Nepeta rtanjensis (Lamiaceae), a plant endemic to the Balkans: Phenolic composition, antioxidant activity, and *in vitro* antigenotoxic effects in triiodothyronine-induced DNA damage in human lymphocytes

Jasna Bošnjak-Neumüller[1]*, Milena Radaković[1], Ninoslav Djelić[1], Branka Vuković-Gačić[2], Zora Dajić Stevanović[3], Stoimir Kolarević[2], Danijela Mišić[4], Milan Stanković[5], Jelena Knežević-Vukčević[2], Biljana Spremo-Potparević[6] and Zoran Stanimirović[1]

[1]Department of Biology, Faculty of Veterinary Medicine, University of Belgrade, Blvd. oslobodjenja Belgrade, Serbia
[2]Department of Microbiology, Faculty of Biology, University of Belgrade, Studentski trg, Belgrade, Serbia
[3]Department of Crop Science, Faculty of Agriculture, University of Belgrade, Nemanjina Belgrade, Serbia
[4]Institute for Biological Research ''Siniša Stanković'', University of Belgrade, Blvd. Despota Stefana, Belgrade, Serbia
[5]Department of Biology and Ecology, University of Kragujevac, Radoja Domanovića, Kragujevac, Serbia
[6]Department of Biology and Human Genetics, Institute of Physiology, Faculty of Pharmacy, University of Belgrade, Vojvode Stepe, Belgrade, Serbia

Abstract: The success of antioxidant therapy in hyperthyroidism implies that disease is mediated by oxidative stress, which is known as one of the causing agents of ageing, degenerative diseases, and cancer. The main objective of our study was to determine possible protective effects of methanolic extract of *N. rtanjensis* in triiodothyronine (T_3)-induced DNA breaks of human lymphocytes under *in vitro* conditions, based upon plant antioxidant capacity related to its phytochemical profile, mainly its polyphenolic complex. The total phenolic and flavonoid content and the antioxidant activity using *in vitro* 1,1-dyphenyl-2- picrylhydrazyl reagent (DPPH) was determined in methanolic extracts of plant leaves and flowers. The phenolic compound content of 62.73±1.80mg of GaA/g, exhibited solid antioxidant activity (IC50= 112.59±0.95µg/ml). The antigenotoxic activity of 0.2, 0.5 and 1.0mg/ml *N. rtanjensis* methanol extracts mixture with 100µM of T_3 was studied in human lymphocytes *in vitro* using the Comet assay. It is supposed that the antigenotoxicity of *N. rtanjensis* methanol extracts was caused by high presence of chlorogenic acid, rosmarinic acid and rutin, all known as efficient antioxidant bioactive compounds, which were determined by ultrahigh-pressure liquid chromatograph with MS/MS Mass Spectroscopy (UHPLC/-HESI-MS / MS).

Keywords: triiodothyronine, DNA damage, *Nepeta rtanjensis,* phenolic compounds and antigenotoxic activity.

INTRODUCTION

Thyroid hormones (TH) are well-known for their profound effects on the metabolic rate, growth and differentiation of nearly all animal and human tissues (Mullur *et al.,* 2014). The metabolic effects of TH, mainly triiodothyronine (T_3), are directly linked to reactive oxygen species (ROS) production due to its capacity to accelerate the basal metabolism and change respiratory rate in mitochondria (Cheng *et al.,* 2010). ROS are essential biomolecules in cell signalizing and regulation, traditionally more recognized with their role in oxidative damage that underlines many human degenerative diseases, aging and cancer (Dickinson and Chang, 2011). Oxidative damage of tissue observed in hyperthyroidism, depends on metabolic response of cell to TH, accordingly, the organ that mainly suffers from oxidative damage in hyperthyroidism is the liver, due to lipid, protein and, DNA oxidation (Messarah *et al.,* 2010). The level of the most common oxidized base 8-oxo-7'8'-dihydroguanine typical for tumours (Azqueta *et al.,* 2009) is increased in hyperthyroid rat liver (Andican *et al.,* 2004). The implication of antioxidants, vitamin E and curcumin, has been used in a prevention of primary liver damage occurring in a hyperthyroid state (Subudhi *et al.,* 2008).

Medicinal plants are considered as a potential source of antioxidants applied in protection from damage induced by oxidative stress through inhibiting or modulating the effects of mutagens and carcinogens (Vukovic-Gacic *et al.,* 2006). *Nepeta rtanjensis* Diklic & Milojević (Lamiaceae) is an endemic perennial plant, growing only on a few sites of a very limited area of dry calcareous grasslands in Southeast Serbia (Diklić *et al.,* 1999). *Nepeta* species are widely used in folk medicine as diuretic, diaphoretic, anti-tussive, anti-spasmodic, anti-asthmatic, febrifuge and sedative agents and also against gastrointestinal and respiratory hyperactive disorders. Since *N. rtanjensis* is a rare plant of a limited distribution, it is important to emphase that its propagation with a 99% survival rate was achieved due to a specially developed micro propagation protocol (Misic *et al.,* 2005). *N.*

**Corresponding author:* e-mail: jasna.bosnjak@patent-co.com

rtanjensis is already known for its antibacterial and antifungal activities due to the presence of nepetalactones in essential oil synthetized in glandular trichomes of leaves and stem (Grbić-Ljaljević *et al.,* 2011). Nepetalactones were shown to have cytotoxic effect on C32 amelanotic melanoma and in renal adenocarcinoma cancer cell (Rigano *et al.,* 2011). The free radical scavenging ability of *N. rtanjensis* have been recently recognized and explained due to the presence of several identified phenolic compounds, dominantly, rosmarinic acid, chlorogenic acid and caffeic acid (Nestorović, 2013). The same phenolic components, rosmarinic acid, caffeic acid and phenolic acids, were also present in *Nepeta menthoides* which had effect on enhancing memory retention and retrieval possibly due to their antioxidant activity, as explained by Sarahroodi *et al.* (2012). Considering that anxiety, as a common symptom in hyperthyroidism, is linked with oxidative stress (Demet *et al.,* 2002; Smaga *et al.,* 2015), there are notes on use of *Nepeta* species in folk medicine as an anxiolytic drug or constituent of herbal preparation with anxiolytic effects (Ishaq, 2014). Sedative and anxiolytic effects of *Nepeta* species are attributed to the main constituents of plants, nepetolactones (Rabbani *et al.,* 2008).

