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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, nepeta-lactones. 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 IC₅₀ value for this cell line was not reached within the tested range of EO concentrations (up to 0.1 μ L/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.

Key words: Anticancer activity, Apoptosis, *Nepeta rtanjensis*, cis-nepetalactone

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-2-yl)-2,5-diphenyltetrazolium bromide.

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 attractant (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 Zivković 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 (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 H₂O (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 H₂ (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 1800 C 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 Wiley 275 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% CO₂ and humidified air atmosphere.

2.4. Cytotoxic activity

Cytotoxicity of the investigated *N. rtanjensis* EO was determined using the 3-(4,5-dimethylthiazol-2-yl)-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 $\mu\text{L/mL}$ and was afterwards diluted with the nutrient medium to the desired final concentrations (in range up to 0.1 $\mu\text{L/mL}$). Solutions of various concentrations of the examined EO were added to the wells, except the control wells where only nutrient medium was added. All treatments were done in triplicate. Cells were incubated (CO₂ incubator 220, Flow Laboratories) for 72 h with the investigated *N. rtanjensis* EO. After incubation, 20 μL of MTT solution, 5 mg/mL in phosphate buffer solution (PBS), pH 7.2 were added to each well. Samples were incubated for 4 h at 37°C, with 5% CO₂ in humidified atmosphere. Formazan crystals were dissolved in 100 μL of 10% sodium dodecylsulfate (SDS). Absorbance was recorded on an enzyme linked immunosorbent assay (ELISA) reader (Thermo Lab systems Multi-skan EX 200–240 V) after 24 h at 570 nm. Concentration IC₅₀ was defined as the concentration of EO producing 50% inhibition of cell survival, and was determined from the representative cell survival diagrams. Cisplatin (CDDP) was used as a standard cytotoxic agent. CDDP stock solution was made in 0.9% NaCl at concentration of 3.3 mM and afterwards diluted with nutrient medium to desired final concentrations (in range up to 100 μM).

2.5. Morphological analysis

Morphological analyses were performed by:

A.) Light microscopy

The morphology of the HeLa, LS-174 and MRC-5 cells was monitored using an inverted microscope (Carl Zeiss, Jena, Germany, magnification 78.75-fold) equipped with a digital camera (Olimpus). Following 72 h treatment with EO or control (nutrition medium), changes in cellular morphology of the cell lines were observed and documented.

•Fluorescent microscopy

Morphological characteristics of HeLa cells' death induced by the treatment with investigated EO were analyzed after staining of the treated cells with a mixture of acridine orange (AO) and ethidium bromide (EB): 0.3 mg/mL AO and 0.3 mg/mL EB in PBS solution (Banada et al., 1996). Briefly, 1×10^5 of HeLa cells were seeded on glass slide in Petri dishes, and treated for 24 h and 48 h with $0.5 \times IC_{50}$, IC_{50} , and $1.5 \times IC_{50}$ concentrations of EO (determined for 48h treatment). The cells were then stained with 10 μ L of a mixture of working concentration of AO/EB, and observed in the next 30 min under a fluorescence microscope Axio Observer Z1, using AxioVision imaging software (Carl Zeiss MicroImaging GmbH). All used chemicals were purchased from Sigma Aldrich.

2.6. Analysis of gene expression by real-time PCR

For total RNA isolation HeLa cells were lysed using TRI REAGENT[®] BD kit (Sigma-Aldrich, St. Louis, USA) 24 h after the treatment with *N. r. tanjensis* EO in concentrations equal

