DOI: 10.1515/cipms-2018-0036

Curr. Issues Pharm. Med. Sci., Vol. 31, No.4, Pages 194-199

sciendo

Current Issues in Pharmacy and Medical Sciences

Formerly ANNALES UNIVERSITATIS MARIAE CURIE-SKLODOWSKA, SECTIO DDD, PHARMACIA

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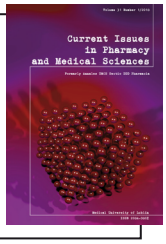

Phenolic compounds of herbal infusions obtained from some species of the *Lamiaceae* family

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ARTICLE INFO

Received 28 February 2018
Accepted 08 June 2018

Keywords:
Dracocephalum moldavica,
Ocimum americanum,
Satureja hortensis,
phenolic compounds,
HPLC,
spectrophotometry.

ABSTRACT

The present investigation was to estimate the total phenolic content and composition of flavonoids and hydroxycinnamic acids in herbal infusions obtained from aerial parts of three *Lamiaceae* species (*Dracocephalum moldavica, Ocimum americanum* and *Satureja hortensis*). The total phenolic content of herbal infusions was determined using a spectrophotometric method, whereas the individual phenolics were assessed by high-performance liquid chromatography (HPLC). The HPLC method was developed and validated. The total phenolic content was measured by applying the Folin-Ciocalteu method with reference to gallic acid. Results were in the range from 29.39 to 65.38 mg estimated as gallic acid equivalents per gram of dry herb. The phenolic profile was, in turn, analysed by HPLC and consisted of gallic acid, hydroxycinnamic acids (caffeic, chlorogenic, ferulic, and rosmarinic) and flavonoids (rutin, hyperoside, quercitrin, quercetin, apigenin, apigenin-7-glucoside and catechin) in different concentrations. Rosmarinic acid was the predominant component among the hydroxycinnamic acids in herbal infusions of all three plants. This was found to be in the range of 3.64 to 5.28 mg per gram of dry herb. Apigenin-7-glucoside, quercitrin and hyperoside were the prevailing flavonoid components of the infusions.

INTRODUCTION

Lamiaceae Martinov is a large botanical family that contains more than 7000 species belonging to 245 genera [1]. The *Nepetoidae* Burnett. subfamily is the most numerous in this family. Its representatives are rich in essential oils and phenolic compounds that manifest obvious antioxidant, anti-inflammatory, antimicrobial or sedative properties, etc. [2-6]. Herein, the phenolic compounds play a key role in preventing the oxidative deterioration induced by various negative factors that arise because human detoxifying mechanisms are not always effective enough against the influence of xenobiotics and metabolic products [7-12].

Many researchers have used different organic solvents to extract phenolic compounds from plant material [9,13,14]. A few publications are, hence, dedicated to the phenolic compounds of the *Nepetoidae* species water extracts [15,16]. Phenolic compounds are partially hydrophilic and therefore water can be used for their extraction. Water infusions are also a convenient dosage form for administration to patients [3,11].

Herbal infusions from officinal medicinal plants belonging to the *Nepetoidae* subfamily of the *Lamiaceae* family, mint, lemon balm, sage, oregano and thyme have long been used since many years to treat respiratory, gastro-intestinal and nervous disorders, etc. [15,17]. We assume that the list of herbal infusions could be expanded by using such species of the *Nepetoidae* subfamily as *Dracocephalum moldavica* L., *Ocimum americanum* L. and *Satureja hortensis* L. Despite their valuable therapeutic and nutritional properties [2,5,11,15,18], these species have not investigated phytochemically enough and are not used in the official medicine of Europe, including Ukraine [19]. Still, they are quite close

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to the taste and chemical composition of the known officinal *Lamiaceae* plants (*Mellissa officinalis* L., *Origanum vulgare* L., *Mentha × piperita, Thymus serpyllum* L., *etc.)*; thus, they have the potential to be added to the herbal teas assortment.

As it is known, the component composition of plant constituents is affected by climatic factors, growth conditions, their genetic chemotypes and extraction procedures [8]. In this regard, the composition of phenolic compounds in the same species from the different region can differ significantly.

The purpose of this study was to estimate the total phenolic content and compound composition of flavonoids and hydroxycinnamic acids in herbal infusions obtained from aerial parts of *D. moldavica*, *O. americanum* and *S. hortensis* (*Lamiaceae*) cultivated on the soil and under the climatic conditions of Ternopil region, Ukraine.

