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Differences in bioactivity of three endemic *Nepeta* species arising from main terpenoid and phenolic constituents

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Abstract: Methanol extracts of three endemic *Nepeta* species were analyzed for their main secondary metabolites, terpenes and phenolics, and further investigated for antioxidant capacity and embryonic toxicity in zebrafish. UHPLC/DAD/(±) HESI-MS/MS analysis showed that the dominant compound in *N. rtanjensis* was *trans,cis*-nepetalactone, the *cis,trans* isomer of this monoterpene lactone was dominant in *N. sibirica*, while nepetalactone was detected only in traces in *N. nervosa*. In all investigated species, rosmarinic acid was the dominant phenolic compound, while other identified phenolic acids (chlorogenic, neochlorogenic and caffeic) were present in considerably lower amounts. ABTS and DPPH assays showed that the methanol extracts of *N. rtanjensis*, *N. sibirica* and especially *N. nervosa* possessed strong antioxidant activities, with the FRAP assay revealing high ferric-reducing abilities for all three tested species. Such a strong antioxidant potential, especially as manifested in the DPPH and FRAP assays, can be attributed to phenolic acids, and in the first place to rosmarinic acid. Increased lethality of zebrafish embryos in any of the treatments was not observed, but several toxic effects on embryonic development were recorded, such as pericardial and yolk sac edema. As in other *Nepeta* species, the three studied endemic species possessed a great potential for food conservation or as medicinal supplements if applied in optimized concentrations; however, alternative sources of plant material (e.g. field cultivation) should be established bearing in mind their vulnerability in nature.

Key words: *Nepeta rtanjensis*; *Nepeta sibirica*; *Nepeta nervosa*; nepetalactone; phenolic acids

INTRODUCTION

Oxidative stress can lead to the development of disorders and diseases in humans. Scientists have become interested in natural sources that can provide active compounds to prevent or reduce oxidative stress-induced damage in cells [1]. The constantly produced free radicals can damage DNA, proteins and lipids, and the resulting cell damage can lead to accelerated ageing and many oxidative stress-related chronic conditions, including cancer, cardiovascular diseases, diabetes, obesity, joint diseases and Alzheimer's disease [2]. In recent years, there is growing interest in the use of natural compounds with antioxidant properties derived from aromatic herbs for improving human health. Previous studies showed that aromatic plants with high contents of phenolic compounds are an important source of natural antioxidants [3,4].

Numerous pharmacological effects have been attributed to *Nepeta* species, including antiinflammatory [5], analgesic [6], cytotoxic [7], antiviral [8] and antimicrobial activities [9]. Furthermore, phytotoxic activities, attractant effects on cats and aphids and repellent activity against a variety of insects and other pests were reported for *Nepeta* species [10-12].

The pharmacological activities of *Nepeta* species are usually ascribed to numerous secondary metabolites, in the first place iridoid monoterpenes – nepetalactones,

and phenolic compounds. Previous phytochemical studies on species belonging to the genus *Nepeta* revealed that monoterpenes, diterpenes, triterpenes and phenolics (flavonoids, phenolic acids and their derivatives) were the major secondary metabolites. Among monoterpenoids, the most frequently determined are nepetalactones [13], which appear as the main constituents in most species. Nepetalactone is actually a cyclopentanoid monoterpene with two fused rings, a cyclopentane and a lactone. There are eight stereoisomers, four diastereomers and their corresponding enantiomers. With some exceptions, the (7S)-diastereomers are found in natural sources [14]. Nepetalactone isomers show different biological activities [15].

N. rtanjensis, N. sibirica and *N. nervosa* have been previously phytochemically characterized according to their phenolic contents, the second most important group of *Nepeta*'s secondary metabolites [13]. Among the identified compounds, derivatives of hydroxybenzoic acids (syringic acid and its *O*-hexoside, protocatechuic acid, hydroxybenzoic acid *O*-hexoside and vanillic acid) and especially derivatives of hydroxycinnamic acids (caffeoylquinic acid, cinnamic acid and its derivatives, caffeic acid and its derivatives: caffeoylquinic acid, dicaffeoylquinic acid, rosmarinic acid, as well as ferulic acid) were abundant in the analyzed *Nepeta* species. Of the flavonoids, the following groups were determined: (1) flavones (luteolin, apigenin, chrysin, acacetin); (2) flavonols (quercetin, rutin, kaempferol, astragalin, methyl derivatives of both quercetin and kaempferol, galangin), and (3) flavanones (naringenin and pinocembrin). The contents of these compounds have been reported to vary between *N. rtanjensis, N. sibirica* and *N. nervosa*; however, rosmarinic and chlorogenic acids were the major phenolic compounds in methanol extracts [13].

Many *Nepeta* species were tested for antioxidant activity and it has been shown that phenolic compounds, in the first place phenolic acids, played an important role in the antioxidant assays [3,5,16]. Tepe et al. [3] have demonstrated a positive correlation between antioxidant activity and the amount of total phenolic compounds in *Nepeta flavida*.

The *Nepeta* species in this study included *N. rtanjensis* Diklić & Milojević, an endemic and critically endangered perennial in Serbia [17], *N. sibirica* L., which is endemic to central Asia, Mongolia and southern Siberia [18], and *N. nervosa* Royle ex Bentham, a plant species endemic to Kashmir [19]. To the best of our knowledge, these species have not previously been described for their antioxidant activities and zebrafish embryo toxicity. Because of the specific phytochemical composition, i.e. qualitative composition of nepetalactone isomers, they represent interesting objects for investigating the biological activities of their extracts. As previously reported, *N. rtanjensis* contains much higher amounts of *trans,cis*-nepetalactone than *cis,trans*-nepetalactone [13,17], while *N. sibirica* possesses high amounts of *cis,trans*-nepetalactone [13,18]. *N. nervosa* contains nepetalactones only and in trace amounts [13,20]. Considering that these species are endemic and extremely endangered, as in the case of *N. rtanjensis*, plant material for the experiments was obtained by micropropagation.