to IC_{50} and $1.5 \times IC_{50}$ values (determined for 48 h treatment). cDNA synthesis from total RNA (2 μ g) was performed using random primers and MultiScribeTM Reverse Transcriptase from High-Capacity cDNA Reverse Transcription kit (50 U/ μ L, Applied Biosystems, CA, USA). Target transcripts *Bax*, *Bcl-2*, *p53* and *Skp2* were detected using quantitative real-time PCR (qRT-PCR) (TaqMan[®] Gene Expression Assays and TaqMan[®] Gene Expression Master Mix, Applied Biosystems, CA, USA). To exclude variations arising from different inputs of total mRNA to the reaction, data were normalized to an internal housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Non-template controls were included in each amplification mixture. PCR reactions were performed on ABI Prism 7700 Sequence Detection System (Applied Biosystems, CA, USA) and the fluorescence the fluorescence was monitored in real time. The thermal cycling conditions comprised of initial denaturation step at 95°C for 10 min followed by 40 cycles of denaturation (15s at 95°C) and annealing/extension (1 min at 60°C) in a final volume of 20 μ L. All thereactions were performed in triplicate and the data represent mean $\pm$ S.E. of three independent experiments. Data were analyzed using the classical delta-delta-Ct method.

2.7. Cell cycle analysis

Quantitative analysis of cell cycle phase distribution was performed by flow-cytometric analysis of DNA content in fixed HeLa cells, after staining with propidium iodide (PI) (Ormerod, 1994).

Cells were seeded at a density of 2×10^5 cells/well in 6-well plates and grown in nutrition medium. After 24 h cells were continually exposed to EO at concentrations that

corresponded to $0.5 \times IC_{50}$, IC_{50} and $1.5 \times IC_{50}$ (determined for 48 h treatment). Control cells were incubated only in nutrient medium. After 48 h of continual treatment cells were collected by trypsinization, washed twice with ice-cold PBS, and fixed for 30 min in 70% EtOH. After fixation cells were washed again with PBS, and incubated with RNaseA (1 mg/mL) for 30 min at 37°C. Cells were then stained with PI (at concentration of 400 µg/mL) 15 min before the flow-cytometric analysis. Cell cycle phase distribution was analyzed using a fluorescence activated sorting cells (FACS) Calibur Becton Dickinson flow cytometer and Cell Quest Pro computer software.

2.8. Statistical analysis

Data of cytotoxic activity are shown as mean $\pm$ S.D. GraphPad Prism V5.04 (GraphPad Software, San Diego, CA) was used to compare means by two way analysis of variance (ANOVA) in analysis of gene expression data. When an overall statistically significant difference was observed, post-tests were performed to compare pairs of treatments, using the Bonferroni method to adjust the p-value for multiple comparisons. Differences were considered significant if $p < 0.05$. P-values for each analysis are indicated in figure legends.

3. Results and discussion

Essential oils from aromatic and medicinal plants are mainly composed of terpenes and terpenoids, phenylpropanoids and aliphatic components (Bakkali et al., 2008; Bhalla et al., 2013). Chemical analysis revealed that *N. rtanjensis* EO is especially rich in monoterpenoids, mainly *trans,cis*-nepetalactone which represents 71.66% of EO (Table 1). Other stereoisomer, *cis,trans*-nepetalactone constitutes 17.21% of the EO. In total, nepetalactone is the major constituent in *N. rtanjensis* EO with 88.87%, and minor components are α -pinene (3.28%), 2-methoxy-para-cresol (1.85%), α -copaene (0.86%), etc. (**Table 1**).

Results of the cytotoxic activity assay (shown in Fig. 1 as representative cell survival diagrams) indicate that the investigated EO after 72 h of incubation showed a dose-related cytotoxic activity on all analyzed cell lines in the tested range of concentrations. The highest cytotoxicity was determined for HeLa ($IC_{50} = 0.050 \pm 0.017 \mu\text{L/mL}$), K562 ($IC_{50} = 0.052 \pm 0.003 \mu\text{L/mL}$) and A549 ($IC_{50} = 0.064 \pm 0.009 \mu\text{L/mL}$) cells after 72 h treatment, while IC_{50} values were $0.093 \pm 0.009 \mu\text{L/mL}$ for LS-174 cells and $0.097 \pm 0.003 \mu\text{L/mL}$ for MDA-MB-231 cells. The MRC-5 cell line was the least sensitive to the treatment with EO and IC_{50} was not reached within the investigated range of concentrations (up to $0.1 \mu\text{L/mL}$). Cisplatin was used as a positive control and for 72 h treatment exhibited the IC_{50} values in the following concentration range $0.99\text{--}4.29 \mu\text{g/mL}$ in the investigated cell lines. IC_{50} values of some other tested EOs obtained from various *Nepeta* species ranged from $10\text{--}50 \mu\text{g/mL}$ (Tsuruoka et al., 2012; Kahkeshani et al., 2014; Shakeri et al., 2014, 2016).

