Comparative study of antioxidant flavonoids in *Lysimachia* species

PhD thesis

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Introduction

The investigation of antioxidant properties of natural compounds has come to the forefront of interest in recent years, due to the compounds' protective effect against oxidative stress mediated deterioration and their beneficial effects in numerous pathological processes. Therefore, the evaluation of effective screening methods for potential naturally occurring antioxidant compounds is crucial. In order to identify and quantitatively determine the active ingredients of herbal medicines, the development of sensitive, accurate, repeatable and validated methods is essential.

Plant phenolics are reported to have notable antioxidant activity. Since herbal samples are complex mixtures, various compounds participate in the pharmacological actions. Thus, contribution of each component to the total antioxidant activity is always a recurring question. However, we cannot ignore, that pharmacological effects are often attributed to more than one certain active substance, due to the occurring interactions between them. Despite this, detection of the main biologically active compounds may assist the targeted selection of fractions to further biological studies. Furthermore, phenolics may serve as chemotaxonomic markers in plants and are considered to be chemical markers for authentication of herbal extracts.

In light of the above, species of *Lysimachia* genus can serve as a good example for investigation, since their known effect may be attributed to their flavonoid content. Literature data on their phenolic composition and antioxidant activity is incomplete, their examination is justified.

The aim of our work was the comparative phytochemical evaluation of three *Lysimachia* species native to Hungary: *L. vulgaris* L., *L. nummularia* L., and *L. punctata* L. Despite their traditional medicinal use, their phytochemical exploration is insufficient. Furthermore, investigation of two cultivated species: *L. clethroides* Duby, L. *ciliata* L. *var.* 'firecracker' and the traditional Chinese medicinal herb *L. christinae* Hance was also planned. There is no report concerning the phytochemical composition of *L. ciliata* L. *var.* 'firecracker'. Our results may help to explore the possible connection between the traditional European and Chinese medicine and may also contribute to reinforcement of taxonomic classification.

Our experiments focused on screening antioxidant phenolics in the selected *Lysimachia* species. HPLC-ESI-MS/MS methods were chosen for structural characterisation of the phenolics present in the *Lysimachia* extracts. HPLC-DAD and UPLC-DAD methods were validated for quantification of the main flavonoids.

Moreover, in order to acquire information on their biological activity, studies on *in vitro* radical scavenging activity were carried out.

Objectives

The primary aim of our study was to characterise the phenolic profile of the *Lysimachia* species native to Hungary: *Lysimachia vulgaris* L, *Lysimachia nummularia* L. and *Lysimachia punctata* L. and the cultivated species: *Lysimachia christinae* Hance, *Lysimachia clethroides* Duby and *Lysimachia ciliata* L. var. 'Firecracker'. Literature data on the phenolic composition and antioxidant activity of the *Lysimachia* species mentioned above is limited. The phenolic composition of *L. ciliata* and has not been studied in details yet. Considering the fact that taxonomy classification of the genus has changed a lot in the last decade, we hoped to find correlation with the classification based on molecular phylogenetic studies and the phenolic compounds accumulation of *Lysimachia* species in the different subgenus.

1. In order to gain preliminary information about the phenolic constitution of the *Lysimachia* herbal drugs, we aimed to analyse their total phenolic-, tannin-, hydroxycinnamic acid- and flavonoid contents according to the methods of Ph. Hg. VIII.
2. For qualitative analysis of the phenolic composition of the *Lysimachia* extracts we aimed to optimise high-performance liquid chromatographic separation of the compounds and acquire structural information about the constituents by different detection methods: diode array detection and electrospray ionisation-tandem mass spectrometry.
3. Our objective was to develop and validate high-performance liquid chromatographic-diode array methods for the quantitative determination of the main phenolic compounds in the herbs of *Lysimachia* species native to Hungary.
4. In order to facilitate the comparison of the studied species, and to reduce the number of components, the glycosides were hydrolysed, allowing the measurements of the main flavonoid aglycone accumulation by utilizing and validating an ultra-high performance liquid chromatographic-diode array method.
5. Plant phenolics are widely reported to act as antioxidants by scavenging free radicals, thus comparison of *in vitro* scavenging activity of the *Lysimachia* extracts, to well-known antioxidant phenolics was planned. To reveal the contribution of certain compounds of the *Lysimachia* hydrolysed extracts to the antioxidant activity we aimed to develop and use a high-performance liquid chromatographic-mass spectrometric method coupled with DPPH free radical scavenging assay.

