COMPARATIVE PHYTOCHEMICAL SCREENING OF PHENOLOIDS IN *LYSIMACHIA* SPECIES

Anita Tóth[a], Eszter Riethmüller[a]*, Ágnes Alberti[a], Krisztina Végh[a] and Ágnes Kéry[a]

Keywords: *Lysimachia vulgaris; Lysimachia nummularia; Lysimachia punctata*; phenoloids; antioxidants; RP-HPLC-DAD-ESI-MS/MS

A comparative qualitative and quantitative screening of phenoloids of *Lysimachia vulgaris* L., *Lysimachia nummularia* L. and *Lysimachia punctata* L. is reported for the first time. The total flavonoid, hydroxycinnamic acid, tannin and polyphenol content of these species were examined by spectroscopic methods (Ph. Eur. 5.). The accumulation of various phenoloids in individual organs was investigated as well. The quantitative analytical measurements revealed that the total flavonoid content expressed in hyperoside of *L. vulgaris* was significantly lower than that of *L. nummularia* and *L. punctata*, but its hydroxycinnamic acid content was outstanding. The accumulation of polyphenols and tannins showed to be homogeneous among the samples. The *in vitro* radical scavenging activities of the plant extracts were tested by DPPH and ABTS methods. Our studies proved that *L. nummularia* extract had the most significant activity. The composition of flavonoids in the samples and their *in vitro* antioxidant effect showed that the dominance of myricetin glycosides in *L. nummularia* is closely related to the most significant antioxidant capacity of this sample. In addition to the quantitative accumulation of flavonoids, significant differences have been observed in the composition of their quality as well. The main flavonoids in *L. nummularia* and *L. vulgaris* were identified myricetin- and myricetin-, quercetin, and kaempferol-glyosides, respectively by HPLC-DAD-ESI-MS/MS method.

* Corresponding Authors
Tel:/Fax: *+ 36-1-459-1500/55206; + 36-1-3172979*
E-Mail: *eszter.riethmuller@gmail.com*
[a] Department of Pharmacognosy, Semmelweis University, H-1085, Hungary

Introduction

The members of the *Lysimachia* genus are at the forefront of interests. The various species of the genus are distributed in meadows, but their versions are planted also extensively in gardens. They can commonly be found in the temperate zone in North America, Asia and Europe. Most of the known species are native in China.[1]

The phytochemical screenings of Far-East native *Lysimachia* species have started in the last two decades. The chemical studies showed the presence of flavone and flavonol type flavonoids and saponins in these plants. The representatives of the genus include free flavonols and flavone: myricetin, quercetin, kaempferol,[1, 2] isorhamnetin and flavonoid mono-, di- and tri-glycosides. The members of *Lysimachiopsis* and *Nummularia* subgenus have been reported to contain also C-glycosides.[2]

The polar compounds of the *Lysimachia* genus such as flavonoids have been found to have potent anti-tumor and cytotoxic activity in human leukemia K562 cells.[3] Their anti-bacterial and excellent anticholecistic potential, antipyretic effect and the availability to increase bile secretion in acute cholecystitis have been reported as well.[4] The alcoholic extract of *L. ramosa* herb could give rise to killing of different parasite tapeworm.[5]

Hungarian folk medicine and Traditional Chinese Medicine (TCM) also use the leaves of different *Lysimachia* species. *L. nummularia* L. can be used in rheumatic pains, gingivitis, stomatitis, paradontosis, and purulent skin diseases treatment due to its antibiotic and anti-inflammatory properties. The herb tea is used for gastrointestinal diseases.[6 - 8] The TCM uses the *L. christinae* herb for treatment of retention of urine, painful urination, kidney stones jaundice, anorexia, gallstones. It has diuretic, anti-inflammatory and hepatoprotective properties too. It can be used externally for skin cooling, ulcers, infected wounds, snake bite treatment, reduces swelling and edema.[9] The *Lysimachia* genus can serve as a good example, helping us to explore the possible links between European folk medicine and Traditional Chinese Medicine.

As the exploration of the European species of the *Lysimachia* genus is incomplete, we aimed their comparing screening. In this paper the first results of the study of the phenolic metabolites of *Lysimachia vulgaris* L., *Lysimachia nummularia* L. and *Lysimachia punctata* L. are summarized.