MATERIALS AND METHODS

Plant material and *in vitro* culture establishment

Seeds of three *Nepeta* species – *N. rtanjensis*, *N. sibirica* and *N. nervosa*, were used to establish the *in vitro* cultures. Seeds of *N. sibirica* and *N. nervosa* were provided by Grugapark Essen (Germany), while those of *N. rtanjensis* were collected in August 2004 from greenhouse-grown plants. After 10 min of surface sterilization in a 20% solution of commercial bleach, the seeds were rinsed 5 times with sterile distilled water and transferred onto 9-cm Petri dishes, each containing 20 mL solid ½ MS [21] culture medium, supplemented with 20 g L^{-1} sucrose, 7 g L^{-1} agar (Torlak, Serbia) and 100 mg L^{-1} myo-inositol (Merck, Germany). Seedlings were transferred into 370-mL glass jars with transparent polycarbonate covers. Each jar contained 100 mL of ½ MS culture medium. Plants were grown in a growth chamber under long day conditions (16/8 h light/dark cycle), at 25±2°C. A photon flux rate of 32.5 μmol m^{-2} s^{-1} was provided to the plant cultures by white fluorescent tubes. Plant material for the analyses and assays was obtained by micropropagation, using nodal segments with one pair of leaves as explants. Following several cycles of subculturing and micropropagation, sufficient plant material was obtained for further analyses.

Preparation of plant methanol extracts

Shoots of four-week-old micropropagated plants were used for the analysis of secondary metabolites. Plant material was air-dried in a thin layer at room temperature until constant weight was attained. The plants were weighed (dry wt) and powdered in liquid nitrogen (LN) until further use. Each sample (0.2 g, dried and powdered) was extracted with 10 mL of methanol (AppliChem GmbH, Germany) in an ultrasonic bath for 15 min. After centrifugation for 20 min at 10000 *g*, the supernatants were filtered through 0.2-μm cellulose filters (Agilent Technologies, Santa Clara, CA) and stored at 4°C until use.

UHPLC/DAD/(+/-)HESI-MS/MS analysis

Methanol extracts of *N. rtanjensis*, *N. sibirica* and *N. nervosa* were subjected to UHPLC/DAD/(+/-) HESI-MS/MS analysis using a Dionex Ultimate 3000 UHPLC system (ThermoFisher Scientific, Bremen, Germany) equipped with a diode array detector (DAD) connected to a TSQ Quantum Access Max triple-quadrupole mass spectrometer (ThermoFisher Scientific, Basel, Switzerland). Separation of components in samples was performed on a Hypersil GOLD C18 column (50 × 2.1 mm) with 1.9 μm particle size (ThermoFisher Scinetific, USA), thermostated at 30°C. Elution and mass spectrometry were performed as described in Mišić et al. [13]. The flow rate was set to 0.4 mL min^{-1} and the detection wavelengths to 225, 260 and 320 nm. The injection volume was 2 μL. All analyses were performed in triplicate.

A TSQ Quantum Access Max triple-quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI) source was used as previously described [13], with slight modifications. The vaporizer temperature was kept at 350°C, and the ion source settings were as follows: spray voltage was 4000 V, sheet gas (N_2) pressure was 50 AU, ion sweep gas pressure was 0 AU and the auxiliary gas (N_2) pressure was 20 AU, capillary temperature was 270°C, skimmer offset was 0 V. The mass spectrometry data were initially acquired in both the positive and negative modes, in the m/z range from 100-1000. Full scanning (FS) and product ion scanning (PIS) mass spectrometric scanning modes were conducted for the qualitative analysis of targeted compounds in samples. Collision-induced fragmentation experiments were performed using argon as the collision gas, with the collision energy was set to 30 eV for both phenolic acids and nepetalactones. The selected reaction monitoring (SRM) experiment for quantitative analysis was performed using two MS2 fragments for each compound, which were previously defined as dominant in the PIS experiments.

Phenolic acids were identified by direct comparisons with commercial standards and literature data. Standards of caffeic acid, chlorogenic acid and rosmarinic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Essential oils of *N. cataria*, containing 90% of *cis,trans*-nepetalactone and *N. rtanjensis* containing 72% of *trans,cis*-nepetalactone and 16% of *cis,trans*-nepetalactone were used as standards for nepetalactone diastereomer identification and quantification, as previously described [13]. Stock standard solutions of phenolics were prepared by dissolving 1 mg of a pure compound in 1 mL of methanol. The stock solution was further diluted with methanol to obtain a concentration of 100 μg mL^{-1}, and subsequent calibration levels were obtained by diluting the solution with methanol. Working standard solutions were prepared at 15 levels, in a concentration range from 100 μg mL^{-1} to 0.001 μg mL^{-1}. Regressions were calculated for each of the calibration curves and they all showed good linearity with correlation coefficients between $r=0.990$ and 0.999, $p<0.001$. The total amount of each compound in a sample was evaluated by calculating the peak areas and is expressed as μg 100 mg^{-1} dry wt.

The solvents for HPLC-MS analyses (acetonitrile and formic acid) were LC-MS grade, obtained from Fisher Scientific (Loughborough, UK). The methanol used for plant extraction (HPLC grade) was purchased from AppliChem (Cheshire, CT, USA). Ultrapure water was generated by deionization (Millipore, Billerica, USA).

Determination of total phenolics and total flavonoids

The total phenolics concentration (TPC) was measured by the Folin-Ciocalteu assay [22], while the total flavonoid concentration (TFC) of the samples was deter-

mined according to Karadeniz et al. [23], as previously described by Šiler et al. [24]. The TPC values were calculated from a standard calibration curve based on gallic acid (Sigma Aldrich, Germany), and expressed as mmol of gallic acid equivalents (GAE) per 1 g of dry wt (mmol GAE g^{-1} dry wt). The TFC in each extract was calculated from the standard curve based on rutin hydrate (Sigma Aldrich, Germany). The results are expressed as mmol of rutin hydrate equivalents (RE) per 1 g of dry wt (mmol RE g^{-1} dry wt).

Antioxidant activity

Preparation of *N. rtanjensis* and *N. cataria* essential oil and phenolic acid standard solutions

For antioxidative activity, the essential oil of *N. rtanjensis* (which contained 72% of *trans,cis*-nepetalactone and 16% of *cis,trans*-nepetalactone) and the essential oil of *N. cataria* (composed of 77% *cis,trans*-nepetalactone) were diluted in methanol (AppliChem, Cheshire, SAD) to obtain a final nepetalactone concentration of 4%. Preparation of essential oils and GC/MS analysis were described previously by Dmitrović et al. [11]. *N. rtanjensis* EO was isolated from air-dried aerial parts by hydrodistillation for 2 h in a Clevenger-type apparatus. The essential oil of *N. cataria* was a gift from Dr. M. Birkett (Biological Chemistry and Crop Protection Department, Rothamsted Research, Harpenden, UK), and is prepared by steam distillation using cyclohexane as the cosolvent. Working solutions of chlorogenic and rosmarinic acids were prepared by adjusting their concentrations to 1 mM.