Material and Methods

Plant material

Aerial parts of *Lysimachia vulgaris* L., *Lysimachia nummularia* L. and *Lysimachia punctata* L. were obtained from Bükk National Park, Hungary (June-July, 2011). Aerial parts of *Lysimachia christinae* Hance were provided from herbal medicine store, Shanghai, China, (2012). Aerial parts of *Lysimachia clethroides* Duby and *Lysimachia ciliata* L. *var.* 'Firecracker' were obtained from a controlled cultivation of the Garden of Medicinal Plants, Jagiellonian University, Cracow, Poland, (2013).

For all the plant materials, 100-100 g samples were collected from several plants during flowering stage. Hungarian native plant samples were authenticated in the Department of Pharmacognosy, Semmelweis University, Budapest, where voucher specimens are deposited. Voucher specimens of Polish samples are deposited in the Department of Pharmacognosy, Pharmaceutical Faculty, Medical College, Jagiellonian University, Cracow, Poland.

Extraction and sample preparation

Extraction

Soxhlet extraction was performed using laboratory-scale apparatus. Dried and milled herb samples (30 g each) were extracted with 250-250 ml of chloroform at 60 ºC and then methanol at 90 ºC, for 6 h each. The extracts were evaporated to dryness under reduced pressure in a rotary evaporator at 50 ºC. The dried extracts were dissolved in HPLC grade methanol to obtain sample solution concentrations of 25-30 mg/ml. The solutions were filtered through Phenex-RC 15 mm, 0.2 µm syringe filters.

Hydrolysis

0.1 ml (containing approximately 0.02 g dried extract) of each redissolved residue solution was mixed with 2.0 ml 25% hydrochloric acid and 10 ml methanol and was heated at 85 °C for 1 h. The mixture was transferred to separating funnel containing 20 ml of water and extracted three times with 15 ml of water-saturated ethyl acetate. The combined ethyl acetate fractions were dried over anhydrous sodium sulphate and evaporated to dryness under reduced pressure in a rotary evaporator at 40 °C. The hydrolysates were redissolved in HPLC grade methanol and filtered through Phenex-RC 15 mm 20-µm syringe filters.

Quantitative phytochemical analysis

Determination of total phenolics, tannin, flavonoid and hydroxycinnamic acid contents of the *Lysimachia* herb samples was performed according to the guidance of Ph. Hg. VIII.

Antioxidant activity assays

Antioxidant activity of *Lysimachia* extracts, their hydrolysates and myricetin, quercetin, kaempferol, myricitrin, rutin, caffeic acid and chlorogenic acid standards was determined using $DPPH^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl) and $ABTS^{\bullet+}$ (2,2'azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) as free radicals. In three parallels, at least 5 different volumes of the diluted samples were added to 2.5 ml $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ solution resulting in different final concentrations and producing inhibition of the radical solutions between 20% - 80%. The decrease of absorbance was recorded against a blank sample (methanol for DPPH and ethanol for ABTS) at 515 and 734 nm, respectively. The concentrations belonging to the half maximal inhibition (IC_{50} value as µg/ml) were determined by linear regression analyses.

HPLC-DAD-ESI-MS/MS evaluation of *Lysimachia* extracts

For chromatographic separation an Agilent 1100 HPLC system was used. The *Lysimachia* samples were separated on a Zorbax SB C18 Plus C18 column (3.0 mm x 150 mm, 3.5 µm) maintained at 25 °C. Injection volume was 1 µl. The following gradient elution program was applied at a flow rate of 0.4 ml/min; where eluent A was 0.3% (v/v) formic acid, eluent B was acetonitrile: 0 min: 10% (v/v) B, 30 min: 40% (v/v) B, 31 min: 10% (v/v) B, 35 min: 100% (v/v) B. Chromatograms were acquired at 280 and 340 nm, as the most selective wavelengths for the detection of flavonoids. UV spectra were recorded between 200 and 400 nm. Tandem mass spectrometric (MS/MS) analyses were performed on an Agilent 6410 triple quadrupole system equipped with a Jet Stream (ESI) ion source. During LC-MS analyses ESI was operated in the negative ion mode, which provided better sensitivity due to the phenolate group of the investigated compounds.