MATERIALS AND METHODS

Plant material

The aerial parts of the investigated species were collected at the flowering stage from the experimental plots in Ternopil region (Ukraine) in 2017. Herbs were dried at a temperature of 30-35°C in shadow, then ground and placed in tightly closed containers. A voucher specimen of each plant was deposited at the Department of Pharmacognosy and Medical Botany of I. Horbachevsky Ternopil State Medical University.

Chemicals

Folin-Ciocalteu's reagent, gallic acid and sodium carbonate for spectrophotometric method were obtained from POCH (Polish Chemical Reagents, Poland). Trifluoroacetic acid, acetonitrile and standards for HPLC (gallic acid, chlorogenic acid, caffeic acid, rosmarinic acid, ferulic acid, catechin, hyperoside, rutin, quercetin, quercitrin, apigenin, apigenin-7-glucoside) were purchased from Merck, Germany. All the chemicals were of analytical grade.

Extraction

Herbal infusions were prepared with the addition of hot water and the forthcoming heating in a boiling water bath as recommended by several authors as effective conditions for the extraction of phenolic compounds [10,11,17,20]. Plant material (5.0 g), ground to 1-2 mm of particle size, was placed in a 500 ml round bottom flask and 200 ml hot distilled water (98-100°C) were added. The flask was placed in a boiled water heater for 15 minutes. After cooling for 45 minutes, the infusion was filtered through a paper filter into a volumetric flask. Purified water was added to obtain a volume of 200 ml that is accepted as the normal amount of consumed herbal tea [3].

Determination of total phenolic content

Total phenolic content was measured using the Folin-Ciocalteu method slightly modified as described by Singleton & Rossi [21]. It was calculated as gallic acid equivalent per gram of dry herb (mg GAE/g). To prepare a sample solution, 0.1 ml of each diluted infusion was transferred to a flask containing 1.5 ml of purified water and 0.1 ml of undiluted Folin-Ciocalteu's reagent. After 3 min, 0.3 ml of 20 % (w/w) Na_2CO_3 was added into each flask. This mixture was shaken up and incubated for 2h at room temperature in darkness to complete the reaction. Gallic acid was dissolved in purified water to obtain 0.02-0.14 mg/ml solutions for the plotting of calibration curve [22]. The absorbance was measured at 760 nm using UV/VIS spectrophotometer Hitachi U-2810 against blank, i.e. purified water. All the experiments were performed in triplicate.

HPLC-analysis of phenolic compounds

The qualitative and quantitative analysis of phenolic compounds in the infusions was performed via the Shimadzu HPLC-DAD system. Separation was achieved by a Phenomenex Luna C18 column (250 × 4.6 mm i.d., 5 µm particle size) at 35°C. The mobile phase consisted of two solutions: trifluoroacetic acid 0.1 % in water (solution A), and trifluoroacetic acid 0.1 % in acetonitrile (solution B) at 1.0 ml/min flow rate, volume injection was 5 µL. The gradient elution was conducted by mixing mobile phases A and B according to Golembiovska's method [23] with minor modifications (Table 1).

Table 1. Gradient of the mobile phases in HPLC-analysis

Time after injection of a sample (min)	Mobile phase A (vol%)	Mobile phase B (vol%)
0–5	95	5
5–35	95 → 75	5 → 25
35–40	75	25
40–60	75 → 50	25 → 50
60–65	50 → 20	50 → 80
65–70	20	80
70–85	95	5

All the solvents were filtered through a 0.45 µm millipore filter before use and degassed in an ultrasonic bath. The UV absorption spectra of the standards, as well as the samples, were recorded in the range of 190-400 nm. The identification of phenolic compounds was achieved by comparison of both the retention time and the absorption spectra obtained for each peak with those obtained for the standards. Quantification of the phenolic compounds was made by measuring the peak area at three different wavelengths (280, 330 and 350 nm) according to absorption maxima of analyzed compounds.

The main validation parameters (linearity, range, limit of detection (LOD), limit of quantitation (LOQ), specificity, accuracy, precision and robustness) were evaluated to ascertain the suitability of the developed chromatographic assay of investigated phenolic compounds using a Luna C18 column. The standard calibration mixtures contained the expected concentration range of the compounds present in the sample extracts. The linearity of the response was determined by calculating the y-intercept and correlation coefficient (r) of each calibration curve. The specificity of the method was determined by evaluating the UV spectra of peaks selected for quantification. The intra-day precision was determined by consecutive repeated injections (n = 6) of the standard mixture.