ABTS radical scavenging activity

The ABTS radical cation decolorization assay was carried out as previously described by Re et al. [25], with some modifications [24]. $ABTS^{\bullet+}$ radical scavenging activity (%) was calculated using the formula:

$$(A_{control}\text{-}A_{sample})/A_{control}] \times 100,$$

where A_{sample} is the absorbance of the solution when the extract has been added, and $A_{control}$ is the absorbance of the $ABTS^{\bullet+}$ solution without added extract. A methanol solution of Trolox was used for construction of the calibration curve and the results are expressed as reducing activity in mmol Trolox equivalents per gram of dry sample wt (mmol TE per g^{-1} dry wt). All analyses were performed in triplicate.

DPPH radical scavenging activity

DPPH assay was performed as previously described by Brand-Williams et al. [26], with slight modifications reported earlier [24]. The radical scavenging activity was calculated as the percentage of $DPPH^{\bullet}$ discoloration using the equation:

$$DPPH^{\bullet} \text{ radical scavenging } (\%) = [(A_{control}\text{-}A_{sample})/ A_{control}] \times 100,$$

where A_{sample} represents the absorption of the solution when extract has been added, and $A_{control}$ is the absorbance of the $DPPH^{\bullet}$ solution without the addition of extract. Trolox was used as the reference compound. All the analyses were run in triplicate and mean values were calculated. Results are expressed as mmol TE per g^{-1}.

Ferric reducing/antioxidant power (FRAP)

The ferric reducing/antioxidant power (FRAP) assay was carried out according to the procedure described by Benzie and Strain [27], with some modifications described by Šiler et al. [24]. The methanol solution of Trolox was used for construction of the calibration curve and the results are expressed as Trolox equivalents antioxidant capacity (mmol TE per g^{-1} dry wt).

Zebrafish husbandry, eggs collection, and embryo exposure

Adult, wild-type Tubingen zebrafish (*Danio rerio*) were used in all experiments and were maintained in fish medium (2 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.7 mM $NaHCO_3$, 0.07 mM KCl). Fish were maintained at a temperature of 26±1°C under continuous water aeration and filtering, and under artificial light with a 12 h dark/light cycle. Female and male individuals were always kept apart and regularly fed twice a day with commercial dry-flake food (TetraMin™ flakes; Tetra Melle, Germany) supplemented with *Artemia nauplii*. The day before spawning, males and females at a ratio of 2:1 were placed in a breeding tank before the onset

Table 1. UHPLC/(-)HESI-MS/MS and UHPLC/(+)HESI-MS/MS data of targeted phenolic acids and monoterpenes quantified in methanol extracts of *N. rtanjensis*, *N. sibirica* and *N. nervosa*. Relative intensities of the main diagnostic MS^2 fragments are presented.

		MS data			DAD data	
Peak No.	**Assignment**	**Rt (min)**	**[M-H]⁻[*m/z***	**MS^2 fragments (m/z (relative abundance))**	**Rt (min)**	**λ_{max} (nm)**
1	**3-*O*-caffeoylquinic acid**[s]	2.39	353	**191** (100), 173 (5), 135 (10), **127** (<5)	2.33	230, 330
2	**caffeic acid**[s]	2.58	179	135 (100), **134 (85)**, 132 (5), **117 (10)**, 109 (10), 106 (<5), 91 (5)	2.52	230, 330
3	**5-O-caffeoylquinic acid**[l]	3.01	353	**191** (100), 173 (5), 161 (<5), 135 (10), **127** (<5)	2.95	230, 330
4	**unidentified caffeic acid derivative**	3.63	521	359 (10), 341 (15), 197 (60), 179 (<5), **161** (<5), 153 (100)	3.57	330
5	**rosmarinic acid**[s]	4.83	359	197 (5), 179 (20), **161** (100), 135 (40), **133** (50), 123 (15)	4.76	330
6	***cis,trans*-nepetalactone**[s]	7.07	167	**111** (5), 105 (10), 95 (5), 93 (15), 91 (55), 81 (25), 79 (55), **77** (100), 69 (5), 67 (15), 65 (10)	6.85	225
7	***trans,cis*-nepetalactone**[s]	7.20	167	**111** (60), 105 (10), 95 (10), 93 (35), 91 (50), 83(15), 81 (15), 79 (50), **77** (100), 69 (5), 67 (10), 65 (20)	7.11	225

[a] – numbers of peaks according to Fig. 1. [S] – confirmed by standard; [l] – confirmed by references. Masses used in the selected reaction monitoring experiments for the quantification of compounds are presented in bold.

of darkness and left undisturbed overnight. At the onset of light, the separators were removed from the breeding tanks. The eggs were collected after 30 min, rinsed twice from debris using fresh fish medium and transferred into Petri dishes with the fish medium.

Within 1.5 h post-fertilization (hpf), fertilized eggs were selected under a binocular stereomicroscope (PXS-VI, Optica) and transferred into 24-well plates, 10 embryos per well, in a fish medium volume of 2 mL. The extracts were diluted 100x and 1000x in fish medium and added to the wells. The fish medium, 1% methanol and 0.1% methanol were used as controls. The embryos were then incubated at 28±0.5°C. Each experiment was performed in duplicate or triplicate and repeated three times from independent breedings.

Fish embryo toxicity assay was performed if fertilization rates were ≥90%. An assay was considered valid if the overall survival of embryos in negative controls was ≥90% until hatching. The apical endpoints for the assessment of embryotoxicity (mortality) and teratogenicity were evaluated at 24, 48, 72 and 96 hpf using an inverted microscope (CKX41; Olympus, Tokyo, Japan).

All embryos were inspected for morphological characteristics at different developmental stages as described by Kimmel [28]. Lethal and teratogenic effects were recorded according to OECD (236) guidelines for testing of chemicals. Teratogenic effects were recorded if the fingerprint endpoint was observed in at least 50% of all embryos showing teratogenic effects in the test group and if a concentration-response was present.