In our study we choose to assess the antigenotoxic potential of *N. rtanjensis* in the Comet assay because it is a simple, rapid, and very sensitive test for the quantification of DNA damage (Tice *et al.,* 1991). Additionally, it was previously reported, using the *in vitro* Comet assay, that genotoxic effects of the T_3 hormone can be reduced with an antioxidant defense system of enzyme catalase (Djelic and Anderson, 2003) and flavonoids, purified kaempferol, and quercetin (Dobrzyńska *et al.,* 2004). The main aim of our study was to provide the first data about possible antigenotoxic potential of *N. rtanjensis*, in attenuating formation of DNA lesions induced by T_3 hormone. Prior to ultrahigh-pressure liquid chromatograph with MS/MS Mass Spectroscopy (UHPLC/-HESI-MS / MS) determination of phenolic compounds in *N. rtanjensis* extract with the most antioxidant potential, we have tested the total flavonoids and phenolics content and antioxidant property of leaves' and flowers' methanolic extracts.

MATERIALS AND METHODS

Preparation of plant material

Aboveground parts of the plant were collected in the flowering phase from its natural habitat, the mountain of Rtanj in Eastern Serbia (N 43°43.657' E 21°54.257'). Since the species is listed in the List of Protected Species of Serbia, special permission for collecting a small amount of plant material was obtained from the Institute of Nature Protection of Serbia, in line with Convention on Biological Diversity and the Convention on the Trade in Endangered Species of Wild Fauna and Flora ratificated in Serbian Law on Nature Protection (Official Gazette of the Republic of Serbia No 36/09 and No 88/2010). Plant material was authenticated by Prof. Zora Dajic Stevanovic and a herbarium voucher was deposited at the Department of Applied Botany, Faculty of Agriculture University of Belgrade (FOA NR 34).

For the spectrophotometric tests and evaluation of *N. rtanjensis* antioxidant activity the air-dried plant material (10g) was coarsely crushed and extracted with methanol. The extract was filtered through a paper filter (Whatman, No. 1, Sigma-Aldrich, Steinheim, Germany) and evaporated under reduced pressure with a rotary evaporator. The obtained leaf and flower extracts were stored in dark glass bottles for further processing.

Preparation of *N. rtanjensis* Methanolic Extract for UHPLC/-HESI-MS/MS analyses (Liquid Chromatography Under Ultra-High Pressure with MS/MS Mass Spectroscopy) and the Comet Assay was carried our separately. Leaf plant parts of *N. rtanjensis* mass ~5.9g per sample were crushed in liquid nitrogen until reaching powder form. Extraction was carried out in 25ml of methanol (99.8%, AppliChem Cheshire, USA) with sonication for 10min. Samples were then centrifuged at 8 000×*g* for 10min, at a temperature of 4°C. The supernatant was filtered and the volume adjusted to 25 ml adding methanol. For the purposes of UHPLC/-HESI-MS/MS analysis, the samples were filtered through a cellulose filter with a pore size of 0.2µm (Agilent Technologies, Santa Clara, CA, USA) and stored at 4°C until use. Evaporation of the samples was performed in a vacuum evaporator (Concentrator 5301, Eppendorf GmbH, Austria), at room temperature. The dry methanolic extract was used for further analysis.

Spectrometric determination of total phenolics and flavonoids in the plant extracts and their antioxidant activity

Chemicals, gallic acid, rutin hydrate, and 2,2-Dyphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma Chemicals Co., St Louis, MO, USA. Folin-Ciocalteu phenol reagent and aluminium chloridehexahydrate was purchased from Fluka Chemie AG, Buchs, Switzerland.

The concentration of phenolics in the plant extracts was measured by using a spectrophotometric method (Singleton *et al.,* 1999). The methanol solution of the plant extracts in a concentration of 1mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5ml of methanol solution of the extract, 2.5ml of Folin-Ciocalteu reagent, and 2ml of 7.5% NaHCO3. The samples were thereafter incubated at 45°C for 15min. The absorbance was determined using a spectrophotometer at λ_{max}=765nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the

standard solution of gallic acid and the calibration line was constructed. Based on the measured absorbance, the concentration of phenolics was read (mg/ml) from the calibration line; then, the content of phenolics in the extracts was expressed in terms of gallic acid equivalent, GAE (mg of GaA/g of extract).

The content of flavonoids in the examined plant extracts was determined using a spectrophotometric method (Quettier *et al.,* 2000). The sample contained 1ml of methanol solution of the plant extracts in a concentration of 1mg/ml and 1ml of 2% AlCl3. The samples were incubated for an hour at room temperature. The absorbance was determined using spectrophotometer at of λ_{max}= 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of rutin and the calibration line was constructed. Based on the measured absorbance, the concentration of flavonoids was read (mg/ml) on the calibration line; then, the content of flavonoids in extracts was expressed in terms of rutin equivalent, RUE (mg of RU/g of extract).

The ability of the plant extract to scavenge 1,1-dyphenyl-2- picrylhydrazyl (DPPH) free radicals was assessed by the standard method (Takao *et al.,* 1994), adopted with suitable modifications (Kumarasamy *et al.,* 2007). The stock solution of the plant extracts was prepared in methanol to achieve the concentration of 1mg/ml. Dilutions were made to obtain concentrations of 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.99, 0.97µg/ml. Diluted solutions (1.00 ml each) were mixed with DPPH (1 ml). After 30 min in the dark at room temperature, the absorbance was recorded at 517 nm. The control samples contained all the reagents except the extract. The percentage inhibition was calculated using equation (1), whilst IC_{50} values were estimated from the percent inhibition versus concentration plot, using a non-linear regression algorithm. The data were presented as mean values ± standard deviation (n = 3).

$$\%\ inhibition = \left(\frac{A \text{ of control} - A \text{ of sample}}{A \text{ of control}} \right) \times 100 \quad \text{Eq. (1)}$$