RESULTS AND DISCUSSION

Polyphenols are known to be responsible for the free radical scavenging and antioxidant activities of plants [3, 15,18]. Their possibility to prevent the free radical oxidation depends mainly on the number and location of hydroxyl groups [12]. Hence, measurements of phenols in infusions may be very important for further study of their free radical scavenging activity [13,21]. The total phenolic content of the prepared herbal infusions is shown in Table 2.

Table 2. The total phenolic content in herbal infusions obtained from some *Lamiaceae* species

Species	Extracted phenols, mgGAE/g dw*	Extracted phenols, mgGAE/200 ml infusion
Dracocephalum moldavica	65.38±1.57	326.9±3.12
Satureja hortensis	44.3±0.92	221.5±2.96
Ocimum americanum	29.39±0.56	146.6±1.17

*dw – dry weight of herb

The total phenolic content of the investigated plants was in range from 29.39 to 65.38 mg GAE/g dry weight. Among the investigated infusions, *D. moldavica* shows the highest contents of phenolic compounds (65.38 mg GAE/g). These results are consistent with those obtained by [10,11,15,17] who studied the total phenolic content of infusions obtained from the *Lamiaceae* family members: lemon balm, common basil, sage, peppermint, etc. The obtained results also correlated with the data of [18]: *Ocimum* species from Manipur (India) accumulate the total phenolic content from 26.15 to 39.31 mg GAE/g dry weight.

HPLC method development and validation for quantitative analysis of phenolic compounds was carried out in accordance with the USP and ICH guidelines [24]. Various parameters have been taken into account to validate the reproducibility of the method, LOD, LOQ, the linearity, the precision and the accuracy (Table 3). The standard mixtures were prepared at six concentration points, by carrying out the specificity of the method dilutions from the working standard solution.

The specificity of the method was evaluated by analysis of blank, standard and sample solution chromatograms. Good separation between the peaks of all investigated phenolic compounds was achieved. LOD and LOQ were calculated using the formula:

$$LOD = 3.3\ (\delta)/S \text{ and } LOQ = 10\ (\delta)/S,$$

where: (δ) = standard deviation of response (peak area),
S = slope of the calibration curve.

The linearity in detector response was observed over the concentration ranges investigated with correlation coefficients greater than 0.990 for all 12 analytes.

The accuracy was evaluated by means of recovery assays carried out by adding known amounts of phenolic compound standards to the samples. The accuracy values were calculated by comparing the back-calculated values with the actual values. The precision of the method was investigated with respect to repeatability, intermediate precision (inter-day variation) and reproducibility by determination of standard solution at 100% of the test concentration. For the robustness investigation, three sample solutions were prepared and analyzed under the established conditions and by changing some method conditions within ± 10%. In this study, the wavelength, column supplier and pH of the mobile phase were changed. None of the modifications induced any significant change in the resolution or response of the all the peaks of phenolic compounds.

All y-intercepts were fairly low and all Pearson's product moment correlation coefficients were close to 1.000. The parameters of the calibration curves and their correlation coefficients show very good linearity in the concentration ranges.

The retention times, wavelengths and validation parameters of 12 phenolic compounds standards are listed in Table 3.

The rutin, hyperoside and ferulic acid peaks were found to be difficult to separate in a chromatogram due to their similar retention properties. However, the use of specific wavelengths both for detection and quantification and spectra comparison improved their identification. Typical

Table 3. Analytical data, results of calibration and sensitivity, including LOD and LOQ of phenolic compounds standards