RESULTS

Metabolic profiling of *N. rtanjensis*, *N. sibirica* and *N. nervosa* methanol extracts

Full scan UHPLC/HESI-MS spectra were acquired in both positive and negative ion modes, showing a series of peaks between *m/z* 100 and 1000. The UHPLC/(-)HESI-MS total ion chromatograms of *N. rtanjensis*, *N. sibirica* and *N. nervosa* methanol extracts had several major peaks. The compounds corresponding to the peaks were identified based on their retention times and by comparison of their MS and MS^2 spectra (Table 1) with commercial standards. Chlorogenic acid (peak 1), showing *m/z* $[M-H]^-$ at 353, was identified at Rt=2.39 min, while caffeic acid (2), with *m/z* $[M-H]^-$ at 179 was recorded at Rt=2.58 min. Peaks at Rt=3.01 and 4.83 min, with *m/z* $[M-H]^-$ at 353 and *m/z* $[M-H]^-$ at 359, respectively, were assigned to neochlorogenic acid (3) and (5) rosmarinic acid, respectively. Peak 4 at Rt=3.63 min, showing *m/z* $[M-H]^-$ at 512, was referred to as an unidentified caffeic acid derivative, as previously described [13]. Identification of phe-

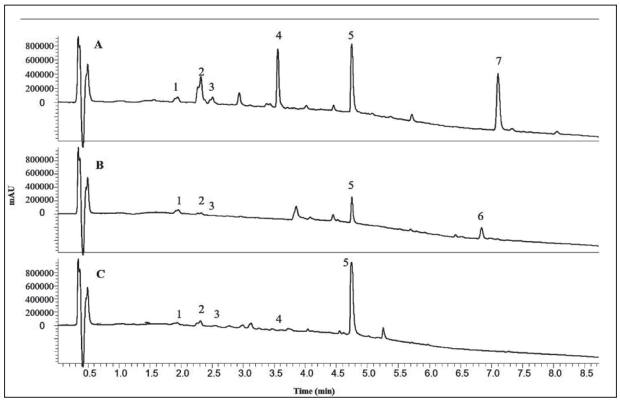

Fig. 1. UHPLC-DAD chromatograms (λ_{max}=225nm) of *N. rtanjensis* (**A**), *N. sibirica* (**B**) and *N. nervosa* (**C**) methanol extracts. Identified compounds are labelled with the following numbers: (1) 3-*O*-caffeoylquinic acid; (2) caffeic acid; (3) 5-*O*-caffeoylquinic acid; (4) unidentified caffeic-acid derivative; (5) rosmarinic acid; (6) *cis, trans*-nepetalactone; (7) *trans, cis*-nepetalactone.

nolic acids was also confirmed by their UHPLC-DAD spectra. UHPLC-DAD chromatograms of *N. rtanjensis*, *N. sibirica* and *N. nervosa* methanol extracts are presented in Fig. 1, while the retention times and λ_{max} values of the corresponding compounds are shown in Table 1.

Targeted phenolic acids (chlorogenic, caffeic, and rosmarinic acid) were quantified in an SRM experiment using two reference MS^2 fragments. Deprotonated caffeic acid ($C_9H_8O_4$) with *m/z* $[M-H]^-$ of 179 was characterized by a typical fragmentation pattern showing the loss of a CO_2 from the carboxylic acid group, providing anion $[M-H-CHO_2]^-$ at *m/z* 134. The other reference MS^2 fragment used for the quantification of caffeic acid was *m/z* $[M-H-CO_2-H_2O]^-$ at 117

Table 2. Concentration [*μg* 100 *mg*$^{-1}$ fresh weight] of targeted compounds (3 phenolic acids and 2 nepetalactones) in methanol extracts of *N. rtanjensis*, *N. sibirica* and *N. nervosa*, as revealed by UHPLC/(±)HESI-MS/MS analysis.

Peak No.	Name of compound	Concentration [*μg* 100 *mg*$^{-1}$ fresh weight]		
		N. rtanjensis	***N. sibirica***	***N. nervosa***
1	**chlorogenic acid**	228.506±7.891	7.357±1.055	51.752±2.746
2	**caffeic acid**	6.058±0.510	3.827±0.222	12.703±0.897
5	**rosmarinic acid**	296.727±2.454	136.980±6.445	391.980±6.304
6	***cis,trans*-nepetalactone**	/	1777.719±296.385	/
7	***trans,cis*-nepetalactone**	2657.233±291.493	/	/

Peak numbers correspond to the numbers indicated in Table 1 and in Fig. 1.

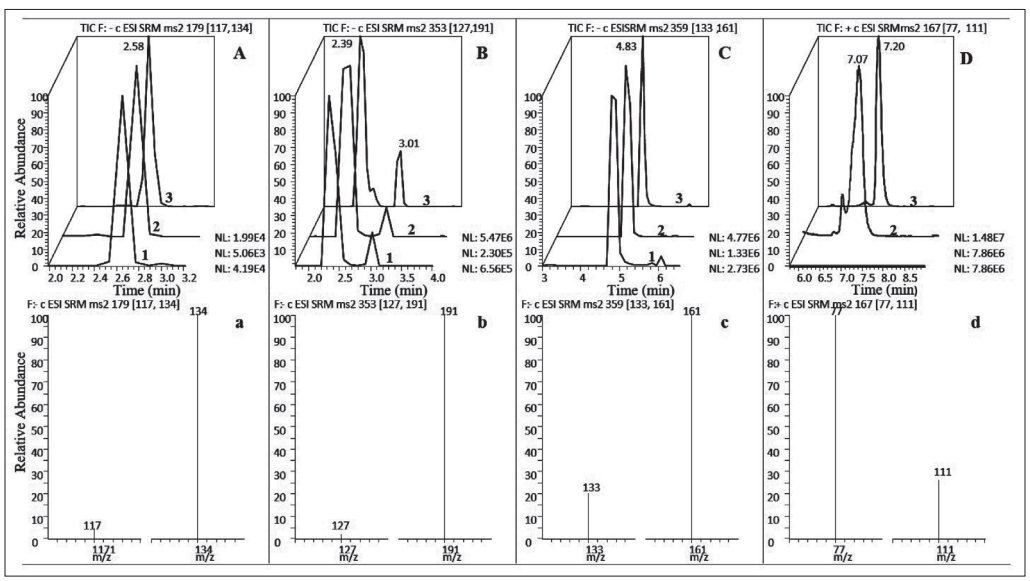

Fig. 2. UHPLC-SRM (Single Reaction Monitoring) chromatograms (capital letters, A-D) and corresponding MS^2 spectra (small letters, a-d) of the compounds quantified in the methanol extracts of *N. rtanjensis* (3), *N. sibirica* (2) and *N. nervosa* (1). (A and a) caffeic acid, (B and b) 3-*O*-caffeoylquinic acid, (C and c) rosmarinic acid, and (D and d) nepetalactone.

