Comparative Polyphenolic Content and Antioxidant Activities of Two Romanian *Lysimachia* Species

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The study of polyphenolic composition and antioxidant capacity of Lysimachia nummularia L. and Lysimachia vulgaris L. extracts were the aim of this study. The polyphenolic profile of these two extracts was carried out using a HPLC-MS method. The total polyphenolic, phenolic acid and flavonoid content were spectrophotometrically determined. The antioxidant activity of these extracts towards various radicals generated in different systems was evaluated using DPPH bleaching method, trolox equivalent antioxidant capacity (TEAC) and Electron Paramagnetic Resonance (EPR) spectroscopic methods. All methods indicated that L. vulgaris extract was more potent antioxidant than L. nummularia extract. That is in good agreement with the total polyphenolic and flavonoidic content. p-coumaric acid, isoquercitrin, rutin, quercitrin, quercetin, luteolin and apigenin were identified in both analyzed extracts. Chlorogenic acid, hyperoside, kaempferol and apigenin were found only in L. vulgaris.

Keywords: Lysimachia, polyphenols, antioxidant capacity

Genus *Lysimachia* L. belonged to *Primulaceae* family. Results from recent phylogenetic analyses suggested the genus *Lysimachia* L. relocation to the family *Myrsinaceae*. The genus *Lysimachia* L. comprises about 200 species, wild and cultivated, native to temperate regions of Eurasia [1,2]. *Lysimachia nummularia* L. and *Lysimachia vulgaris* L. are two of five representatives of *Lysimachia* genus found on natural stands in Romania [3].

The medicinal value of many *Lysimachia* species is well known. There are reports on their analgesic, anti-leishmanial, anti-helminthic properties, and their uses as agents to treat cholecystitis [4,5,6,7]. Also, *L. nummularia* has been used in medicine since antiquity for indications such as diarrhea, fever, arthritis, tuberculosis, skin diseases. Extracts of *L. nummularia* aerial part were found active against a number of microorganisms, including *Streptococcus pyogenes*, *Staphylococcus aureus*, *Salmonella sp.* and *Shigella sp* [8].

In terms of chemical composition *Lysimachia* genus species contain: flavonoids and phenolic acids in the aerial part, respectively benzoquinones and tannins in the whole plant [9,10,11,12,13].The main flavonoids in *L. nummularia* and *L.vulgaris* were identified to be myricetin and myricetin-, quercetin-, and kaempferol-glycosides, respectively by chromatographically methods. According to Toth et al., 2012, *L. nummularia* had the most significant antioxidant activity determinate by DPPH and ABTS method [14].

The polyphenolic composition of Romanian *Lysimachia* species has not been investigated; chemical and pharmacological data are limited too. In addition, there is no data to evaluate the antioxidant potential of these Romanian species.

The aim of this comparative study was to determine the polyphenolic composition of extracts from the aerial parts of these *two Romanian Lysimachia species: L. nummularia* L. and *L. vulgaris* L. and to evaluate their *in vitro* antioxidant properties, for a better characterization and therapeutical exploitation of these species.

Experimental part

Preparation of samples.

Vegetal materials (aerial parts) from these two species were collected in 2014, during the blooming period (July-August) from the Cluj-Napoca surroundings (NW of Romania). Voucher specimens (No. 959, 960) were deposited in the Herbarium of the Department of Pharmacognosy of the Faculty of Pharmacy, Cluj-Napoca, Romania. The vegetal material was air dried at room temperature in shade, separated and grinded to fine powder (300 μm).To 2.0 g of the material were added 20 mL of 70% ethanol (Merck, Darmstadt, Germany), maintained for 30 min on a water bath, at 60°C. The samples were then cooled down and centrifuged at 4500 rpm for 15 min, and the supernatant was recovered and used for the studies [15,16].

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Chemicals

Ferulic, sinapic, gentisic, gallic acids, patuletin, luteolin were purchased from Roth (Karlsruhe, Germany), cichoric, caftaric acids from Dalton (Toronto, ON, Canada), chlorogenic, *p*-coumaric, caffeic acids, rutoside, apigenin, quercetin, isoquercitrin, quercitrin, hyperoside, kaempferol, myricetol, fisetin from Sigma (St. Louis, MO, USA). HPLC grade methanol, analytical grade orthophosphoric acid, hydrochloric acid, aluminum chloride, sodium acetate, sodium carbonate, ethanol and Folin-Ciocalteu reagent were purchased from Merck (Darmstadt, Germany). Hydrogen peroxide, ABTS (2,2'-azinobis-3-ethyl-benzotiazoline-6-sulphonic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), butyl-hydroxytoluene (BHT) were obtained from Alfa-Aesar (Germany). 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH), fluorescein, potassium persulfate were purchased from Sigma–Aldrich (Germany).