Fig. 1. Representative diagrams of cells survival after 72 h of continual *N. rtanjensis* EO action. Data represent mean $\pm$ S.D. of a representative experiment performed intriplicates

Table 1

The chemical composition of *N. rtanjensis* essential oil detected by GC–MS and GC–FID analysis.

Constituents	KIE	KIL	% m/m
1 α -pinene	931.5	932	3.28
2 β -pinene	974.7	974	0.40
3 1,8-cineole	1030.3	1026	0.30
4 α -campholenal	1127.9	1122	0.18
5 2-metoxo-para-cresol	1218.0	1188	1.85
6 <i>cis,trans</i> -nepetalactone	1364.2	1357	17.21
7 α -copaene	1374.7	1374	0.86
8 <i>trans,cis</i> -nepetalactone	1400.3	1391	71.66
9 1,3,3-trimethyl-5-oxobicyclo[2.2.1]hept-2-yl acetate	1460.8	1449	0.30
10 γ -cadinene	1513.9	1513	0.27
11 δ -cadinene	1523.5	1522	0.74
12 α -calacorene	1543.8	1544	0.23
13 α -cadinol	1657.7	1652	0.16
14 <i>cis</i> -14-nor-muurool-5-en-4-one	1688.8	1688	0.21
Total			97.65

KIE – experimentally determined Kovats index

KIL – Kovats index from literature

% m/m – percentage of component in extract (mass on mass)

Essential oil from *N.sibirica*, rich in *cis,trans*-nepetalactone, showed cytotoxic effect on human leukemia myeloid-derived cell line HL-60 and on stomach adenocarcinoma Kato III, but was not cytotoxic to the lung adenocarcinoma cell line A549 (Tsuruoka et al., 2012). *N.rtanjens* EO exhibited a significant cytotoxic effect on the same cancer cell line (A549), as well as on the other type of myeloid leukemia cancer cell line K562. Essential oils of *N. sintesii* and *N.menthoides* which mainly consists of oxygenated monoterpenes, mostly nepetalactones and 1,8 cineol, showed a potent antiproliferative and strong cytotoxic activity on various tested human carcinoma cell lines, including HeLa (cervical cancer) and MCF-7 (breast adenocarcinoma) cells (Kahkeshani et al., 2014; Shakeri et al., 2016). Similar effects on breast carcinoma cell line MCF, as well as on ovarian cancer A2780 cell line was demonstrated for EO of *N. ucrainica* rich in germacrene-D (Shakeri et al., 2014). According to the reports in the literature, extracts with cytotoxic activity values lower than 30 µg/mL ($IC_{50} < 30 \text{ µg/mL}$), should be considered as good candidates for future anticancer drug development (Suffness and Pezzuto, 1990; de Oliveira et al., 2015; Shakeri et al., 2016). It is important to point out the observed selectivity of *N. rtanjensis* EO towards some tumor cell lines (HeLa, K562 and A549) in comparison to investigated normal fibroblast cells (MRC-5, $IC_{50} > 0.1 \text{ µL/mL}$). A similar selectivity between cancer cell lines and a normal cell line (HUVEC) was demonstrated by Shakeri et al. (2016) for EO of *N. sintenisii*, while EO of *N. menthoides* was cytotoxic on cancer and normal cells as well (Kahkeshani et al., 2014). It is considered as important that chemotherapeutic drugs should target only tumor cells exhibiting cytotoxic and/or cytostatic effects with minimal damage to normal cells (Johnstone et al., 2002). Therefore, EOs with a proven in vitro selectivity toward cancer cells are favorable candidates for future investigations. Thus, the presented results emphasize the significance of

the possible use of *N. rtanjensis* EO as a candidate for further research and antitumor drug development.