(Fig. 2a). Chlorogenic acid ($C_{16}H_{18}O_9$) was quantified in the methanol extracts of the three *Nepeta* species using the referent product ion *m/z* [M-H-$C_9H_6O_3$]$^-$ at 191, resulting from the loss of caffeoyl moiety. The minor MS^2 ion of chlorogenic acid with *m/z* [M-H-$C_9H_6O_3$-H_2O-CH_2O_2]$^-$ at 127 can arise from deprotonation and subsequent decarboxylation of the ion *m/z* [M-H-$C_9H_6O_3$-H_2O]$^-$ at 173, which is a product of dehydration of quinic acid (*m/z* 191) (Fig 2b). Rosmarinic acid ($C_{18}H_{16}O_8$), the ester of caffeic acid and 3,4-dihydroxyphenyl lactic acid, showed the major product ion at [$C_9H_7O_4$–H_2O]$^-$ at *m/z* 161, resulting from the loss of a water molecule from the caffeic part (*m/z* 179) of rosmarinic acid. Fragment m/z [$C_9H_7O_4$–CO_2-2H]$^-$ at 133 occurred after the loss of the CO_2 group from the caffeic part of the molecule and its deprotonation (Fig 2c).

Quantitative analysis of targeted phenolic acids in the methanol extracts showed that rosmarinic acid was dominant in all three species (Table 2). The maximum amount of rosmarinic acid was observed in *N. nervosa* (~392 μg 100 mg^{-1} FW), followed by *N. rtanjensis* (~297 μg 100 mg^{-1} FW), while in *N. sibirica* rosmarinic acid was present at only one third of the amount in *N. nervosa* (~137 μg 100 mg^{-1} FW). A significant amount of chlorogenic acid was detected in the methanol extract of *N. rtanjensis* (around 229 μg 100 mg^{-1} FW), while considerably lower amounts of this compound were detected in the methanol extract of *N. nervosa* (~52 μg 100 mg^{-1} FW) and especially of *N. sibirica* (~7 μg 100 mg^{-1} FW) (Table 2).

UHPLC/(+)HESI-MS total ion chromatogram of *N. rtanjensis* methanol extracts showed a clearly separated dominant peak at Rt=7.20 min (Table 1), which was identified as *trans,cis*-nepetalactone (7), with *m/z* $[M+H]^+$ at 167. The same analysis of the *N. sibirica* methanol extract revealed the presence of another nepetalactone stereoisomer, *cis,trans*-nepetalactone (6) at Rt=7.07 min, which also showed *m/z* $[M+H]^+$ at 167 (Table 2). Identification of nepetalactones was also confirmed by UHPLC-DAD analysis after comparison of their UV spectra with standards and literature data (Table 1, Fig. 1). UHPLC/DAD/(+)HESI-MS and UHPLC-DAD analysis of *N. nervosa* methanol extracts did not confirm the presence of nepetalactone.

UHPLC/(+)HESI-MS^2 spectra of *trans,cis*-nepetalactone and *cis,trans*-nepetalactone (Fig. 3) exhibited very similar fragmentation profiles. Both isomers were characterized by the presence of fragments *m/z* $[M+H-C_4H_8]^+$ at 111 and *m/z* $[M+H-C_4H_8-C_2H_4-6H]^+$ at 77. These two fragments were utilized in the SRM experiment for the quantification of nepetalactones (Fig. 3D). The methanol extract of *N. rtanjensis* was

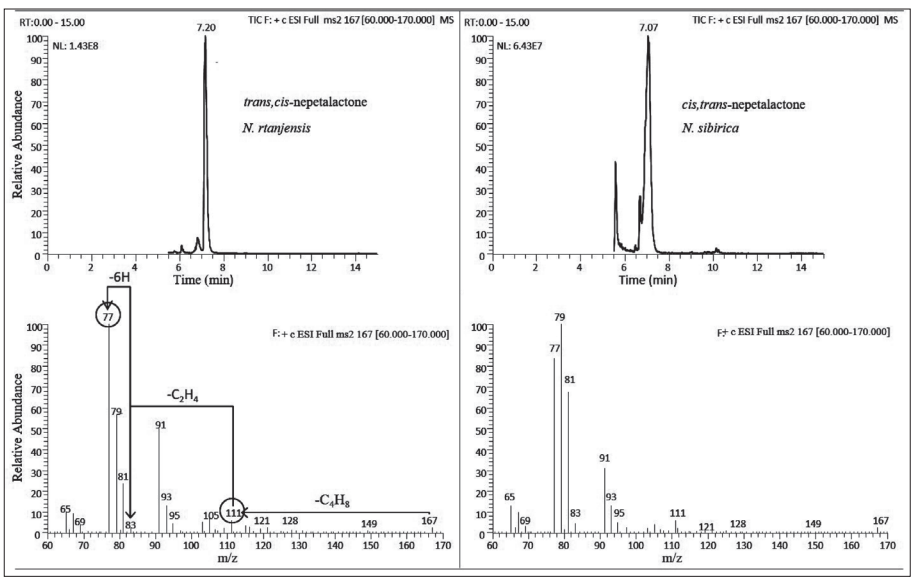

Fig. 3. UHPLC-PIS (Product Ion Scanning) chromatograms (upper row) and corresponding MS^2 spectra (lower row) of *trans, cis*-nepetalactone in the methanol extract of *N. rtanjensis*, and *cis, trans*-nepetalactone in the methanol extract of *N. sibirica*.

very rich in *trans,cis*-nepetalactone, and its concentration reached about 2.7 mg 100 mg^{-1} FW, which represents 2.7% FW (Table 2). *Cis,trans*-nepetalactone was abundant in the *N. sibirica* methanol extract (Table 2), with concentrations of ~1.7 mg 100 mg^{-1} FW (1.7%).

Total phenolic and total flavonoid contents in the methanol extracts of *N. rtanjensis*, *N. sibirica* and *N. nervosa*

Table 3 shows the total phenolic and total flavonoid contents in the methanol extracts of the three studied *Nepeta* species. The methanol extract of *N. rtanjensis* was characterized by a higher content of total phenolics (~18 mmol GAE g^{-1} dry wt) than *N. nervosa* (~14 mmol GAE g^{-1} dry wt) and *N. sibirica* (~13 mmol GAE g^{-1} dry wt). The content of total flavonoids followed the same trend. The highest amount of total flavonoids was recorded in the methanol extract of *N. rtanjensis* (around 1 mmol RE g^{-1} dry wt), while the extract of *N. nervosa* (~0.8 mmol RE g^{-1} dry wt) contained more total flavonoids than the extract of *N. sibirica* (~0.6 mmol RE g^{-1} dry wt).

Antioxidant activity of *N. rtanjensis*, *N. sibirica* and *N. nervosa* methanol extracts

Considering that antioxidants can act through different mechanisms, the antioxidant potential of *N. rtanjensis*, *N. sibirica* and *N. nervosa* was determined using three different assays: DPPH, ABTS and FRAP. Table 4 shows Trolox equivalent antioxidant capacity (TEAC) values of *N. rtanjensis*, *N. sibirica* and *N. nervosa* methanol extracts, and of the dominant reference chemicals present in the tested species: the essential oil of *N. rtanjensis* (containing 72% of *trans,cis*-nepetalactone and 16% of *cis,trans*-nepetalactone), the essential oil of *N. cataria* (containing 90% of *cis,trans*-nepetalactone), and standards of rosmarinic and chlorogenic acids.