UHPLC/-HESI-MS/MS analysis of phenolic Compounds in N. rtanjensis methanol extract

The analysis was performed on a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Bremen, Germany) which includes a binary pump, a vacuum degasser, a thermostat for columns and an auto-sampler. UHPLC system is configured with a triple-quadrupole mass spectrophotometer (TSQ Quantum Access MAX, Thermo Fisher Scientific, Bremen, Germany) with an electron spray ionization (HESI, eng. Heated electro spray ionization). Chromatographic separation was carried out on a Hypersil Gold C18 column with dimensions of 50 mm × 2.1mm, with a particle size of 1.9µm (Thermo Fisher Scientific, USA). The mobile phase consists of 0.1% solution of formic acid (A) and acetonitrile (B) and samples were eluted as previously described by Mišić *et al*. (2015). The flow rate was adjusted to 0.400 ml/min. All analysis were conducted with MS-quality solutions (Fisher Scientific UK, Leics, UK). The column was thermo stated at 30°C, while the injection volume was 10µl. To control the UHPLC instrument Chromeleon Xpress software was used (Thermo Fisher Scientific, Bremen, Germany). Targeted phenolics were quantified based on the calibration curves of pure compounds, which all revealed good linearity, with r^2 values exceeding 0.99, p<0.001. Standards of chlorogenic acid, rosmarinic acid, caffeic acid, ferulic acid, aesculin, rutin, luteolin, apigenin, kaempferol and quercetin were purchased from Sigma-Aldrich (Steinheim, Germany).

Study Design: Antigenotoxic potential of methanol extract of N. rtanjensis in T_3-Treated human lymphocytes

The genotoxic effects of several concentrations of 3,3',5-trijodo-L-thyronine sodium salt (ICN Biomedicals Inc, Irvine, California, USA) (T_3) was preexamed. T_3 was dissolved in PBS (Torlak, Belgrade, Serbia) to experimental concentrations 1.5, 5.0, 15, 50, 100, and 200 µM. We used 100µM hydrogen peroxide as the positive control (Galafarm, Skopje, Macedonia), and the solvent (PBS) for T_3 was the negative control.

For the antigenotoxicity assay, the T_3 hormone was used as genotoxic agent at 100µM of final concentration since it caused a significant level of DNA damage in treated cells, but also retained good cell viability. The same concentration of T_3 was used in previous studies where hormone genotoxicity was evaluated [22, 23].

The dry methanol extract of *N. rtanjensis* was freshly dissolved and diluted dimethyl sulfoxide (DMSO)(Merck KGaA, Darmstadt, Germany) to obtain final concentrations of 0.2, 0.5 and 1mg/ml and the solvent was used as a negative control. For positive control, we used 100µM of hydrogen peroxide (Galafarm, Skopje, Macedonia).

Human lymphocytes were exposed to *N. rtanjensis* extracts in all studied concentrations, together with 100 µM of T_3 and were incubated for 30 min. Genotoxicity of 1mg/ml of *N. rtanjensis* was tested under the same treatment conditions.

Isolation of lymphocytes

For the Comet assay, human lymphocytes were obtained and immediately used by venepuncture from three healthy male donors (25-35 years of age). All of them were healthy at the moment of blood sampling and non-smokers. Donors who reported alcohol consumption,

medicinal usage, exposure to diagnostic X-rays and, severe aerobic physical training were excluded. Human blood sampling was performed in accordance with the Declaration of Helsinki and approved by the Ethical Committee for clinical research at the Faculty of Pharmacy in Belgrade Serbia on 27.04.2015. (846/2). Informed donor consent was also obtained before they participated in the study.

Isolation of lymphocytes from whole blood was performed with a Ficoll-Paque PLUS medium (GE Healthcare Life Sciences, NJ, USA). After centrifugation at 1900×g 15min, the lymphocytes formed a layer directly above the Ficoll-Paque. The isolated lymphocytes were washed twice in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma Chemical Co., St. Louis, MO, USA), each wash was followed by a centrifugation of 10 min at 1800×g. Finally, the supernatant was removed as carefully as possible without disturbing the pellet. An aliquot of 1ml of RPMI 1640 was added and the pellet was resuspended. A manual cell count and an estimate of cell viability were performed using the Trypan blue exclusion test.

The comet assay

All chemicals used in the Comet assay were obtained from Sigma (Saint Louis, MO, USA) if not stated differently. An alkaline Comet assay was performed according to the technique of Singh *et al.* (1988) and Tice *et al.* (1991), with slight modifications. All experiments were repeated three times on lymphocytes isolated from different donors. Prior to the Comet assay, the viability of lymphocytes cells was evaluated after incubation for 30 min with 100 and 500μM of T_3 and 0.2, 0.5 and 1.0 mg/ml of *N. rtanjensis* methanol extract using the Tripan blue dye assay. Microscope slides were precoated with 1% normal melting point agarose and allowed to air dry at room temperature for at least 48 h. After centrifugation (5 min at 2000 rpm), 100μl of cell suspension was mixed with 100μl of 1% low melting point agarose (LMPA). Ninety micro litres of the suspension was rapidly pipetted onto the first agarose layer and spread using a coverslip, and refrigerated to solidify. After removal of the cover slip, the 90μl of 0.5% LMPA was added as the third layer, spread using a cover slip and allowed to solidify at 4°C for 5 min. Afterwards, the slides were immersed in cold lysis solution at pH 10 [2.5M NaCl, 100mM EDTA, 10mMTris pH 10, 1% Triton X-100 (Amersham Biosciences, Freiburg, Germany), 10% DMSO] overnight at 4°C. After lysis, the slides were placed in a horizontal gel electrophoresis tank to allow DNA unwinding in cold (4°C) alkaline electrophoresis buffer [300mMNaOH (MercK GaA, Damstadt, Germany), 1mM EDTA, pH > 13] for 30 min. Electrophoresis was done at 4°C under an electric current of 25 V and 300mA for 30min. All these steps were performed under dimmed light (tank was covered with a black cloth) to prevent the occurrence of additional DNA damage. The slides were then neutralized with 400mM Tris-HCl (pH 7.5), three times for 5 min. Then, the slides were fixed with cold methanol, dried and, stored. Before analysis, the slides were rehydrated with ice cold distilled water and stained with 50μL of ethidium bromide (Serva, Heidelberg, Germany) (EtBr, final concentration 20μg/ml,).