Materials and methods

Plant material

Samples of *Lysimachia nummularia* L., *Lysimachia vulgaris* L. and *Lysimachia punctata* L. were obtained from Bükk National Park in early and late flowering stage. The samples were dried at room temperature and authenticated in the Department of Pharmacognosy, Semmelweis University, Budapest, where voucher specimen are deposited.

Table 1.: Phytochemical characteristics of *Lysimachia* samples (*****average of 3 measurements)

	Total flavonoid* (g/100g, in hyperoside)	Total polyphenol* (g/100g. in pyrogallol)	Tannin* (g/100g)	Hydroxycinnamic acid* (g/100g, in rosmarinic acid)
L. vulgaris L.				
herba	0.55±0.01	3.42±0.15	1.82±0.11	2.43±0.10
leaf	1.14±0.04	3.26±0.18	1.96±0.12	2.45±0.08
stem	0.10±0.02	0.99±0.13	0.32±0.04	0.53±0.04
L. nummularia L.				
herba	1.02±0.02	3.00±0.12	1.95±0.10	1.25±0.03
leaf	1.77±0.05	2.34±0.11	1.26±0.11	1.16±0.07
stem	0.36±0.05	1.77±0.14	0.97±0.10	0.63±0.06
L. punctata L.				
herba	0.87±0.07	4.05±0.13	2.81±0.14	1.78±0.08
leaf	1.32±0.05	3.52±0.14	2.63±0.12	1.69±0.08
stem	0.19±0.03	1.31±0.14	0.99±0.12	0.38±0.02
flower	0.61±0.05	2.98±0.12	1.89±0.12	2.35±0.10

Extraction

Soxhlet extraction was performed in laboratory-scale apparatus according to the guidance of Ph. Eur. 5. Ten g of plant material was extracted with methanol. After filtration, the extract was evaporated under reduced pressure with a rotary evaporator at 60 ^{o}C.

Quantitative and qualitative methods

Total phenoloid, tannin, flavonoid and hydroxycinnamic acid content

Contents of polyphenols, tannins, flavonoids and hydroxycinnamic acids were determined in the dried plant samples by applying the methods of Ph. Eur. 5.[10]

DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) method

Antioxidant activities of the *Lysimachia* extracts were determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) as free radicals.[11-13] Ten mg of DPPH (Sigma-Aldrich, Budapest, Hungary) was dissolved in 25.0 mL HPLC grade methanol. Ten mg ABTS (Fluka) was dissolved in 2.6 mL HPLC grade water. To produce ABTS radical cation, stock solution was reacted with 1.72 mg potassium persulfate. Stock solutions were diluted right before measuring by HPLC methanol and spectroscopic ethanol, respectively, so that absorbance of the diluted free radical solutions was approximately 0.900. In their radical forms DPPH and ABTS have absorption maximums at characteristic wavelenghts, at 515 nm and 734 nm, respectively. During the reaction with antioxidants, the radicals are converted back to their colorless neutral form. This decolourisation is stoichiometric with respect to the concentration of antioxidant and can be measured spectrophotometrically. During the assay 50 µL of the samples of 5 different concentrations were added to the free radical solutions and the decrease of the absorbance was measured by Hitachi U-2000 spectrophotometer at the characteristic wavelengths for 6 minutes. From the decrease of the absorbance, percentage of inhibition was calculated. The percentages of inhibition were plotted vs. the concentrations. The concentrations belonging to the 50% inhibition (IC_{50}) were determined by linear regression.

RP-HPLC-DAD-ESI-MS/MS method

Samples were redissolved in super gradient grade methanol (Sigma-Aldrich, Budapest, Hungary), were purified on Supelco SPE Cartridge LC18 (500 mg / 3 mL) and after evaporation, were redissolved in super gradient grade methanol prior to analysis.

For chromatographic separation an Agilent 1200 HPLC system (G1379A degasser, G1312A binary gradient pump, G1329A autosampler, G1316A column thermostat and G1315C diode array detector) was used (Agilent Technologies, Waldbronn, Germany). Samples were separated on a Supelco Ascentis Express C18 column (50 x 3,0 mm, 2,7 µm; Agilent Technologies, Waldbronn, Germany) maintained at 40 °C, injection volume was 10 µL.