The methanol extracts of all three studied *Nepeta* species exhibited scavenging activities against both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals and possessed a high capacity for the reduction of Fe^{3+} in the FRAP assay (Table 4). The highest neutralizing activity of $ABTS^{\bullet+}$ radicals was characteristic of the *N. nervosa* methanol extract (with a TEAC value of 1.217 mmol TE g^{-1} dry wt), while the methanol extract of *N. sibirica* displayed the lowest activity (0.706 mmol TE g^{-1} dry wt). The methanol extracts of *N. rtanjensis* and *N. nervosa* (0.835 and 0.870 mmol TE g^{-1} dry wt, respectively) showed higher $DPPH^{\bullet}$ radical scavenging activities than *N. sibirica* (0.768 mmol TE g^{-1} dry wt). In the FRAP assay, the methanol extract of *N. sibirica* showed the lowest capacity for reduction of Fe^{3+} (1.935 mmol TE g^{-1} dry wt), while no statistically significant difference between the methanol extracts of *N. rtanjensis* and *N. nervosa* was observed (TEAC values of 2.939 and 2.968 mmol TE g^{-1} dry wt, respectively). The tested essential oils showed similar $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical scavenging activities and no activity in the FRAP assay (Table 4). Among the tested phenolic acids, rosmarinic acid showed a higher neutralizing capacity of $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals (TEAC values were 1.695 and 40.326 mmol TE g^{-1} respectively) than chlorogenic acid (TEAC values were 0.823 and 23.311 mmol TE g^{-1}, respectively). The FRAP test showed that rosmarinic

Table 3. Total phenolic and total flavonoid contents in the methanol extracts of *N. rtanjensis*, *N. sibirica* and *N. nervosa* revealed spectrophotometrically.

	N. rtanjensis	***N. sibirica***	***N. nervosa***
TPC [mmol EGA g^{-1}]	17.59 ± 0.17^{b}	13.02 ± 0.12^{a}	13.94 ± 0.13^{a}
TFC [mmol RE g^{-1}]	1.02 ± 0.05^{c}	0.56 ± 0.02^{a}	0.78 ± 0.22^{b}

Table 4. $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical scavenging activity and FRAP capacity of *N. rtanjensis*, *N. sibirica* and *N. nervosa* methanol extracts and selected reference compounds. Values are presented as Trolox equivalent antioxidant capacity (TEAC) and expressed as mmol TE g^{-1} FW.

	TEAC [mmol TE g^{-1} FW]		
	$DPPH^{\bullet}$	**$ABTS^{\bullet+}$**	**FRAP**
N. rtanjensis	0.835 ± 0.008^{b}	1.073 ± 0.103^{b}	2.939 ± 0.096^{b}
N. sibirica	0.768 ± 0.007^{a}	0.706 ± 0.057^{a}	1.935 ± 0.098^{a}
N. nervosa	0.870 ± 0.002^{b}	1.217 ± 0.014^{c}	2.968 ± 0.093^{b}
	TEAC [mmol TE g^{-1} DW]		
***N. rtanjensis* EO**	17.016 ± 0.943^{a}	0.806 ± 0.003^{a}	/
***N. cataria* EO**	13.678 ± 1.050^{a}	0.706 ± 0.003^{a}	/
Rosmarinic acid	40.326 ± 1.153^{c}	1.695 ± 0.011^{b}	1.523 ± 0.081^{a}
Chlorogenic acid	23.311 ± 1.081^{b}	0.823 ± 0.003^{a}	1.403 ± 0.141^{a}

All determinations of antioxidant capacity by ABTS, DPPH, and FRAP assays were conducted in triplicate. The reported value for each sample was calculated as the mean of three measurements. Within each parameter, for methanol extracts and reference chemicals separately, values with the same letter are not significantly different at the $p \leq 0.05$ level according to the LSD test.

Abbreviations: TE – Trolox Equivalents; DPPH – 1,1-diphenyl-2-picrylhydrazil; ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate); FRAP – ferric reducing/antioxidant power; EO – essential oil.

acid, as in other antioxidant tests, exhibited a higher potential for the reduction of Fe^{3+} (1.523 mmol TE g^{-1}) than chlorogenic acid (1.403 mmol TE g^{-1}).

Toxicity evaluation of *N. rtanjensis*, *N. sibirica* and *N. nervosa* methanol extracts

An increase in lethality by any of the treatments compared to the controls was not observed. There were several toxic effects on development that were observed after the treatment of zebrafish embryos with all three extracts, but only pericardial and yolk sac edema were observed in 50.7±2.5% of all treated embryos, and only at the 48 hpf stage and in 100x diluted extracts (Fig. 4). We observed premature hatching (increased hatching rate) in embryos treated with 100x dilution of *N. nervosa* extracts, with 59.3%±4.2% of embryos hatching at 48 hpf as compared to only 14.3%±3.4% hatching in all other treatments and controls.

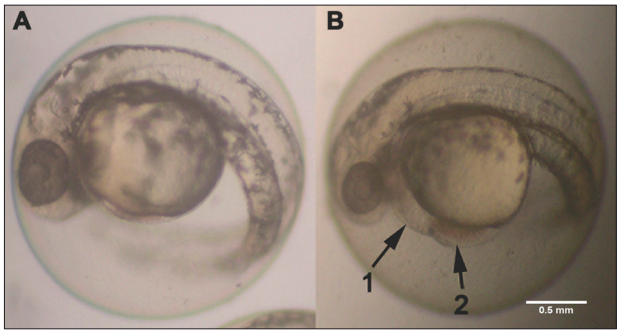

Fig. 4. Representative pictures showing zebrafish embryo toxicity assay of *Nepeta* extracts. **A** – Control embryo, 48 hpf. **B** – Embryo treated with a 100× dilution of *N. rtanjensis* methanol extract, 48 hpf. Arrows indicate pericardial edema (1) and yolk sac swelling (2).