From each replicate slide, 50 nuclei were scored (a total of 100 nuclei per donor) and a tail intensity- percentage of tail DNA (Ti) was used to evaluate the extent of DNA migration. Slides were examined at 400× magnification on a fluorescent microscope (Leica, UK) and image analysis software (Comet Assay IV Image Analysis system, PI, UK. Undamaged cells have an intact nucleus without a tail and damaged cells have the appearance of a comet (fig. 1).

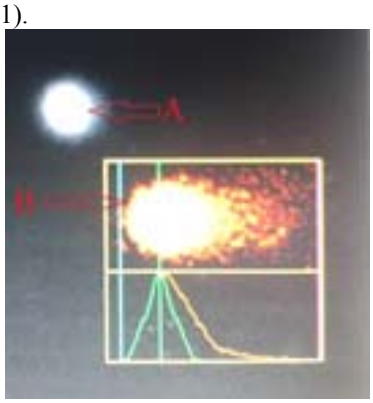

Fig. 1: Fluorescent microscope (Leica, UK) images of human lymphocytes nucleus: (A) intact nucleus without a tail; (B) damaged cell nucleus with the appearance as a comet analyzed by software (Comet Assay IV Image Analysis system, PI, UK)

STATISTICAL ANALYSIS

All experimental measurements related to the determination of total phenolic and flavonoid content, as well as antioxidant activity, were carried out in triplicate and are expressed as the average of three analyses ± standard deviation. Statistical analysis of the Comet assay results for both treatments was performed using Statistica 7.0 Software [StatSoft, Inc., 2001, STATISTICA for Windows (Computer Program Manual). Stat Soft, Inc., Tulsa, OK, USA]. Mean values of Ti for each group and each of 3 donors in genotoxic and antigenotoxic treatments were calculated. Afterwards, groups of 3 values were formed in both treatments and compared using t-test. A difference at $p<0.05$ was considered as statistically significant.

RESULTS

Total phenolic and flavonoid content in the methanol extracts of N. rtanjensis leaves and flowers and their antioxidant activity

The results of the total phenolic and flavonoid content, and antioxidant activity determination in the methanol

extracts of *N. rtanjensis* leaves and flowers are presented in table 1. The content of total phenolic compounds, expressed as gallic acid equivalents per gram of dry extract, as well as flavonoid content, expressed in terms of rutin equivalents per mg of Ru/g of extract, were higher in methanolic leaf extract compared to in flowers extract. The investigated leaf and flower methanolic extracts of *N. rtanjensis* demonstrated very different radical-scavenging activities, respectively; IC_{50} of DPPH scavenging activity values were 112.59±0.95 and 507.70 ± 1.47µg/ml.

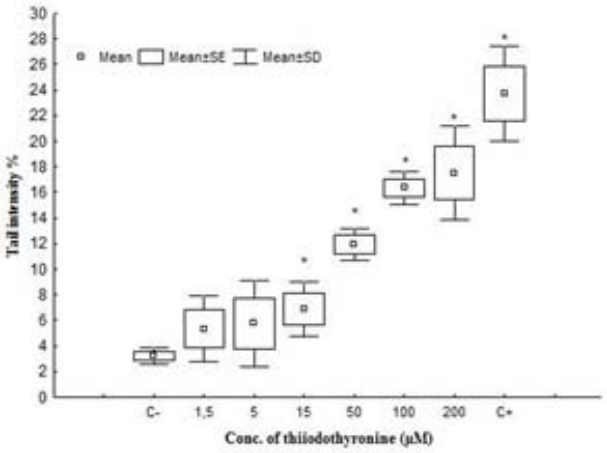

Fig. 2: Effects of T_3 in concentration range of 1.5-200µM on the DNA integrity of human lymphocytes; C^-, negative control; C^+, positive control (100µM hydrogen peroxide). *significance in comparison with negative control ($p<0.05$)

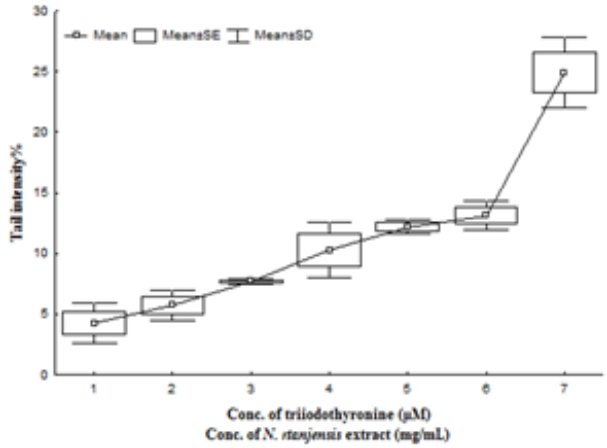

Fig. 3: Reduction in Ti by *N. rtanjensis* methanol extracts against triiodothyronine-treated human lymphocytes 1: C^-, negative control; 2: N.r. (1.0mg/ml); 3: N.r. (0.2mg/ml) + T_3 (100µM); 4: N.r. (0.5mg/ml) + T_3 (100µM); 5: N.r. (1.0mg/ml) + T_3 (100µM); 6: T_3 (100µM); and 7: C^+, positive control (100µM hydrogen peroxide).

Chemical characterization of phenolic complex of N. Rtanjensis leaf methanol extract

Ten phenolic compounds belonging to the groups of phenolic acids (chlorogenic acid, rosmarinic acid, caffeic acid, ferulic acid, and aesculin) and flavonoids (rutin, luteolin, apigenin, kaempferol and quercetin) have been quantified in *N. rtanjensis* leaf methanol extract. UHPLC/DAD/±HESI-MS and UHPLC/DAD/±MS/MS data of targeted phenolics quantified in the methanol extract of *N. rtanjensis* are presented in table 2. Hydroxycinnamic acids, chlorogenic and rosmarinic acid were the dominant phenolic compounds in the methanol extract, with concentrations of 13.32 and 10.07µg/ml of dry extract, respectively. Among flavonoids, flavanol rutin and flavone luteolin were the most abundant (table 2).