Compound*	t_R ±SD (min)	λ_{det}, nm*	Range, µg/ml	Linearity	LOD, µg/ml	LOQ, µg/ml	Accuracy, %	Precision, %
Gallic acid	6,8±0,09	280	1.0-20.0	y = 1984.6x + 1824 r = 0,9921	0.01	0.2	100,2	0,23
Chlorogenic acid	20,1±0,10	280	0.1-100.0	y = 2089.4x + 74576 r = 0,9953	0.005	0.01	99,3	0,31
Caffeic acid	21,9±0,12	330	1.0-100.0	y = 1384x + 16334 r = 0,9979	0.005	0.01	97,3	0,77
Rosmarinic acid	37,9±0,05	330	10.0-1000.0	y = 94787x - 278 r = 0,9998	0.005	0.01	101,5	0,12
Ferulic acid	31,5±0,14	330	10.0-500.0	y = 83774x + 29001 r = 0,9994	0.007	0.01	103,3	0,33
Catechin	19,4±0,01	280	1.0-50.0	y = 39394x + 66831 r = 0,9990	0.003	0.01	100,4	0,29
Hyperoside	31,5±0,23	350	1.0-500.0	y = 9932x + 7347 r = 0,9992	0.005	0.01	99,2	0.55
Rutin	31,0±0,25	350	1.0-500.0	y = 185509x + 5568 r = 0,9963	0.01	0.05	99,7	0,48
Quercitrin	35,7±0,30	330	1.0-500.0	y = 77589x - 4280 r = 0,9926	0.02	0.05	98,4	0,79
Quercetin	46,8±0,12	350	1.0-100.0	y = 99763x + 1233 r = 0,9988	0.03	0.05	98,9	0,63
Apigenin	51,9±0,21	330	1.0-100.0	y = 145087x – 2428 r = 0,9993	0.02	0.05	99,5	0,25
Apigenin-7-glucoside	36,3±0,22	330	10.0-500.0	y = 250407x - 2421 r = 0,9987	0,08	0,1	100,3	0,43