Since this is, to our knowledge, the first time that cytotoxic activity of *N. rtanjensis* EO has been demonstrated, further investigations were conducted toward determination of the type of cancer cell death it induces. Analysis of morphology of HeLa, LS-174, and MRC-5 cells revealed the appearance of a reduction in cell number as early as 72 h after addition of the EO (Fig. 2). Cells lost their normal morphology and became rounded (as observed for HeLa and LS-174 cells) which might indicate apoptosis as a type of cellular death (Fig. 2). Apoptosis is a preferable type of cell death to necrosis, since it does not produce inflammation or damage to normal tissue surrounding the tumor (Johnstone et al., 2002). In order to determine the type of cell death induced by the investigated *N. rtanjensis* EO, HeLa cells were stained with acridine orange and ethidium bromide and observed under a fluorescence microscope. Representative microphotographs after 24 h of treatment with the $1.5 \times \text{IC}_{50}$ concentration of EO determined for 48 h ($\text{IC}_{50} = 0.084 \mu\text{L/mL}$) are presented in Fig. 3. Untreated control cells were dense and light green colored. After the 24 h treatment with the investigated concentration of EO the number of cells was reduced compared to control, cells lost their normal morphology and became rounded. This was followed by the cytoplasmic shrinkage and nuclear condensation and fragmentation, cell membrane blebbing and occurrence of apoptotic bodies (Fig. 3), which are characteristic for apoptosis (Marino et al., 2014; Sharma et al., 2010; Westhoff et al., 2014).

Fig. 2. Morphological changes of treated HeLa, LS-174 and MRC-5 (normal) cells for a 72 h treatment with *N. rtanjensis* EO detected by inverted microscope.

Different types of stress may induce cell cycle arrest at different phases and enable maintenance of genomic integrity by initiating apoptosis (Guichard et al., 2015). In order to

further clarify the potential influence of the investigated EO on cell cycle progression, analysis of cell cycle has been assessed on HeLa cells by flow cytometry. Results presented in Fig. 4 indicated that *N.rtanjensis* EO induced perturbations of the cell cycle of HeLa cells, only at the highest concentration tested ($1.5 \times \text{IC}_{50}$ determined for 48 h). The obtained results showed an increase in the percentage of cells in sub-G1 phase (apoptotic), a decrease of percentage of cells in G1 and an increase of percentage of cells in the S phase of the cell cycle after 48 h of continual EO action. The observed potential of *N. rtanjensis* EO to induce cell cycle perturbations and to increase the number of cells with fragmented DNA indicated its ability to induce tumor cell apoptosis.

There is increasing evidence that changes in the expression of many genes occur due to the cancer cell's response to chemotherapeutic drugs, and according to the numerous literature data, these molecular changes are involved in the induction of apoptosis in cancer cell lines (Johnstone et al., 2002; Chipuk and Green, 2006; Mariño et al., 2014).

Antitumor properties of monoterpenes involve activation of apoptotic pathways, reduction of pro-survival signals via interruption of the MAPK cascade at several levels, cell cycle arrest and efficiency improvement of radiotherapy (Fernandes, 2015). Apoptosis induced by monoterpenes occurs through both extrinsic (death receptor) and intrinsic (mitochondrial) pathways (Fernandes, 2015). Members of the Bcl-2 family proteins including proapoptotic (e.g. Bax, Bak, p53 upregulated modulator of apoptosis – PUMA, Bad, Bcl-Xs, Bid, Bik, Bim) and antiapoptotic (Bcl-2, Bcl-XL, Bcl-W, Bfl-1, Mcl-1) have a major role in cell survival and drug sensitivity (Fernandes, 2015), usually by upregulation of proapoptotic and/or downregulation of antiapoptotic members of this family (Kalimuthu and Se-Kwon, 2013; Verhaegen et al., 2014).

Fig. 3. Morphological changes of HeLa cells treated for 24 h with *N. rtanjensis* EO detected by fluorescence microscope. Arrows indicate characteristic cell membrane blebbing and occurrence of apoptotic bodies.