Antigenotoxic potential of methanol extract of N. rtanjensis in triiodothyronine-treated human lymphocytes

Prior to antigenotoxicity testing of *N. rtanjensis*, Tripan blue dye assay was used to test the viability of human lymphocytes of cells after 30 min of incubation with 100 and 500µM of T_3. Cell viability after the treatment at above-mentioned doses was mostly >90%. Secondly, the effects of a wide spectrum of T_3 concentrations (1.5 to 200µM) on DNA integrity in human lymphocytes was tested (fig. 2). Treatments with 1.5µM and 5µM of T_3 increased average Tail intensity values by 1.7- and 1.8-fold respectively, compared with the negative control, but without statistical significance. DNA damage in lymphocytes treated with 15µM of T_3 and all other higher concentrations gave rise to significant DNA damage in dose-dependent manner (fig. 2). Nevertheless, for the examination of the antigenotoxic potential of the methanol extract of *N. rtanjensis*, we choose T_3 at final concentration of 100µM, since the viability of cells was above >90% in Trypan blue dye exclusion assay and DNA damage was about four-fold greater compared to the negative control group (fig. 2). The results of the tested antigenotoxic property of *N. rtanjensis* leaf methanol extracts at three final concentrations (0.2mg/mL, 0.5 mg/ml, and 1.0mg/ml) are presented in table 3 and fig. 3. All plant concentrations reduced tail intensity in Comet assay in some extent. The smallest concentration of the plant extract reduced the tail intesity for 41,33% and showed significant antigenotoxic effects ($p<0.05$), while 1.0mg/ml and 0,5mg/ml reduced the tail intensity for 7,07% and 21,67% compared to the negative control, respectively ($p>0.05$). The highest concentrations of the plant extract did not cause significant DNA damage (table 4, fig. 3) and all concentrations of extract did not reduce cell viability in Trypan blue dye exclusion assay (table 4).

DISCUSSION

Increased mitochondrial oxygen consumption in hyperthyroidism leads to the presence of excessive amounts of ROS including H2O2 (Magsino *et al.,* 2000), which further leads to the development of oxidative stress and DNA strand breaks (Žukovec Topalović *et al.,* 2015).

Table 1: Total phenolic and flavonoid content in the methanol extracts of *N. rtanjensis* and their ability for free radical scavenging

Plant extracts	Total phenolic content[1]	Total flavonoid content[2]	Antioxidant activity[3]
Leaf	62.73 ± 1.80	83.30 ± 1.40	112.59 ± 0.95
Flower	25.63 ± 1.12	21.98 ± 1.10	507.70 ± 1.47

[1]Total phenolic content expressed as gallic acid equivalent (mg GA/g of extract); [2]Total flavonoid content expressed as rutin equivalent (mg Ru/g of extract);[3]Antioxidant activity expressed as IC_{50} values of DPPH scavenging activity of *N. rtanjensis* extracts (µg/mL);*Each value in the table was obtained by calculating the average of three analysis ± standard deviation.

In patients with Graves hyperthyroidism there is evidence of an imbalanced prooxidant-antioxidant status (Rybus-Kalinowska *et al.,* 2008) and increased oxidative DNA damage (Tsai *et al.,* 2009). Oxidative stress is also considered as the key mechanism of genotoxic effects of hormones with phenolic groups, which easily undergo redox cycling that leads to the generation of ROS (Djelic *et al*., 2007). Increased thyroid hormone levels can also indirectly act as genotoxic agents, possibly due to the depression of the endogenous enzymatic antioxidant defense (Villanueva *et al.,* 2013).

In previous studies it was observed that T_3 hormone can cause oxidative DNA damage in isolated human lymphocytes (Djelic and Anderson, 2003) and sperm (Dobrzyńska *et al*., 2004) at concentrations higher than 10 µM, which is in accordance to our results (fig. 1). Evaluation of a higher T_3 concentrations genotoxicity than generally recorded in humans is justified due to difficulties in extrapolating longer periods of exposure in laboratory *in vitro* conditions, as in previous studies (Cemeli and Anderson, 2011). Bearing in mind that T_3 can induce clear oxidative damage on DNA of human lymphocytes, we investigated possible desmutagenic effects of three final concentrations of *N. rtanjensis* (0.2 mg/ml, 0.5mg/mL, and 1.0mg/ml) in ameliorating DNA damage caused by 100 µM of T_3.

The findings in our study shows that methanolic extract of *N. rtanjensis* can protect DNA of human lymphocytes from oxidative damage induced by the T_3 hormone. All three concentrations showed protective effects in some extent, but the significant decrease of DNA damage was observed only with the treatment using 0.2mg/ml of the examined extract (fig. 2). The phenomenon when lower dosage of antimutagen exhibits the highest antigenotoxic activity, and the highest concentration of shows antimutagen less or no activity, has been already described as a hormetic-like effect (Moretti *et al.,* 2013). Like in our study, the antigenotoxic, hormetic-like effect of plant extract was also observed in study by Žukovec Topalović *et al.* (2015), where the commercial dry olive leaf extract showed the most significant efficacy in decreasing DNA breakage of human lymphocytes induced by the T_4 hormone when used in lower dosage. Hormesis is a biphasic dose response to an environmental agent characterized by low dose stimulation or beneficial effect, whereas higher doses sometimes cause inhibitory or toxic effects (Mattson, 2008). However, the 1.0 mg/ml of *N. rtanjensis* extract did not show genotoxic effects in our study conditions. Moreover, all concentration of *N. rtanjensis* extract did not interfere human lymphocytes viability in the Trypan blue exclusion test (table 4).