HPLC-DAD condition for quantitative analysis of Hungarian native *Lysimachia* extracts

Quantification of kaempferol, quercetin, myricetin-3-*O*-rhamnoside (myricitrin), rutin and chlorogenic acid in *L. vulgaris, L. nummularia,* and *L. punctata* extracts was performed by the external standard method. Quantities of phenolic compounds of *Lysimachia* species were determined by LC-DAD. For chromatographic separation an Agilent 1260 Infinity HPLC system was used. The *Lysimachia* samples were separated on a Zorbax Eclipse Plus C18

column (4.6 mmm x 100 mm, 3.6 μm) maintained at 25 °C. Injection volume was 1 μl. The following gradient elution program was applied at a flow rate of 0.6 ml/min; where eluent A was 0.1% (v/v) formic acid, eluent B was methanol: 0 min: 20% (v/v) B, 30 min: 100% (v/v) B, 31 min: 100% (v/v) B, 33 min: 20% (v/v) B, 37 min: 20%, (v/v) B. Chromatograms were acquired at 280 and 350 nm, as the most selective wavelengths for the detection of flavonoids. UV spectra were recorded between 200 and 400 nm.

Standard solutions for the calibration were prepared at five different concentrations using kaempferol, quercetin, rutin, myricetin-3-*O*-rhamnoside and chlorogenic acid standards in 80% v/v methanol. Linearity was determined by analysing the standards at five concentrations, each in triplicate. Slope, intercept and correlation coefficient were determined by least squares polynomial regression analysis. LOD and LOQ parameters were determined at 3/1 and 10/1 signal to noise ratios, respectively. Retention time repeatability was checked with six successive runs of the *Lysimachia* extracts. Quality control samples were prepared in three different concentrations for each standard solution. These were used to determine both the intra-day and inter-day precision and accuracy (low, mid and high concentrations of the standards in three parallel runs on the same day and on three successive days, respectively).

UPLC-DAD condition for quantitative analysis of hydrolysed *Lysimachia* extracts

Quantification of kaempferol, quercetin and myricetin in the hydrolysed *Lysimachia* extracts was performed by the external standard method. Quantities of flavonoid compounds of *Lysimachia* species were determined by UPLC-DAD. For chromatographic separation an UPLC Acquity system was used. The *Lysimachia* samples were separated on an Acquity UPLC HSS C18 column (2.1 mm × 100 mm, 1.8 μm) maintained at 40 °C. Injection volume was 3 μl. The following gradient elution program was applied at a flow rate of 0.65 ml/min; where eluent A was 0.2% (*v/v*) acetic acid, eluent B was acetonitrile: 0 min: 22% (*v/v*) B, 3 min: 40% (*v/v*) B, 3.5 min: 100% (*v/v*) B, 4 min: 22% (v/v) B. Chromatograms were acquired at 254 nm. UV spectra were recorded between 200 and 400 nm.

Standard solutions for the calibration were prepared at six concentrations using kaempferol, quercetin and myricetin standards in gradient grade methanol. Linearity was determined by analysing the standards at six concentrations, each in triplicate. Slope, intercept and correlation coefficient were determined by least squares polynomial regression analysis. LOD and LOQ parameters were determined at 3/1 and 10/1 signal to noise ratios, respectively. Retention time repeatability was checked with six successive runs of the hydrolysed *Lysimachia* extracts. Quality control samples were prepared in three different concentrations for each standard

solution. These were used to determine both the intra-day and inter-day precision and accuracy (low, mid and high concentrations of the standards in three parallel runs on the same day and on three successive days, respectively).

HPLC-based DPPH scavenging assay

Sample preparation

100 µl of the *Lysimachia* extracts of known concentrations dissolved in methanol and 100 µl of the DPPH solution (1.5 mg/ml in methanol) were mixed and incubated for 30 minutes at room temperature, protected from light. Then the reaction mixture was directly analysed by HPLC-DAD-QMS. The concentration of the extract used for the analyses was adjusted by the evaluation of the chromatograms after mixing them with the DPPH solution in various ratios to set in the range of 1.0-5.0 mg/ml. The concentration, where the decrease in the peak are of myricetin reached the maximum was chosen. The control samples were prepared by the addition of 100 µl methanol to 100 µl of the extracts.