* wavelength chosen for quantification

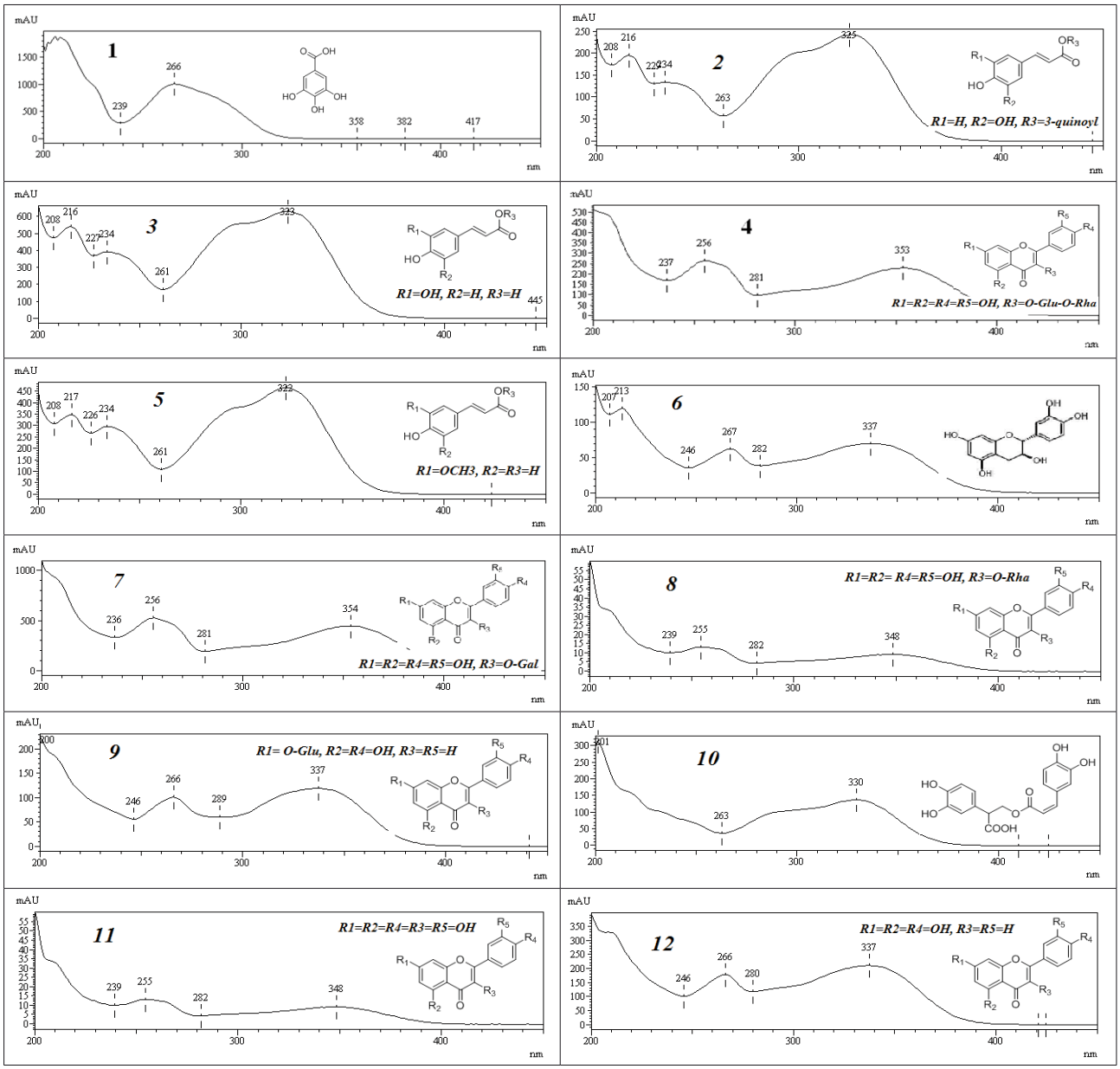

Figure 1. Typical UV-Vis spectral properties of the different phenolic compounds present in *D. moldavica*, *S. hortensis* and *O. americanum* herbal infusions: 1 – gallic acid, 2 – chlorogenic acid, 3 – caffeic acid, 4 – rutin, 5 – ferulic acid, 6 – catechin, 7 – hyperoside, 8 – quercitrin, 9 – apigenin-7-glucoside, 10 – rosmarinic acid, 11 – quercetin, 12 – apigenin

Table 4. Quantification of phenolic acids and flavonoids in herbal infusions of some *Lamiaceae* species (mg/g dw)

Compound	*Dracocephalum moldavica*	*Satureja hortensis*	*Ocimum americanum*
Gallic acid	0.08±0.01	0.08±0.01	0.07±0.01
Chlorogenic acid	0.07±0.01	0.04±0.01	0.05±0.001
Caffeic acid	0.74±0.05	0.4±0.05	0.55±0.01
Rosmarinic acid	5.28±0.43	3.64±0.23	5.16±0.23
Ferulic acid	0.21±0.01	0.22±0.01	2.46±0.09
Catechin	<0.01	0.09±0.01	<0.01
Hyperoside	3.58±0.22	0.49±0.03	2.66±0.14
Rutin	0.13±0.01	0.08±0.01	0.05±0.001
Quercitrin	2.03±0.19	2.6±0.12	4.34±0.41
Quercetin	0.62±0.01	0.03±0.002	0.09±0.01
Apigenin	0.51±0.01	0.02±0.001	0.06±0.005
Apigenin-7-glucoside	4.25±0.32	2.17±0.18	0.38±0.03

UV-Vis spectra for all the investigated phenolic compounds are illustrated in Figure 1.

The HPLC method was developed and validated for the quantification of the major phenolic compounds present in *D. moldavica, S. hortensis* and *O. americanum* herbal infusions (Table 4). The HPLC analysis showed the presence of gallic acid, hydroxycinnamic acids (caffeic, chlorogenic, ferulic, and rosmarinic), and flavonoids (rutin, hyperoside, quercitrin, apigenin, quercetin, catechin, and apigenin-7-glucoside) in different concentrations. The chromatograms of the phenolic samples are shown in Figure 2.

It should be noted that we found that rosmarinic acid was the most common phenolic compound in the infusions obtained from the aerial parts of the investigated plants. Our results correlate with the data of other researchers of the *Nepetoidae* subfamily of the *Lamiaceae* family, i.e. *Melissa officinalis*, *Ocimum gratissimum* etc. [8,9,20,25].