Although previous results on *Nepeta* spp. antigenotoxic activity are very limited, the genus property to reduce DPPH radicals was previously reported (Yazici *et al.,* 2012). Our results are in accordance with other studies where positive correlation between antioxidant activity and total phenol content in the plant extracts was observed (Yazici *et al.,* 2012). The solid antioxidant potential of the *N. rtanjensis* methanolic extract is related to its phytochemical profile and confirming a comparison of obtained values for leaf extract (112.59±0.95µg/ml) with the values for the antioxidant activity of chlorogenic acid (11.65±0.52µg/ml) and rutin (9.28±0.27µg/ml) determined by the same method (Takao *et al.,* 1994). The antigenotoxic effects of the *N. rtanjensis* leaf methanolic extract can be attributed to the presence of phenolic and flavonoid compounds (table 1). The total phenolic and flavonoid concentrations are not necessary indicators of the antioxidant activity of some extract, the synergetic and antagonistic interaction of single components need to be considered (Stanković *et al.,* 2010). The most abundant phenolic compounds determined by UHPLC/-HESI-MS/MS in *N. rtanjensis* methanol extract were chlorogenic acid, rosmarinic acid, and rutin (table 2). Synergetic antimutagenic properties in the Comet assay of chlorogenic acid, rosmarinic acid and rutin have been reported for leaf ethanolic extract of *Melissa officinalis* (Kamdem *et al.,* 2013). Single chlorogenic acid is known as an excellent natural scavenger and antigenotoxic agent due to the fact that the one-electron oxidation product of chlorogenic acid formed by the reaction with ROS is rapidly broken down to further products that cannot generate any free radicals (Shibata *et al.,* 1999). The ability of rosmarinic acid to act as an antioxidant is linked with penetration through the cell membrane without interfering in its structure and permeability, while decreasing lipid per oxidation (Fadel *et al.,* 2011).

Table 2: UHPLC/D AD/±HESI-MS and UHPLC/DAD/±HESI-MS/MS data of targeted phenolics quantified in methanol extract *Nepeta rtanjensis*. Relative intensities of the main diagnostic MS^2 fragments of each targeted compound are presented. Quantitative data are presented as means ± se, and expressed as μg/mg dry extract.

Phenolic acids		-HESI-MS		-HESI-MS/MS	Quantification data
Peak No	Assignment	Rt (min)	$[M-H]^-$ [m/z]	MS^2 fragments [m/z(relative intensity in %)]	Concentration [μg/mg dry extract]
1	3-O-Caffeoylquinic acid[S,R]	2.32	353	191 (100), 179 (<5), 161 (<5), 136 (5), 127 (10)	13.3180 ± 0.0554
2	Caffeic acid[S,R]	2.57	179	135 (100), 134 (85), 133 (5), 117 (10), 109 (10), 107 (5), 91 (<5), 89 (20)	0.1477 ± 0.0006
3	Ferulic acid[S,R]	3.70	193	178 (70), 149 (100), 134 (40)	0.0566 ± 0.0002
4	Rutin[S,R]	3.88	609	301 (30), 300 (100), 255 (5), 178 (<5), 150 (<5)	2.7979 ± 0.0116
5	Rosmarinic acid[S,R]	4.77	359	197 (5), 179 (20), 161 (100), 135 (40), 133 (50), 123 (15)	10.0661 ± 0.0419
6	Luteolin[S,R]	5.39	285	217 (<5), 199 (5), 175 (5), 151 (10), 133 (100), 121 (<5), 107 (8)	0.2094 ± 0.0009
7	Quercetin[S,R]	5.39	301	272 (15), 211 (20), 179 (20), 151 (80), 121 (100), 107 (90), 83 (15)	0.0002 ± 0.0001
8	Apigenin[S,R]	5.80	269	225 (5), 179 (<5), 151 (20), 149 (10), 121 (5), 117 (100), 107 (10)	0.0905 ± 0,0001
9	Kaempferol[S,R]	5.92	285	268 (10), 255 (15), 239 (20), 211 (10), 187 (25), 157 (10), 143 (30), 93 (10)	0.0148 ± 0,0001

Masses used in SRM (selected reaction monitoring) experiments for the quantification of compounds are presented in bold. Abbreviations: S-confirmed by standard; R-confirmed by references; HESI- heated electrospray ionisation; MS-mass spectrometry; Rt-retention time; $[M-H]^-$ - mass in negative ion mode; m/z-mass-to-charge ratio

Table 3: Protective effect of *N. rtanjensis* methanol extract against T_3 hormone induced DNA damage

Treatments	Concentrations	Tain intesity (mean values ± SD)
C^{-*}	untreated	4.25±1.63
T_3/N.r.	100 µM/0.2 mg/ml	7.70 ± 0.19[a]
T_3/N.r.	100 µM/0.5 mg/ml	10.28 ± 2.27*
T_3/N.r.	100 µM/1.0 mg/ml	12.19 ± 0.57*
T_3[a]	100 µM	13.12 ± 1.22*
C^+	100 µM	24.91 ± 2.91*[a]

C^- : negative control; T_3: triiodothyronine; N.r: *Nepeta rtanjensis* methanol extract; C^+: positive control (hydrogen peroxide); * significance in comparison with the negative control ($p<0.05$), [a] significance in comparison with the T_3 group.

Table 4: Viability of human lymphocytes treated with different methanol extracts of *Nepeta rtanjensis* and non-mutagenic effects of the highest concentration.

Type of test	Treatments	Concentrations	Tail intesity (mean values ± SD) /Viability of cells (%)
Comet Assay	C^-	untreated	4.25 ± 1.63
	N.r.	1.0 mg/ml	5.75 ± 1.26
	T_3	100 µM	13.12 ± 1.22*
	C^+	100 µM	24.91 ± 2.91*
Trypan Blue	N.r.	0.2 mg/ml	≥90
	N.r.	0.5 mg/ml	≥90
	N.r.	1.0 mg/ml	≥90

C^-: negative control; T_3: triiodothyronine; N.r.: *Nepeta rtanjensis* methanol extract; C^+: Positive control (hydrogen peroxide); *significance in comparison with C- ($p<0.05$).