MS/MS analyses were performed on an Agilent 6460 triple quadrupole system equipped with an electrospray ionization source (ESI) (Agilent Technologies, Palo Alto, CA). ESI was operated in the positive ion mode, conditions were as follows: temperature: 350 °C, nebulizer pressure: 40 psi (N_2), drying gas flow: 13 L/min (N_2), capillary voltage: 4000 V, fragmentor voltage: 80 V. High purity nitrogen was used as collision gas, collision energy was changed between 10 and 25 eV according to differences in molecule structures. Full mass scan spectra were recorded over a range of 100–1000 m/z. The Masshunter B.01.03 software was used for data acquisition and qualitative analysis.

Results and discussion

Quantitative studies: total flavonoid, polyphenol, tannin and hydroxycinnamic acid content

The total flavonoid content expressed in hyperoside found to be considerably lower in herb of *L. vulgaris* than in herb of *L.nummularia* and *L. punctata* cultivars.

The stem contained much smaller amount of flavonoids than the leaves in the case of all three species, which is important information on the quality of herbal drugs. The higher rate of leaves in the drug is favourable (Table 1.).

More similar accumulations were observed in the total polyphenol and tannin content of the samples. The total polyphenol content was around 3-4%. The highest accumulation was in *L. punctata*, and the lowest in *L. nummularia* herb extract. Tannins' accumulation was also highest in *L. punctata*, while the lowest tannin content was measured in *L. vulgaris*. The accumulation in individual organs was examined as well. The stem contained much less polyphenol and tannin than the leaves in the case of all three species (Table 1.).

The hydroxycinnamic acid content expressed in rosmarinic acid was found to be the highest in the *L. vulgaris*, while the lowest in *L. nummularia*. The *L. punctata* herb's hydroxycinnamic acid content was between. Among the individual organs, the stem contained much less content than the leaves in the case of all three species (Table 1.).

In vitro antioxidant activity assay

Results of the study on *in vitro* DPPH and ABTS antioxidant activity of the samples are summarized in Table **2**., indicating also well-known antioxidant compounds' 50% inhibition value (IC_{50}). The *L. nummularia* extract had the highest activity in both systems, which exceeded the antioxidant effect of kaempferol. *L. vulgaris* extract had lower scavenger capacity than the reference materials and than the other two species. The cultivar of *Lysimachia punctata* L. had moderate activity both in DPPH and ABTS tests.

Table 2.: DPPH and ABTS radical-scavenging activities of the *Lysimachia* extracts and the standards

	DPPH IC_{50} (10^{-3} mg/mL)	ABTS IC_{50} (10^{-3} mg/mL)
L. vulgaris L.	35.3±0.3	11.2±0.3
L. nummularia L.	16.3±0.2	3.57±0.2
L. punctata L.	22.8±0.3	6.84±0.2
Gallic acid	2.7±0.1	0.95±0.1
Rutin	7.3±0.1	3.26±0.2
Quercetin	3.4±0.1	1.22±0.1
Kaempferol	19.1±0.2	8.56±0.3

We aimed to find answers for the connection between the flavonoid composition and the bioactivity of the samples, therefore the individual flavonoid compounds were also investigated.

RP-HPLC-DAD-ESI-MS/MS analysis

A new method was improved for the separation of the compounds of the investigated samples, where eluent A was 0.1 %(v/v) TFA - water, eluent B was acetonitrile. The following gradient elution program was applied at a flow rate of 0.9 mL/min; 0 min: 0 % (v/v) B, 10 min: 50 % (v/v) B, 12 min: 100 % (v/v) B. Chromatograms were acquired at 280 and 340 nm, UV spectra were recorded between 200 and 400 nm. Totalion and UV chromatograms at wavelength of 280 nm and 340 nm were recorded of the methanolic extracts of *L. nummularia* and *L. vulgaris.* In the extract of *L. vulgaris* four, in the extract of *L. nummularia* one flavonoid-type structure was detected. Due to the fact that the MS/MS method is not suitable for accurate identification of the sugar molecules, neither to establish their connection, compounds were tentatively identified based on the structures of the flavonoid aglycones.[14]