HPLC–MS analysis was performed on an Agilent 1100 HPLC Series system using the chromatographic conditions previously described [15,16]. Quantitative determinations were performed using an external standard method. Calibration curves in the 0.5-50 mg/mL range with good linearity (R^2=0.999) for a five points plot were used to determine the concentration of polyphenols.

Determination of total polyphenols, phenylpropanic derivatives and flavonoids content

The total polyphenolic content (TPC) of these extracts was determined by the Folin-Ciocalteau method with some modifications [17-0]. A spectrophotometric aluminum chloride method was performed for quantitative determination of flavonoids [16,20,21]. The total content of phenylpropanic derivatives was determined by using the spectrophotometric method with Arnow's reagent [20]. All spectrophotometric data were acquired using a Jasco V-530 UV-Vis spectrophotometer (Jasco International Co., Ltd., Japan).

DPPH• Radical Scavenging Assay. The antioxidant potential of the ethanolic extracts was quantified using the stable DPPH radical method. The DPPH solution (25 mM) was prepared in methanol and 5.0 m of this solution was added to 5.0 mL of extract solution (or standard) in methanol at different concentrations (10-50 µL for *L. nummularia* extract and 4-10 µL for *L. vulgaris* extract and 10-50 µg/mL standard). After 30 min of incubation at 40°C in a thermostatic bath, the decrease in the absorbance (n = 3) was measured at 517 nm. The percent of DPPH scavenging ability was calculated as: DPPH scavenging ability = $(A_{control} - A_{sample}/A_{control}) \times 100$, where $A_{control}$ is the absorbance of DPPH radical + methanol (containing all reagents except the sample) and A_{sample} is the absorbance of DPPH radical + sample extract or standard. The antiradical activity (three replicates per treatment) was expressed as IC_{50} (µg/mL), the concentration of vegetal material required to cause a 50% DPPH inhibition [17,22, 23]. The positive controls were quercetin and butyl-hydroxytoluene (BHT).

Trolox equivalent antioxidant capacity (TEAC) assay

A stable stock solution of $ABTS^{\cdot+}$ was produced by treating a 7.5 mmol/L methanolic solution of ABTS with 2.6 mmol/L potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12 h before use. At the beginning of the analysis day, an ABTSÿ$^{+}$ working solution was obtained by the dilution in ethanol of the stock solution to an absorbance of 0.70 ± 0.02 AU at 734 nm, verified at spectrophotometer. Than 1 mL of this mixture was mixed with 60 mL of methanol and used for determinations, as ABTS reagent solution. At 1.5 and 3.0 µL from each extract was added 6 mL ABTS reagent solution and incubated at room temperature, at dark for 2 h. For each extract were determined the IC_{50} values. There was used the following formula to determine the inhibition percentage: $\%I = (A_r - A_s)*100/A_r$, where A_r is the absorbance of reference solution and A_s is the absorbance of the solutions with samples [24,25]

Electron paramagnetic resonance (EPR) spectroscopy method

EPR measurements were performed on a Bruker Elexsys E500 spectrometer (Bruker, Billerica, MA, USA) operating in X band (~9.4 GHz) with 100 kHz modulation frequency, at room temperature. The sample was scanned using the following parameters: centre field, 3360 G; sweep width, 60 G; power, 2 mW; receiver gain, 1 × 103; modulation amplitude, 2 G; time of conversion, 15 ms; time constant, 30.72 ms; sweep time 60 s. A solution of 4.5 mM DPPH was added in liquid samples of antioxidant extracts and quickly mixed with 10 µL of extract and transferred in EPR quartz capillary. The EPR spectra were recorded at different time intervals. The variations of the relative concentration of paramagnetic species were obtained through double integration of experimental spectra using XEPR Bruker software [26,27].

Statistical analysis

Data were presented as means and standard deviation (SD). All the samples were analyzed in triplicate; the average and the relative SD were calculated using the Excel software package.