Upregulation of *p53* is well documented in apoptosis as well (Chipuk and Green, 2006; Kalimuthu and Se-Kwon, 2013). In fact, *p53* represents a major regulator of apoptosis through several mechanisms including transcription-dependent functions and transcription-independent activities (Ryan et al., 2001; Johnstone et al., 2002; Chipuk and Green, 2006; Kalimuthu and Se-Kwon, 2013). *p53* could initiate apoptosis through activation of

the proapoptotic Bcl-2 family members or some other genes (i.e. *PTEN*, Apaf-1, PERP), and by repression of antiapoptotic Bcl-2 proteins (Johnstone et al., 2002; Chipuk and Green, 2006; Kalimuthu and Se-Kwon, 2013). Skp2, an E3 ligase, acts as an oncoprotein by targeting p27 for degradation, and is usually overexpressed in tumor cells (Wang et al., 2012; Chan et al., 2014; Chung et al., 2015). Skp2 has been considered as a promising novel therapeutic target for several types of human cancers (Wang et al., 2012; Chan et al., 2014). Recent studies reported down regulation of *Skp2* gene expression in drug-induced apoptosis (Huang et al., 2011; Sun et al., 2012; Chung et al., 2015).

In order to elucidate the mechanisms underlying the apoptosis induced by *N. ratanjensis* EO, the changes in *Bax*, *Bcl-2*, *p53* and *Skp2* gene expression in HeLa cells were analyzed by real timePCR (Fig. 5). The quantitative gene expression analysis showed a general upregulation of *Bax* mRNA expression, downregulation of *Bcl-2*, upregulation of *p53* and downregulation of *Skp2*, all confirming an apoptosis-inducing effect of *N. ratanjensis* EO oil on HeLa cells. Similarly to nepetalactone-rich EO, curcumin was shown to reduce the level of *Skp2* mRNA in a breast cancer cell line (MDA-MB-231/Her2) (Sun et al., 2012). In human squamous cell lung carcinoma H520 cells, curcumin induced apoptosis by increasing the expression of pro-apoptotic Bax, and by downregulation of anti-apoptotic Bcl-2 and Bcl-X_L, leading to the activation of caspases (Sen et al., 2005). Perillyl alcohol and d-limonene, both belonging to monoterpenes, also influenced the apoptotic pathway causing an increased expression of *Bax* and decreased expression of Bcl-2 (Chaudhary et al., 2009, 2012). Linalool, a natural small molecule monoterpene, induced apoptosis with activation of p53 and several cyclin-dependent kinase inhibitors (Gu et al., 2010). Lingaiah et al. (2013) demonstrated that the monoterpene myrtenal considerably increased p53 expression in cancer-bearing rats and suggested that the shown overexpression of p53 plays a crucial role in

thymyrtenal-induced apoptosis. Thymoquinone, monoterpene from *Nigella sativa*, induced apoptosis in HeLa cells by upregulation of p53 expression (Yazan et al., 2009), while citral from *Zingiber officinale* (ginger) induced an increase of p53, decrease of Bcl-2, with no effect on Bax, leading to the apoptosis in endometrial cancer cells (Liu et al., 2012).

Essential oils represent the mixture of numerous constituents hence their cytotoxic activity actually represents a sum of each individual activity and all possible impacts including synergies and antagonisms (Lesgards et al., 2014; Bhalla et al., 2013; Shakeri et al., 2016). Easy isolation of monoterpenes from natural sources, their low toxicity, as well as the presence of increasing reports of their anticancer activities and the elucidation of their principal mode of action in tumor cells make a good rationale for their potential use in clinical trials (Fernandes, 2015). In this regard, it is noteworthy that even when monoterpenes do not exhibit antitumor effects they can enhance the activity of other natural products or drugs used in clinical treatment (Fernandes, 2015).