CONCLUSION

Antioxidants could be a new therapeutic tool to improve the clinical manifestation of Graves disease (Guerra *et al.,* 2001). Considering that *N. rtanjensis* methanol extract decreased DNA damage of human lymphocytes induced by T_3-generated oxidative stress, the plant extract can be placed in a group of desmutagens with antioxidant activity. Desmutagens are compounds that interact directly with mutagens, or their precursors, in order to deactivate them (Kada *et al*., 1982). Antioxidant activity of *N. rtanjensis* as well as its antigenotoxic, can be attributed to determined polyphenol complex in methanolic extract. To the best of our knowledge, this research also provided the first information about human lymphocytes DNA damage-reducing ability and non-genotoxic behavior of *Nepeta* species. In general, this study supports the idea (Misic *et al.,* 2005) of the protection and/or reintroduction of rare plants, through scientific exploration of possible commercial applications. Further investigation of *N. rtanjensis* methanol extract in different genotoxicological test systems need to be conducted in order to better understand the underlying chemopreventive effects against oxidative stress induced by ROS-provoking genotoxic agents, such as high concentrations of thyroid hormones.

ACKNOWLEDGEMENT

This research was supported by the Serbian Ministry of Education and Science (Grant Numbers III 46002 and OI 173034). Authors thank Mr. Nick Brierley, Professor at Brierley & Co Language Center, Belgrade, for review and correction of English style and grammar.

REFERENCES

Andican G, Gelişgen R, Civelek S, Seven A, Seymen O, Altug T, Yigit G and Burcak G (2004). Oxidative damage to nuclear DNA in hyperthyroid rat liver: inability of vitamin C to prevent the damage. *J. Toxicol. Environ. Health*, **67**(5): 413-420.

Azqueta A, Shaposhnikov S and Collins AR (2009). Detection of oxidized DNA using DNA repair enzymes. In: Anderson D and Dhawan A editors. The Comet Assay in Toxicology, Royal Society of Chemistry, London, U.K., pp.57-78.

Cemeli E and Anderson D (2011). Mechanistic investigation of ROS-induced DNA damage by oestrogenic compounds in lymphocytes and sperm using the comet assay. *Int. J. Mol. Sci*., **12**(5): 2783-2796.

Cheng SY, Leonard JL and Davis PJ (2010). Molecular aspects of thyroid hormone actions. *Endocr. Rev*., **31**(2): 139-170.

Demet MM, Ozmen B, Deveci A, Boyvada S, Adıgüzel, H and Aydemir O (2011). Depression and anxiety in hyperthyroidism. *Arch. Med. Res.*, **33**(6): 552-556.

Dickinson BC and Chang CJ (2011). Chemistry and biology of reactive oxygen species in signaling or stress responses. *Nat. Chem. Biol.*, **7**(8): 504-511.

Diklic N (1999). *Nepeta rtanjensis* Diklić & Milojević. In: Stevanovic V editor. The Red Data Book of Flora of Serbia 1. Extinct and Critically Endagered Taxa, Beograd, Serbia, pp.153-155 (488).

Djelic N and Anderson D (2003). The effect of the antioxidant catalase on oestrogens, triiodothyronine, and noradrenaline in the Comet assay. *Teratog. Carcinog. Mutagen*., **23**(S2): 69-81.

Djelic N, Djelic D, Spremo-Potparevic B, Zivkovic L, Markovic B, Lozance O and Blagojevic M (2007). Lack of clastogenic effects of L-thyroxine in whole-blood cultured human lymphocytes. *Genet. Mol. Biol.*, **30**(4): 1144-1149.

Dobrzyńska MM, Baumgartner A and Anderson D (2004). Antioxidants modulate thyroid hormone and noradrenaline induced DNA damage in human sperm. *Mutagenesis*, **19**(4): 325-330.

Fadel O, El Kirat K and Morandat S (2011). The natural antioxidant rosmarinic acid spontaneously penetrates membranes to inhibit lipid per oxidation in situ. *B.B.A. Biomemb*., **12**: 2973-2980.

Grbic-Ljaljevic M, Stupar M, Vukojevic J and Grubisic D (2011). Inhibitory effect of essential oil from *Nepeta rtanjensis* on fungal spore germination. *Cent. Eur. J. Biol*., **6**(4): 583-586.

Guerra LN, Moiguer S, Karner M, de Molina MDCR, Sreider CM and Burdman JA (2001). Antioxidants in the treatment of Graves disease. *IUBMB life*, **51**(2): 105-109.

Ishaq H (2014). Anxiolytic effect of herbal medicine, Khamira Gaozaban Ambri Jadwar Ood Salib Wala (KGJ) in experimental rat models. *Pak. J. Pharm. Sci.*, **27**(2): 289-294.

Kada T, Inoue T and Namiki N (1982). Environmental desmutagens and antimutagens. *In*: Klekowski EJ editor. Environmental Mutagenesis and Plant Biology, Praeger, New York, NJ, pp.137-151.

Kamdem JP, Adeniran A, Boligon AA, Klimaczewski CV, Elekofehinti OO, Hassan W, Ibrahim M, Waczuk EP, Meinerza DF and Athaydec ML (2013). Antioxidant activity, genotoxicity and cytotoxicity evaluation of lemon balm (*Melissa officinalis* L.) ethanolic extract: its potential role in neuroprotection. *Ind. Crops. Prod.*, **51**: 26-34.

Kumarasamy Y, Byres M, Cox PJ, Jasapars M, Nahar L and Sarker SD (2007). Screening seeds of some Scottish plants for free-radical scavenging activity. *Phytother. Re*s., **21**: 615-621.

Magsino CH, Hamouda W, Ghanim H, Browne R, Aljada A and Dandona P (2000). Effect of triiodothyronine on reactive oxygen species generation by leukocytes, indices of oxidative damage, and antioxidant reserve. *Metabolism*, **49**(6): 799-803.

Mattson MP (2008). Hormesis defined. *Ageing Res. Rev*., **7**(1): 1-7.

Messarah M, Boumendjel A, Chouabia A, Klibet F, Abdennour C, Boulakoud MS and El Feki A (2010). Influence of thyroid dysfunction on liver lipid peroxidation and antioxidant status in experimental rats. *Exp. Toxicol. Pathol*., **62**(3): 301-310.

Misic D, Ghalawenji NA, Grubisic DV and Konjevic RM (2005). Micropropagation and reintroduction of *Nepeta rtanjensis*, an endemic and critically endangered perennial of Serbia. *Phyton.*, **45**(1): 9-20.