Results and discussions

The HPLC-MS method has been developed for the identification and quantification of 19 phenolic compounds (table 1). The concentrations of identified polyphenolic compounds in both analyzed samples are shown in table 2 and organized in the order of their retention time. HPLC profiles of polyphenolic compounds allowed the possibility of identification of ten polyphenolic compounds in *L. vulgaris*

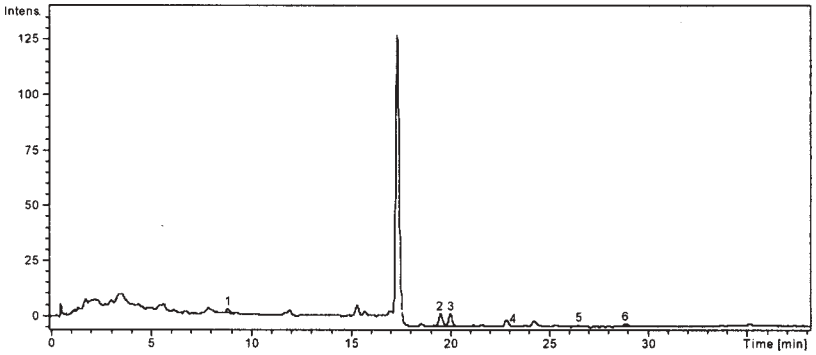

Fig. 1.HPLC chromatogram of *L. nummularia.*
Notes: 1.*p*-Coumaric acid, 2.Isoquercitrin, 3.Rutin, 4.Quercitrin, 5.Quercetin, 6.Luteolin

Peak no.	Phenolic compounds	*m/z*	R_T ±SD (min)	Peak no.	Phenolic compounds	*m/z*	R_T ±SD (min)
1.	Caftaric acid	311	3.54 ± 0.05	11.	Rutoside	609	20.20 ± 0.15
2.	Gentisic acid	179	3.52 ± 0.04	12.	Myricetin	317	21.13 ± 0.12
3.	Caffeic acid	179	5.60 ± 0.04	13.	Fisetin	285	22.91 ± 0.15
4.	Chlorogenic acid	353	5.62 ± 0.05	14.	Quercitrin	447	23.64 ± 0.13
5.	*p*-Coumaric acid	163	9.48 ± 0.08	15.	Quercetin	301	26.80 ± 0.15
6.	Ferulic acid	193	12.8 ± 0.10	16.	Patuletin	331	29.41 ± 0.12
7.	Sinapic acid	223	15.00 ± 0.10	17.	Luteolin	285	29.10 ± 0.19
8.	Cichoric acid	473	15.96 ± 0.13	18.	Kaempferol	285	32.48 ± 0.17
9.	Hyperoside	463	18.60 ± 0.12	19.	Apigenin	279	33.10 ± 0.15
10.	Isoquercitrin	463	19.60 ± 0.10				

Table 1
RETENTION TIMES (R_T) OF STANDARD POLYPHENOLIC COMPOUNDS (MIN)

and only six compounds in *L. nummularia* (fig, 1-2, table 1-2).

In the ethanolic extract of *L. vulgaris*, two phenolic acids, namely chlorogenic and *p*-coumaric acids were identified and quantified (78.00±0.05μg/g and 49.55±0.04 μg/g, respectivelly) (table 2). Four flavonoid glycosides, hyperoside (quercetin-3-*O*-galactoside), isoquercitrin (quercetin 3-glucoside), rutoside (quercetin-3-*O*-rutinoside) and quercitrin (quercetin 3-rhamnoside) were identified and quantified (table 2). Hyperoside was the compound found in the largest amount (2431.74 ± 0.12μg/g), followed by isoquercitrin (495.11± 0.25μg/g), quercitrin (474.78±0.15μg/g) and rutoside (29.35±0.15μg/g). Four free flavonoid aglycons: quercetin, luteolin, kaempferol and apigenin, were identified and quantified. Luteolin was found in the largest quantities (125.67±0.11μg/g) followed by quercetin (46.33±0.03μg/g), kaempferol (13.45± 0.05μg/g) and apigenine (4.81± 0.05μg/g).

In the ethanolic extract of *L. nummularia,* one phenolic acid was identified and quantified: *p*-coumaric acid (15.84±0.12μg/g), among flavonoid glycosides, only isoquercitrin, quercitrin and rutoside were identified and quantified (112.92±0.45μg/g, 5.52±055μg/g and 111.00±0.17μg/g, respectively). There were identified and quantified only two flavonoid aglycons: quercetin (6.14 ± 0.06μg/g) and luteolin (15.74±0.45 μg/g).