DISCUSSION

Previous phytochemical studies on *N. rtanjensis*, *N. sibirica* and *N. nervosa* have revealed that phenolic acids were the most abundant group of phenolic compounds in the methanol extracts, while terpenoids were represented chiefly by nepetalactones [9,13]. Our results confirmed previous studies that showed rosmarinic acid to be the major phenolic acid in *N. rtanjensis*, *N. sibirica* and *N. nervosa, followed by chlorogenic and caffeic acid [13,29]. High amounts* of rosmarinic acid were also reported for other *Nepeta* species, such as *N. bucharica*, *N. cataria*, *N. nepetoides*, *N. ernesti-mayeri*, *N.* x *faassenii* 'Walker's Low', *N. grandiflora* 'Dawn to Dusk', *N. mussinii*, *N. pannonica*, *N. parnassica*, *N. sibthorpii*, *N. spicata*, *N. transcaucasica*, *N. cadmea*, *N. prattii, N. kotschyi, N. menthoides, N. crassifolia* [13,29,30,31]. Chlorogenic acid has also been reported for *N. parnassica, N. mussinii* [32] and *N. sibthorpii* [5], while caffeic acid has been recorded in *N. cataria, N. kotschyi, N. menthoides* and *N. crassifolia [31,32].*

In addition to the phenolic compounds in *Nepeta* species, one more important group of biologically active secondary metabolites are monoterpenoids. The subfamily *Nepetoideae* is divided into two groups based on the presence or absence of nepetalactone [33]. The largest number of *Nepeta* species possess some of the eight stereoisomers of nepetalactone [29]. In the present study, UHPLC/DAD/(+)HESI-MS/MS analysis confirmed previous phytochemical studies which revealed the *tran,cis*-isomer of nepetalactone as the major monoterpenoid in *N. rtanjensis* [9,13,17,34], and *cis,trans*-nepetalactone as the major isomer in *N. sibirica* [9,13,18]. On the other hand, species such as *N. bracteata* [35], *N. cilicia* [36], *N. curviflora* [37] and *N. daenensis* [38] lack nepetalactone. In the case of *N. nervosa*, some researchers report no nepetalactone [9] while others detected this monoterpene lactone in trace amounts [13,20]. Differences in reported data might be due to the detection limitations of the analytical methods applied and of the devices' performances, extraction protocols, plant growth conditions, developmental stages of plants, etc. In the present study, nepetalactone was not detected in the methanol extracts of *N. nervosa* plants grown *in vitro*, either by UHPLC-DAD or UHPLC/(+)HESI-MS^2 analysis.

Previous studies have shown that aromatic plants containing large amounts of phenolic compounds are a good source of natural antioxidants [3]. The vast array of medicinal properties of phenolic compounds, such as anticancer, antimutagenic and protective activities, may be associated with their antioxidant capacity, during which the elimination of free radicals and prevention of lipid peroxidation occur [39]. The present study reports for the first time the antioxidant properties of *N. rtanjensis*, *N. sibirica* and *N. nervosa* methanol extracts. Since antioxidants may act through different mechanisms, the antioxidant potential of the methanol extracts was determined using three differ-

ent assays, DPPH, ABTS and FRAP. The DPPH assay is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and is applicable in the evaluation of antioxidant activity of lipophilic compounds. The ABTS decolorization assay is, however, applicable to both lipophilic and hydrophilic antioxidants. The preformed radical monocation $ABTS^{\cdot+}$ is generated by the oxidation of ABTS with potassium persulfate and is reduced in the presence of hydrogen-donating antioxidants [25]. The FRAP assay measures the absorption change that appears when the ferric-tripyridyltriazine complex (TPTZ-Fe^{3+}) is reduced to the ferrous-tripyridyltriazine (TPTZ-Fe^{2+}) in the presence of antioxidant compounds [40].

Generally, a considerable antioxidant activity in the ABTS assay was recorded for methanol extracts of all three *Nepeta* species analyzed here. The *N. nervosa* methanol extract was the most efficient, followed by the *N. rtanjensis* and *N. sibirica* extracts. In the case of DPPH and FRAP assays, *N. rtanjensis* and *N nervosa* methanol extracts were more efficient than those of *N. sibirica*. The more efficient antioxidant activity of *N. nervosa* and *N. rtanjensis* methanol extracts might be attributed to the higher content of phenolic compounds, especially of rosmarinic and chlorogenic acids. *N. sibirica* is characterized by a lower phenolic content than the other two species and, therefore, by lower $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ radical scavenging activities and FRAP capacity.

When major chemical constituents of the methanol extracts were tested separately, rosmarinic acid showed the highest neutralizing capacity to $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ radicals and a high capacity for iron reduction in the FRAP assay. The antiradical activity of phenolic compounds directly depends on the number of hydroxyl groups, but also on their molecular structure which determines the availability of phenolic hydrogens and the potential for stabilization of the resulting phenoxyl radicals formed by hydrogen donation [41]. Rosmarinic acid is a dimer of caffeic acid and contains two aromatic rings, both carrying two *ortho*-hydroxyl groups. Rosmarinic acid, with its four hydroxyl groups, showed a stronger scavenging potential than other phenolic acids that have only one or two hydroxyl groups [42]. Fadel et al. [43] have proposed a possible mechanism of antioxidative activity of rosmarinic acid according to which its presence in the cell membrane in association with polar lipids could prevent lipid peroxidation in the presence of reactive oxygen species, without disturbing the structure and permeability of the membrane.

As stated before, chlorogenic acid was the second most abundant phenolic acid in the analyzed samples, while caffeic acid was present in significantly lower amounts than chlorogenic acid, especially in *N. rtanjensis* and *N. nervosa*. On the other hand, caffeic acid was reported to possess stronger antioxidative activity in comparison to chlorogenic acid [42]. This can be explained by the fact that the esterification of quinic acid decreases the activity of the acid-phenol in chlorogenic acid. Furthermore, the introduction of a second hydroxyl group in the *ortho* position is known to increase the antioxidative activity, which is the case for caffeic acid (*ortho*-diphenols).

Many authors have ascribed the antioxidant potential of *Lamiaceae* species to rosmarinic acid, as in the case of *N. menthoides* [4], *Salvia virgata*, *S. staminea* and *S. verbenaca* [44]. Some *Nepeta* species have also been investigated for their antioxidant activity, and phenolic compounds were presumed to play an important role there. Kraujalis et al. [30] showed that the antioxidant potential of the methanol extract of *N. cataria*, *N. cataria* var. *citriodora*, *N. transcaucasica* and *N. bulgaricum* originated from rosmarinic acid, which is the dominant phenolic compound in these species. The same authors showed that there was a high degree of correlation between the amount of rosmarinic acid in the tested extracts and their antioxidant potential. Yazici et al. [16] determined the total phenolic compounds and antioxidant potential of *N. italica*, *N. cilicia* and *N. caesarea*, and the species which contained the highest amounts of phenolic compounds (*N. italica*) displayed the highest antioxidant potential. However, besides phenolic acids, other phenolic compounds such as flavonoids also affect the total antioxidant potential of an extract. Flavonoids were considerably less abundant in the studied *Nepeta* species and therefore were not qualitatively analyzed. However, taking into account the content of total flavonoids, the contribution of these compounds to the overall antioxidant capacity should not be ignored.