Mišić D, Šiler B, Gašić U, Avramov S, Živković S, Nestorović-Živković J, Šlutinović M and Tešić Ž (2015). Simultaneous UHPLC/DAD/(+/−) HESI–MS/MS Analysis of Phenolic Acids and Nepetalactones in Methanol Extracts of *Nepeta* Species: A Possible Application in Chemotaxonomic Studies. *Phytochem. Anal*., **26**(1): 72-85.

Moretti M, Cossignani L, Messina F, Dominici L, Villarini M, Curini M and Marcotullio MC (2013). Antigenotoxic effect, composition and antioxidant activity of *Dendrobium speciosum*. *Food Chem*., **140**(4): 660-665.

Mullur R, Liu YY and Brent GA (2014). Thyroid hormone regulation of metabolism. *Physiol Rev*., **94**(2): 355-382.

Nestorović-Živković J (2013). Antioxidative, antimicrobial and allelopathic effects of three endemic *Nepeta* species (Lamiaceae), Ph.D. theses. University of Belgrade, Belgrade, 27.5.

Official Gazette of the Republic of Serbia No 36/09 and No 88/2010, Law on Nature Protection.

Quettier DC, Gressier B, Vasseur J, Dine T, Brunet C, Luyckx MC, Cayin JC, Bailleul F and Trotin F (2000). Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum* Moench) hulls and flour. *J. Ethnopharmacol*., **72**: 35-42.

Rabbani M, Sajjadi SE and Mohammadi A (2008). Evaluation of the anxiolytic effect of *Nepeta persica* Boiss in mice. *Evid. Based Complement. Alternat. Med.,* **5**(2): 181-186.

Rigano D, Arnold NA, Conforti F, Menichini F, Formisano C, Piozzi F and Senatore F (2011). Characterisation of the essential oil of *Nepeta glomerata* Montbret et Aucher ex Bentham from Lebanon and its biological activities. *Nat Prod Res*., **25**(6): 614-626.

Rybus-Kalinowska B, Zwirska-Korczala K, Kalinowski M, Kukla M, Bikner E and Jochem J (2008). Activity of antioxidative enzymes and concentration of malondialdehyde as oxidative status markers in women with newly diagnosed Graves-Basedow disease and after thiamazole therapy leading to euthyroidism. *Pol. Arch. Med. Wewn*., **118**: 420-425.

Subudhi U, Das K, Paital B, Bhanja S and Chainy GBN (2008). Alleviation of enhanced oxidative stress and oxygen consumption of L-thyroxine induced hyperthyroid rat liver mitochondria by vitamin E and curcumin. *Chem. Biol. Interact*., **173**(2): 105-114.

Sarahroodi S, Jafari-Najafi R, Nasri S, Rohampour K, Maleki-Jamshid A and Esmaeili S (2012). Effects of

Nepeta menthoides aqueous extract on retention and retrieval of memory in mice. *Pak. J. Biol. Sci.*, **15**(22): 1085-1089.

Smaga I, Niedzielska E, Gawlik M, Moniczewski A, Krzek J, Przegaliński E, Pera J and Filip M (2015). Oxidative stress as an etiological factor and a potential treatment target of psychiatric disorders. Part 2. Depression, anxiety, schizophrenia and autism. *Pharmacol. Rep.*, **67**(3): 569-580.

Singh NP, McCoy T, Tice R and Schneider L (1988). A simple technique for quantification of low levels of DNA damage in individual cells. *Exp. Cell. Res*., **175**: 184-191.

Singleton VL, Orthofer R and Lamuela RRM (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol*., **299**: 152-178.

Shibata H, Sakamoto Y, Oka M and Kono Y (1999). Natural antioxidant, chlorogenic acid, protects against DNA breakage caused by monochloramine. *Biosci. Biotechnol, Biochem.*, **63**(7): 1295-1297.

Stanković MS, Topuzović M, Solujić S and Mihailović V (2010). Antioxidant activity and concentration of phenols and flavonoids in the whole plant and plant parts of *Teucrium chamaedrys* L. var. *glanduliferum* Haussk. *J. Med. Plant. Res*., **4**: 2092-2098.

Takao T, Watanabe N, Yagi I and Sakata K (1994). A simple screening method for antioxidant and isolation of several antioxidants produced by marine bacteria from fish and shellfish. *Biosci. Biotechnol. Biochem*., **58**: 1780-1783.

Tice RR, Andrews PW, Hirai O and Singh NP (1991). The single cell gel (SCG) assay: An electrophoretic technique for the detection of DNA damage in individual cells. *Adv. Exp. Med. Biol*., **283**: 157-164.

Tsai CC, Cheng CY, Liu CY, Kao SC, Kau HC, Hsu WM and Wei YH (2009). Oxidative stress in patients with Graves' ophthalmopathy: Relationship between oxidative DNA damage and clinical evolution. *Eye* **23**(8): 1725-1730.

Villanueva I, Alva-Sancez C and Pacheco-Rosado J (2013). The role of thyroid hormons as inductors of oxidative stress and neurodegeneration. *Oxid. med. cell longev*., p.15.

Vukovic-Gacic B, Nikcevic S, Beric-Bjedov T, Knezevic-Vukcevic J and Simic D (2006). Antimutagenic effect of essential oil of sage (*Salvia officinalis* L.) and its monoterpenes against UV-induced mutations in *Escherichia coli* and *Saccharomyces cerevisiae*. *Food Chem. Toxicol.*, **44**(10): 1730-1738.

Yazici SO, Ozmen I, Celkoglu U, Oluzcel H and Genc H (2012). *In vitro* antioxidant activities of extracts from some *Nepeta* species. *Int. J. Health Nutr.*, **3**(1): 8-12.

Zukovec Topalovic D, Zivkovic L, Cabarkapa A, Djelic N, Bajic, V, Dekanski D and Spremo-Potparevic B (2015). Dry olive leaf extract counteracts L-thyroxine-induced genotoxicity in human peripheral blood leukocytes *in vitro*. *Oxid. Med. Cell Longev*., Article ID 762192 8.