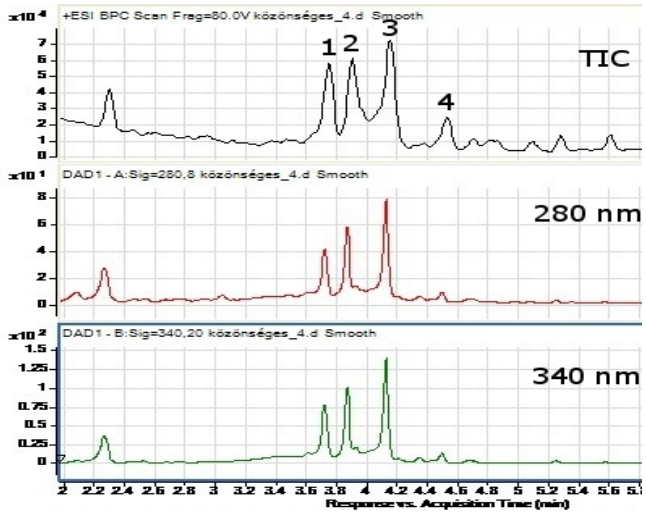

Figure 1.: Chromatograms of *Lysimachaia vulgaris* L. (total ion, 280 and 340 nm) **1** myricetin-hexosyl-desoxyhexoside, **2** quercetin-hexosyl-di(desoxyhexoside), **3** quercetin-hexosyl-desoxyhexoside /rutin, **4** kaempferol-hexosyl- desoxyhexoside

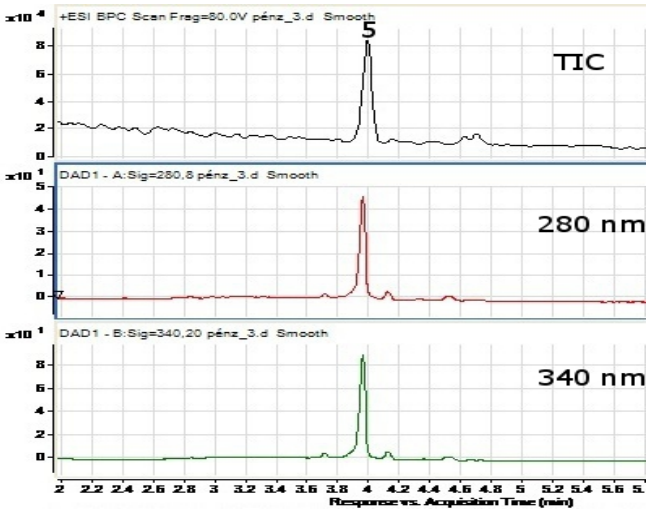

Figure 2.: Chromatograms of *Lysimachaia nummularia* L. (total ion, 280 and 340 nm) **5** myricetin-rhamnoside

The following compounds were identified in *L. vulgaris*:

Compound **1**: Retention time: 3.72 min. The molecule ion $[M+H]^+$ was detected at *m/z* **627**. The mass spectrum exhibited fragments at *m/z* 319, 217, and 153. The pronounced products correspond to myricetin aglycone, while the loss of 308 amu to desoxyhexose-hexose sugar part. Thus compound **1** characterized as myricetin-hexosyl-desoxyhexoside.

Compound **2**: Retention time: 3.87 min. The molecule ion at *m/z* **757** provided pronounced fragments at *m/z* 449, 303, 275, and 153. Product ion at *m/z* 303 determined the type of aglycone to be quercetin, while the neutral loss of 308 amu corresponds to desoxyhexose-hexose, and the neutral loss of 146 amu to desoxyhexose sugar parts. Thus compound **2** was tentatively identified as quercetin-hexosyl-di(desoxyhexoside).

Compound **3**: Retention time: 4.12 min. Molecule ion was detected $[M+H]^+$ at *m/z* 611. Product ion at *m/z* 303 determined the type of aglycone to be quercetin and the loss of 308 amu pointed to a characteristic sugar part (desoxyhexose-hexose). Therefore compund **3** was characterized as quercetin-hexosyl-desoxyhexoside (rutin).

Compound **4**: Retention time: 4.51 min. The compound exhibited molecule ion at *m/z* 595 and fragments at *m/z* 287, 165 and 153. The product at *m/z* 287 indicates kaempferol aglycone, and the neutral loss of 308 amu a desoxyhexosyl-hexosyl group. Thus compound **4** was tentatively identified as kempferol-hexosyl-desoxyhexoside.