Some flavonoidic compounds: hyperoside, kaempferol and apigenine were detected only in *L. vulgaris* and isoquercitrin, rutoside, quercitrin, quercetin, luteolin were found both two species. Chlorogenic acid was found only in *L. vulgaris.* The difference between the separated compounds from these two taxa could serve as a differentiation method and as chemotaxonomic markers to detect the adulterations of these species.

Scientific data on the polyphenolic compounds of these species revealed only the presence of myricetin-rhamnoside in *L. nummularia* and myricetin-hexosyl-desoxyhexoside, quercetin-hexosyl-di(desoxyhexoside), quercetin-hexosyl-desoxyhexoside/rutin, and kaempferol-hexosyl-desoxyhexoside in *L. vulgaris* [14].

The highest amount of polyphenols was found for *L.vulgaris* (76.122±0.35 mg/g) and also the content of flavonoids (26.42±1.3mg/g). This observation is not in agreement with the content of flavonoides determined on the same Hungarian samples [14]. Different Hungarian

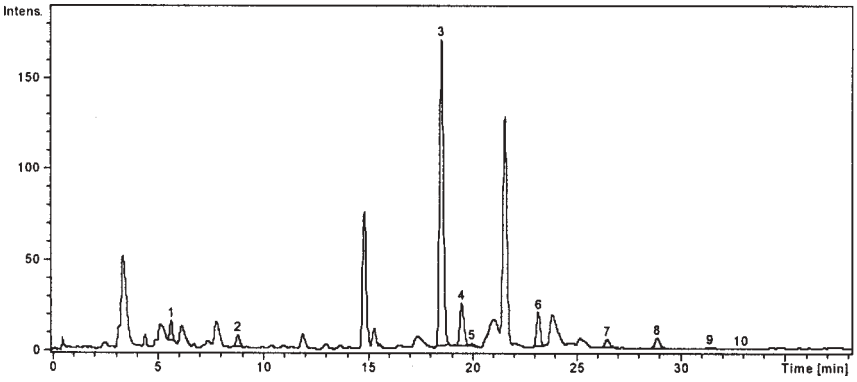

Fig. 2.HPLC chromatogram of *L. vulgaris.*
Notes: 1.Chlorogenic acid, 2.*p*-Coumaric acid, 3.Hyperoside, 4.Isoquercitrin, 5.Rutoside, 6.Quercitrin, 7.Quercetin, 8.Luteolin, 9.Kaempferol, 10.Apigenin.

Polyphenolic compounds	*m/z* value	t_R±SD (min)	*L. nummularia*	*L. vulgaris*
Chlorogenic acid	353	5.62 ±0.05	NF	78.00±0.05
p-Coumaric acid	163	9.08 ± 0.08	15.84 ± 0.12	49.55 ± 0.04
Hyperoside	463	19.00 ± 0,12	NF	2431.74 ±0.12
Isoquercitrin	463	19.60 ± 0.10	112.92 ± 0.45	495.11 ± 0.25
Rutoside	609	20.70 ± 0.15	111.00 ±0.17	29.35 ±0.15
Quercitrin	447	23.64 ± 0.13	5.52 ± 0.55	474.78 ±0.15
Quercetin	301	26.80 ± 0.15	6.14 ±0.06	46.33 ± 0.03
Luteolin	285	29.10 ± 0.19	15.74 ± 0.45	125.67 ±0.11
Kaempferol	285	32.48 ± 0.17	NF	13.45 ±0.05
Apigenin	279	33.00 ± 0.15	NF	4.81 ± 0.05

Note: NF - not found, below limit of detection

Table 2
POLYPHENOLIC COMPOUNDS CONTENT (μg/ g PLANT MATERIAL

Samples	TPC(mg GAE/g vegetal material)	Flavonoids (mg RE/g vegetal material)	Phenylpropan derivatives (mg CA/g vegetal material)
L.nummularia	35.512 ± 0.21	11.26 ± 0.4	16.412± 0.32
L.vulgaris	76.122± 0.35	26.42 ± 1.3	36.63± 0.53

