literature (Adams, 2007).

2.3. Cell culture

Human cervix carcinoma cells (HeLa), human breast cancer cells (MDA-MB-231), human colon carcinoma cells (LS-174), lung adenocarcinoma cells (A549), human myelogenous leukemia cells (K562) and human fetal lung fibroblast cells (MRC-5) were maintained in the Roswell Park Memorial Institute (RPMI) 1640 nutrient medium (Sigma Chemicals Co., USA). RPMI 1640 nutrient medium was prepared in sterile deionized water, supplemented with penicillin (192 U/mL), streptomycin (200 μg/mL), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) (25 mM), L-glutamine (3 mM) and 10% of heat-inactivated fetal calf serum (FCS) (pH 7.2). The cells were grown at 37 °C in 5% CO_2 and humidified air atmosphere.

2.4. Cytotoxic activity

Cytotoxicity of the investigated *N. rtanjensis* EO was determined using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Supino, 1995). Experiments were performed on five tumor cell lines (HeLa – human cervix carcinoma cells, A549 – lung adenocarcinoma cells, LS-174 – human colon cancer cells, K562 – human myelogenous leukemia cells, MDA-MB-231 – human breast cancer cells) and one normal cell line (MRC-5 – human fetal lung fibroblast cells). Briefly, cells were seeded in 96-well cell culture plates in culture medium (RPMI 1640) and grown for 24 h. Stock solution of the investigated EO was made in DMSO at concentration of 10 μL/mL and was afterwards diluted