In addition to phenolic compounds, in the methanolic extracts of *N. rtanjensis* and *N. sibirica*, monot-

erpenoid compounds, *trans,cis*-nepetalactone and *cis,trans*-nepetalactone, respectively, were identified, and they accounted for a significant share of the total secondary metabolites in the methanol extracts. To test the antioxidant activity of nepetalactones, we analyzed *N. rtanjensis* and *N. cataria* essential oils, which consisted of high amounts of *trans,cis*-nepetalactone and *cis,trans*-nepetalactone, respectively. Our results showed that in the DPPH test these two essential oils displayed significantly lower antioxidant potentials than the standards of rosmarinic and chlorogenic acid. There was no statistical difference between the two essential oils tested. In the case of the ABTS assay, the essential oils displayed similar $ABTS^{\cdot+}$ radical scavenging activity to chlorogenic acid. Neither the *N. rtanjensis* nor *N. cataria* essential oil could reduce iron in the FRAP assay, indicating that *trans,cis*-nepetalactone and *cis,trans*-nepetalactone did not contribute to the FRAP capacity of the corresponding methanol extracts. Our results are in agreement with previously published literature data that reported a very weak antioxidant potential of *Nepeta* essential oils in comparison to extracts containing phenolic compounds [3,5,45,46]. Mahboubi et al. [46] showed that the essential oil of *N. persica,* with *cis,trans*-nepetalactone (80%) as the dominant compound, exhibited a very weak antioxidant potential, while the ethanol extract of the same species showed a high capacity for reduction of $DPPH^{\cdot}$ radicals. Also, the essential oil of *N. cataria,* in which the dominant compound contained is *cis,trans*- (70%) and *trans,cis*-nepetalactone (6%), did not exhibit a capacity for $DPPH^{\cdot}$ radical reduction, while the methanol extract of this species, which contained a very small amount of total phenolic compounds, showed a weak antioxidant potential [45]. However, the essential oil of *N. flavida* had significant antioxidant activity, which was attributed to 1,8-cineole and linalool, both compounds being present in the oil at relatively high amounts [3]. The essential oils of the species studied here contained 1,8-cineol and linalool in concentrations below one percent [17,18]. The *N. nervosa* methanol extract, which contained no nepetalactone, generally showed slightly higher antioxidant activity than *N. rtanjensis*, and significantly higher activity than *N. sibirica*. This was further confirmation that the contribution of nepetalactones in the overall antioxidant potential of the analyzed methanol extracts was not of crucial importance. Phenolics most likely represent the major antioxidants in the samples. Synergistic or antagonistic actions between the individual components of extracts, which largely depend on their structure, i.e. between different phenolic compounds and between phenolic compounds and nepetalactone, should not be neglected. Iacopini et al. [47] showed that the antioxidant capacities of catechin, epicatechin, quercetin and rutin differed when applied as separate compounds in comparison with the assay when they were used as a mixture, leading the authors to conclude that the phenols can have synergistic or antagonistic effects. Furthermore, the minor constituents that are present in the methanol extracts of *N. rtanjensis*, *N. sibirica* and *N. nervosa* and which have not been analyzed here (e.g. flavonoids and other terpenoids) contribute to the bioactive potential of the extracts to a certain extent. A high total amount of phenolic compounds in an extract is not necessarily indicative of high antioxidant activity, but it should be taken into consideration that synergistic or antagonistic interactions may exist [48].

Natural products represent a significant reservoir of unexplored chemical diversity for early-stage drug discovery. Due to the strong antioxidant potentials of the tested species, they could be of great interest to the food and pharmaceutical industries. However, prior to use in the human diet, it is necessary to examine their potential toxicity. Zebrafish have been used predominantly in developmental biology and molecular genetics, but their value in toxicology and drug discovery has been recognized [49]. To evaluate the toxicity of a compound or a plant extract, it is essential to identify the endpoints of toxicity and their dose-response relationships, to elucidate the mechanisms of toxicity and to determine the toxicodynamics of the compound/extract [50]. When the extracts of *N. rtanjensis*, *N. sibirica* and *N. nervosa* were diluted 100x and applied to zebrafish embryos, they developed a severe phenotype, consisting of pericardial and yolk sac edema, which may lead to reduced heart size, impaired cardiovascular function resulting in reduced cardiac output and ischemia of peripheral tissues. Only the extract of *N. nervosa* induced premature hatching (increased hatching rate). To our knowledge, this is the first attempt to characterize *N. rtanjensis*, *N. sibirica* and *N. nervosa* methanol extracts for their embryotoxicity. No *Nepeta* species were previously analyzed for their

zebrafish embryo toxicity. A literature search revealed *N. juncea* cytotoxicity in the brine shrimp lethality bioassay [51], in which the water fraction was more effective than the chloroform one, while n-hexane and n-butanol fractions showed no significant cytotoxic activity. The methanol extracts of *N. scrophularioides* [52] and *N. praetervisa* [53] also showed significant cytotoxicity in the brine shrimp lethality assay. Numerous investigations revealed cytotoxicity of *Nepeta* species against animal and human cancer cell lines [54-56]. Shakeri et al. [57] showed cytotoxic effects of the essential oil of *Nepeta ucrainica* L. spp. *kopetdaghensis* on the human ovarian carcinoma A2780 cell line and human breast adenocarcinoma MCF-7 cell line, while Dar et al. [58] showed that the essential oil of *Nepeta govaniana* inhibited the growth of lung and colon cancer cell lines. The hatching rate can be very heterogenous (it normally occurs between 48 and 72 hpf), but an increased hatching rate could be the result of changed physicochemical properties due to treatment or the influence of the treatment on embryonic motility inside the chorion or the hatching gland of zebrafish embryos [59]. The observed increased hatching rate after the treatment with 100x diluted *N. nervosa* warrants further investigation into which constituents of the extract could cause the differences.

Being a plant genus of particular interest to pharmaceutical and food industries [29,54], the presented results contribute to an improved understanding of the chemical diversity of catnips. The three studied endemic species were shown to possess great potential for food conservation or as medicinal supplements if applied in optimized concentrations. However, alternative sources of plant material (e.g. field cultivation) should be established bearing in mind their vulnerability in nature.

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Conflict of interest disclosure: The authors declare that there is no conflict of interest.

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