Fig. 4. Concentration-dependent effect of *N. rtanjensis* EO on cell cycle distribution of HeLa cells. Data represent mean of a representative experiment performed in triplicates. A) Control B) $0.5 \times IC_{50}$ C) IC_{50} D) $1.5 \times IC_{50}$

Fig. 5. Analysis of Bax, Bcl-2, p53 and Skp2 mRNA expression after 24 h of treatment with *N. rtanjensis* EO at concentrations equal to IC₅₀ and 1.5 × IC₅₀. Gene expression levels were determined by qPCR and normalized to GAPDH. Data represent mean ± S.E. of three independent experiments performed in triplicates. CDDP – cisplatin (1.8 μg/mL). Asterisks denote statistical significance compared to control (nontreated) cells (*p < 0.05; **p < 0.01; ***p < 0.001).

The presented data that selective apoptosis induced by EO of *N. rtanjensis*, which is rich in *trans,cis*-nepetalactone, involves cell cycle phase perturbations and changes in expression levels of Bax, Bcl-2, Skp2 and p53, clearly indicate that *N. rtanjensis* EO and its major constituent (*trans,cis*-nepetalactone) should be marked attractive for further investigation and potential anticancer therapy. Considering the obtained results, the concentration of 0.05–0.1 μL/mL of *N. rtanjensis* EO could be recommended for further in vitro investigations, especially on human cervix carcinoma cells (HeLa) and human myelogenous leukemia cells (K562), followed by the evaluation of the changes in gene and protein expression patterns in cancer-bearing animals.

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Fig 1.

Fig. 1. Representative diagrams of cells survival after 72 h of continual *N. rtanjensis* EO action. Data represent mean $\pm$ S.D. of a representative experiment performed intriplicates

Fig. 2

Fig. 2. Morphological changes of treated HeLa, LS-174 and MRC-5 (normal) cells for a 72 h treatment with *N. rtanjensis* EO detected by inverted microscope

Fig. 3

Fig. 3. Morphological changes of HeLa cells treated for 24 h with *N. rtanjensis* EO detected by fluorescence microscope. Arrows indicate characteristic cell membrane blebbing and occurrence of apoptotic bodies.

Fig 4.

Fig. 4. Concentration-dependent effect of *N. rtanjensis* EO on cell cycle distribution of HeLa cells. Data represent mean of a representative experiment performed in triplicates. A) Control B) $0.5 \times IC_{50}$ C) IC_{50} D) $1.5 \times IC_{50}$

Fig.5

Fig. 5. Analysis of *Bax*, *Bcl-2*, *p53* and *Skp2* mRNA expression after 24 h of treatment with *N. rtanjensis* EO at concentrations equal to IC₅₀ and 1.5 × IC₅₀. Gene expression levels were determined by qPCR and normalized to GAPDH. Data represent mean ± S.E. of three independent experiments performed in triplicates. CDDP – cisplatin (1.8 µg/mL). Asterisks denote statistical significance compared to control (nontreated) cells (*p < 0.05; **p < 0.01; ***p < 0.001).

Table 1.

Table 1

The chemical composition of *N. rtanjensis* essential oil detected by GC–MS and GC–FID analysis.

Constituents		KIE	KIL	% m/m
1	α -pinene	931.5	932	3.28
2	β -pinene	974.7	974	0.40
3	1,8-cineole	1030.3	1026	0.30
4	α -campholenal	1127.9	1122	0.18
5	2-methoxy-para-cresol	1218.0	1188	1.85
6	<i>cis,trans</i> -nepetalactone	1364.2	1357	17.21
7	α -copaene	1374.7	1374	0.86
8	<i>trans,cis</i> -nepetalactone	1400.3	1391	71.66
9	1,3,3-trimethyl-5-oxobicyclo[2.2.1]hept-2-yl acetate	1460.8	1449	0.30
10	γ -cadinene	1513.9	1513	0.27
11	δ -cadinene	1523.5	1522	0.74
12	α -calacorene	1543.8	1544	0.23
13	α -cadinol	1657.7	1652	0.16
14	<i>cis</i> -14-nor-muurol-5-en-4-one	1688.8	1688	0.21
Total				97.65

KIE – experimentally determined Kovats index

KIL – Kovats index from literature

% m/m – percentage of component in extract (mass on mass)