Table 3
THE CONTENT OF TOTAL POLYPHENOLS, PHENYLPROPANIC DERIVATIVES AND FLAVONOIDS IN *L. NUMMULARIA* AND *L. VULGARIS*

Table 4
ANTIOXIDANT ACTIVITY PARAMETERS OBTAINED USING SEVERAL METHODS FOR STUDIED *L.NUMMULARIA* AND *L.VULGARIS* SAMPLES

Samples	DPPH IC_{50} (mg/mL)	TEAC IC_{50} (mg/ml)
L.nummularia	178.14 ± 0.75	94.45 ± 5.09
L.vulgaris	72.826 ± 0.65	40.49 ± 4.88
Quercetin	5.40 ± 0.32	-
BHT	15.6 ± 0.44	-

samples of *L.nummularia* and *L.vulgaris* harvested in early and late flowering stage showed the highest flavonoidic content in *L.nummularia* herba (10.2±0.02mg/g and 5.5±0.01mg/g respectively), but there are not major differences in the total polyphenolic content of these two species (34.2±0.15mg/g and 30.0±0.12mg/g respectively). The variations of content may be related to the soil and climatic conditions and to the harvesting period (in relation with different phenologic states of the plants) [14].

The antioxidant capacity of the ethanolic extracts of *L.nummularia* and *L.vulgaris* was determined by several methods (table 4).

The DPPH scavenging ability of the extract obtained from *L.vulgaris* was larger than that of *L.nummularia* (IC_{50} = 72.826±0.65μg.mL^{-1} and IC_{50} = 178.14±0.75μg.mL^{-1}, respectively) (table 4). Also, according to TEAC method, the extract of *L.vulgaris* has significantly higher antioxidant capacity than the extract of *L.nummularia*. These are in good agreement with the TPC, total phenylpropanic derivatives and total flavonoides values. Compared to the reference compounds, quercetin (IC_{50} = 5.60±0.35 μg . mL^{-1}) and BHT (IC_{50} = 16±0.54 μg . mL^{-1}), the extracts of *L.vulgaris* showed lower antioxidant capacity. Contrary,

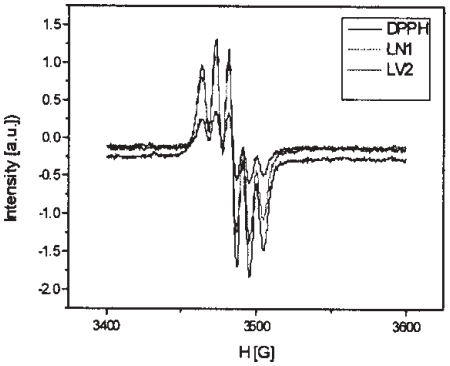

Fig 4**.** The rate of reaction between antioxidant compounds from *L.nummularia* (LN1), *L.vulgaris* (LV2) and DPPH radical

Hungarian authors reported that the DPPH scavenging ability of *L.nummularia* extract is higher than *L.vulgaris* extract [14].

In EPR spectroscopy study, there were made a mixture of free radical (DPPH) and these two antioxidant extracts. The rate of reaction between antioxidant compounds and DPPH radical was monitored by using normalized double integrated residual EPR signal which is correlated with the number of paramagnetic species (fig. 4).

One can observe that integral intensity of DPPH in mixture with different antioxidant extracts decreases compared with DPPH solution without antioxidant extract. The EPR spectra presented in the figure 4 show that a smaller intensity of the signal function of the antioxidant extracts. It represents the oxido-reduction rate of the DPPH radical. Comparing the calculated rates of the both samples, one can observe that *L. vulgaris* extract has a higher antioxidant capacity than the *L.nummularia*. The values of the integral intensity of both samples are represented in table 5 compared with DPPH.

Table 5
THE VALUE OF INTEGRAL INTENSITIES FOR THE ANALYZED SAMPLES

DPPH	*L. nummularia*	*L.vulgaris*
796±12.5	468±9.34	284±4.65

Conclusions

There were determined the polyphenolic profile and the antioxidant activity for two Romanian species of *Lysimachia* genus, *L. nummularia* and *L. vulgaris*, providing important new data concerning the chemical composition and biological activities of these medicinal plants. The results revealed qualitative and quantitative differences of polyphenolic compounds from these species that could avoid adulterations among these two taxa. *L. vulgaris* species contains larger amounts of polyphenols than *L. nummularia,* presenting a relevant higher antioxidant activity.

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