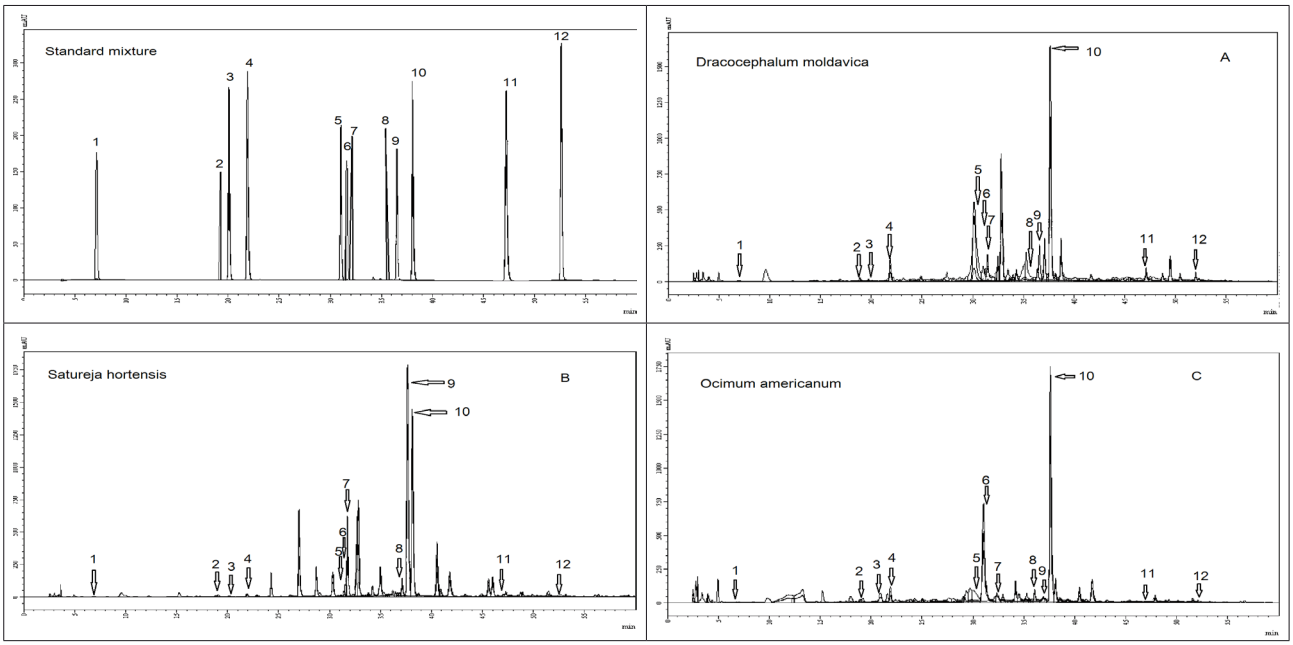

Figure 2. Typical HPLC chromatogram of standard mixture, *D. moldavica* (A), *S. hortensis* (B) and *O. americanum* (C) herbal infusions: 1 – gallic acid; 2 – catechin; 3 – chlorogenic acid; 4 – caffeic acid; 5 – rutin; 6 – ferulic acid; 7 – hyperoside; 8 – quercitrin; 9 – apigenin-7-glucoside; 10 – rosmarinic acid; 11 – quercetin; 12 – apigenin

The infusions of *D. moldavica*, *O. americanum* and *S. hortensis* were found to contain the highest amount of rosmarinic acid (5.28 mg/g dw, 5.16 mg/g dw and 3.64 mg/g dw, respectively) among the phenolic compounds. The presence of rosmarinic acid in high quantities can be closely related to its high free-radicals scavenging potential – which is due to the presence of four hydroxyl groups in its molecule [12]. According to the obtained results, it can be confirmed that levels of rosmarinic acid can be responsible for antioxidant activities of the investigated infusions. Rosmarinic acid is famous for its antiviral, antibacterial and antiinflammatory activities [26].

The other hydroxycinnamic acids were in much lower amounts. The aqueous extract of *O. americanum* contained the highest concentration of ferulic acid (2.46 mg/g dw). The results correlate with [16]. In contrast, *D. moldavica* infusion had the highest level of caffeic acid amounting (0.74 mg/g dw). Appreciable quantities of caffeic acid were also detected in the aqueous extracts of *O. americanum* (0.55 mg/g dw) and *S. hortensis* (0.4 mg/g dw). The presence of fewer hydroxyl groups in their molecules indicates their lower antioxidant potential [8].

Apigenin-7-glucoside was the most common flavonoid in *D. moldavica* and *S. hortensis* infusions (4.25 mg/g dw and 2.17 mg/g dw, respectively). Moreover, high hyperoside content was found in *D. moldavica* and *O. americanum* infusions (3.58 mg/g dw and 2.66 mg/g dw, respectively). Appreciable amount of quercitrin also discovered in all the investigated infusions. Since the listed flavonoids contain from 2 to 4 hydroxyl groups in their molecules, they certainly contribute to the manifestation of the antioxidant activity of the investigated infusions [8,12]. Of note, many differences in the flavonoids profile can be seen between the chemotypes of *O. americanum* collected from their natural habitats throughout Africa and Asia [16].