The following compound was identified in *L. nummularia:*

Compound **5**: Retention time 3.96 min. Molecule ion $[M+H]^+$ detected at *m/z* 487 provided fragments at m/z 341, 319 and 153. The pronounced products correspond to myricetin aglycone, while the neutral loss of 146 amu to a desoxyhexose sugar part. Thus compound **5** was characterized as myricetin-desoxyhexoside.

Table 3.: Compounds identified in *Lysimachia* samples by RP-HPLC-DAD-ESI-MS/MS

Lysimachia vulgaris L.				
Compound No.	Rt (min.)	$[M+H]^+$ (m/z)	Fragment ions(m/z)	Assumed structure
1	3.72	626	319, 217, 153	Myricetin-hexosyl-desoxyhexoside
2	3.87	757	449, 303, 275, 153	Quercetin-hexosyl-di(desoxyhexoside)
3	4.12	611	465, 303, 257, 165, 153	Quercetin-hexosyl-desoxyhexosyde /Rutin
4	4.51	595	287, 165, 153	Kaempferol-hexosyl-desoxyhexoside
Lysimachia nummularia L.				
5	3.96	487	341, 319, 153	Myricetin-rhamnoside

Conclusion

As the phytochemical exploration of European species of *Lysimachia* is incomplete, we aimed their comparative investigation. This study summarizes the first results of the examination of phenolic metabolites of native Hungarian species: *Lysimachia vulgaris* L., *Lysimachia nummularia* L. and *Lysimachia punctata* L. for the first time.

In the DPPH and ABTS *in vitro* antioxidant test systems the extract of *L. nummularia* had the highest activity. The flavonoid content of *L. nummularia* found to be significantly higher than that of *L. vulgaris*. We were looking for answers to relationship between the flavonoid composition and the bioactivity of the samples. Significant differences were found in quantitative accumulation of flavonoids in the different species and their quality as well.

Based on literature data, we tentatively identified the flavonoids of *L. vulgaris* and *L. nummularia* by RP-HPLC-ESI-DAD-MS/MS method for the first time: the dominance of myricetin glycoside in *L. nummularia* is in good accordance with its most significant antioxidant capacity.

References

[1] Hänsel, R., Keller, K., Rimpler, H., Schneider, G. *Hager's Handbuch der Pharmazeutischen Praxis, Drogen E-O*, Springer-Verlag, Berlin, **1976,** 615.

[2] Marr, K., Bohm, B., Cooke, C., Gunning, P., *Phytochemistry* **1998,** *49,* 553.

[3] Liu, Y., Tang, L., Liang, Z., You, B., Yang, S., *J. Ethnopharm.,* **2010,** *131,* 1.

[4] Yang, X., Wang, B., Zhang, X., Liu, W., Qian, J., Li, W., Deng, J., Singh, G., Su, H., *J. Ethnopharm.*, **2011,** *137*, 57.

[5] Challam, M., Roy, B., Tandon, V., *Veterinary Parasitol.*, **2010,** *169*, 214.

[6] Szabó, L. Gy; G*yógynövények és élelmiszernövények A-tól Z-ig – Adatbázis*, Melius Alapítvány, **2010.**

[7] Makai, A; Házipatika: Pénzlevel lizinka. *Természetgyógyász Magazin Online*, **2005**

[8] Zelenyák, J., *A gyógynövények hatása és használata*. Stephaneum, Budapest, **1908,** 45 and 108

[9] Hempen, C.-H, Fischer, T., *A materia medica for chinese medicine plants, minerals and animal products*. Churchill Livingstone elsevier, Oxford, **2009,** 322.

[10] *European Pharmacopoeia V*., EDQM, **2004,** *2*, 1103 and 2377., **2004**, *1*, 221.

[11] Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., *Free Rad. Biol. Med*., **2009,** *26*, 1231.

[12] Arts, M. J., Haenen, G. R., Voss, H. P., Bast, A., *Food Chem. Toxicol.*, **2004,** *42*, 45.

[13] Brand-Williams, W., Cuvelier, M. E., Berset, C., *Lebensm. Wiss. Technol*., **1995,** *28*, 25.

[14] Ma, Y. L., Li, Q. M., Van den Heuvel, H., Claeys, M., *Rapid Comm. Mass Spectr*., **1997,** *11*, 1357.

Received: 25.05.2012.

Accepted: 30.05.2012.