Other phytochemical investigations have shown that the aboveground parts of such representatives of the *Lamiaceae* family as *Mentha*, *Melissa*, *Salvia*, *Origanum*, *Hyssopus*, *Ocimum*, *Satureja* and *Dracocephalum* are rich in phenolic compounds with pronounced antioxidant properties [3,6,7,9,14]. For example, Gougoulias and Mashev [3] on studying seven plants from Greek and Bulgaria revealed that infusions obtained from *Origanum vulgare* herb had the highest amount of total phenols (13.55 mg GAE/g dw). In addition, phenols concentrations in *Melissa officinalis* leaves from Croatia was 443.6 mg mgGAE/200 ml of infusion, while that in Mentha piperita leaves was 141.2 mgGAE/200 ml [10]. Furthermore, *Melissa officinalis* and Mentha piperita harvested in Brasil showed high contents of phenolic compounds (>30 mgGAE/g dw of herb) after water extraction [11].

Beyond the aforementioned, total phenolic content of dry extracts of leaves of the *Ocimum* species obtained with methanol was found to vary from 42.1 to 168.2 mgGAE/g dw [9]. Moreover, large amounts of polyphenols were noted to be in *Hyssopus officinalis* and *Ocimum basilicum* ethanolic extracts (77.72 and 175.57 mg GAE/g dw, respectively) [6]. The total phenolic content in the *Satureja hortensis* water extract calculated as mg of catechol equivalent was 183.0 mg catechol/g extract [14]. Finally, the total phenols yield in *Ocimum americanum* herb water extract harvested in Kenya [13] was very small (1.54 mgGAE/g dw) while in Manipur (India), its yield was 26.15 mgGAE/g dw [18].

Thus, the content of phenolic compounds in the extracts depends to a large extent on the chemotype and origin of the plant raw material, as well as the choice of the solvent and the extraction procedures. Methanol, ethanol, ethyl acetate and other organic solvents are used for extraction of polyphenols from plants [6,7,9,14], but using water as solvent is the most ecologically and economically justified approach. Furthermore, water molecules possess high polarity which

provides a high degree of extracted components [3,8]. Effect of time and temperature of preparation, ratio of dry herbal material to solvent are also very important for extracting of phenols and other biologically active compounds from plant raw material [3,4,7,27].

Thus, studies of the qualitative and quantitative composition of the investigated infusions have revealed the presence of high concentrations of total phenols which decreased in the direction of *D. moldavica* > *S. hortensis* > *O. americanum*. The phenolic compounds found in this study via the HPLC method are known for their anti-inflammatory, antiseptic and antioxidant properties [8,17,24], which can thus be correlated with the popular use of these plants as spices and in beverages [4,18].

CONCLUSIONS

The total phenolic content, quantitative and qualitative composition of flavonoids and phenolic acids in herbal infusions obtained from three species belonging to the *Lamiaceae* family (*D. moldavica*, *S. hortensis* and *O. americanum*) were determined in this study. The total phenolic content of all the three *Lamiaceae* species varied from 29.39 to 68.35 mg GAE/g of dried plant material.

A simultaneous determination of a wide range of phenolic compounds was performed by way of the HPLC method, and this method for the analysis and means of components quantitation in *D. moldavica*, *S. hortensis* and *O. americanum* herbs was developed and validated. Through its use, we found, for the first time, 12 phenolic compounds in the investigated herbal infusions (with ratio of dry herbal material to water 1:40) that were obtained from *D. moldavica*, *S. hortensis* and *O. americanum* aerial parts.

The comparative study showed significant differences in phenolic compound content in the investigated aqueous extracts of three species. Therein, rosmarinic acid was the most abundant phenolic component in the infusions of all the plants (its content is in the range of 3.64 to 5.28 mg/g dw).

As hydroxycinnamic acids and flavonoids are known to possess anti-inflammatory, antioxidant, antimicrobial, and the other value properties, results of the study indicate the possible benefits of *D. moldavica*, *S. hortensis* and *O. americanum* herbs infusions to human health and can be used in the planning of appropriate pharmacological studies.

CONFLICT OF INTERESTS

None declared.

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