HPLC-DAD-QMS condition

For chromatographic separation an Agilent 1100 HPLC system was used. The *Lysimachia* samples were separated on a Kinetex-XB C18 column (4.6 mm × 150 mm, 2.6 µm) maintained at 40 °C. Injection volume was 10 µl. The following gradient elution program was applied at a flow rate of 0.5 ml/min; where eluent A was 0.1% (v/v) TFA in water, eluent B was 0.1% (v/v) TFA in acetonitrile:water (95:5): 0 min: 0% (v/v) B, 20 min: 35% (v/v) B, 40 min: 100% (v/v) B, 45 min: 100% (v/v) B. Chromatograms were acquired at 254, 280 and 350 nm as the most selective wavelengths for the detection of flavonoids. UV spectra were recorded between 200 and 400 nm. Mass spectrometric analyses were performed on an Agilent 6120 Single Quadrupole Mass Spectrometer (QMS) system equipped with an electrospray ion source (ESI).

Results

Quantitative phytochemical analyses

All the Hungarian native species contained notable amounts of polyphenol compounds (3-4%), while in cultivated species large variance (1-5%) was observed, with *Lysimachia clethroides* herb being the richest in polyphenols, tannins and hydroxycinnamic acids, too. However, the flavonoids were not presented in such outstanding quantities compared to the other species. The total polyphenol (~1%) and tannin (~0.5%) content found to be considerably lower in herb of *L. christinae* and *L. ciliata*, than other cultivars. The flavonoid (<0.3%) and hydroxycinnamic acid (<0.8%) content was lower in these herb samples, too. The lowest flavonoid content was

measured in *L. ciliata* herb among all the crude drugs, while flavonoids were accumulated in the *L. nummularia* herb in the highest amount. The methanolic extract of herbs were 3-4 times richer in these metabolites in most cases.

Characterisation of phenolics in *Lysimachia* extracts by HPLC-MS

RP-HPLC method was utilised for the separation of phenolics in the six *Lysimachia* extracts, which provided appropriate platform for the investigation of flavonoid-type compounds. For the characterisation of the compounds UV spectral data obtained by LC-DAD and fragmentation pattern acquired by LC-ESI-MS/MS analyses were compared to those of authentic standards and to literature data. The qualitative LC-MS/MS analyses of six *Lysimachia* species resulted in identification of 86 components. Altogether seventeen organic plant acids, six catechin derivatives, seventeen flavonol-*O*-glycosides, two flavonol aglycones, twenty-seven flavone-*C*-glycosides and further seventeen other flavonoids were detected in the samples. *Lysimachia* species contain these components in various combinations and ratios.

L. vulgaris methanolic extracts comprised twelve tentatively identified compounds. Presence of caffeic acid derivative and chlorogenic acid, altogether seven flavonol- mono- di- and tri– *O* –glycosides and two flavonol aglycones were described. The main compounds were quercetin-3-*O*-hexosyl-desoxyhexoside-7-*O*-desoxyhexoside and rutin. Seven phenolic compounds were identified in *L. nummularia* herbal extract. It contained one caffeic acid derivative, three quercetin- two myricetin- and one kaempferol-*O*-glycoside. The main compound was myricitrin. In the *Lysimachia punctata* herbal extract nine phenolic compounds were detected. Presence of a caffeic acid derivative, six flavonol-*O*-glycosides and two flavonol aglycones were proven. The main compound was myricitrin. In the *L. christinae* extract sixty seven phenolic compounds were detected, among them fourteen hydroxycinnamic acid derivatives, nine flavonol-*O*-glycosides, one flavonol aglycone, twenty six flavone-*C*-glycosides, nine methylated flavone derivatives, further six other flavonoid components, quinic acid and gallic acid-hexoside derivative were identified. The main compound was apigenin-6-*C*-hexosyl-8-*C*-pentoside. In the *L. clethroides* extracts nineteen phenolic compounds were detected, among them caffeic acid derivative, and caffeic acid-hexoside, six catechin derivatives, dihydrokaempferol-hexoside, and ten flavonol-*O*-glycosides were tentatively characterised. The main compounds was quercetin-3-*O*-hexoside. Twelve phenolic compounds were tentatively identified in the *Lysimachia ciliata* methanolic extracts. The presence of a caffeic acid derivative and a caffeic acid-hexoside, three flavone-*C*-glycosides, and seven flavonol-*O*-glycosides were described.

The main compound of the *L. ciliata* extract was quercetin-3-*O*-hexoside. According to literature data, phenolics of *L. ciliata* were described here for the first time.

Quantitative analysis of Hungarian native species by HPLC-DAD

Due to the incomplete scientific investigations and the more detailed phytochemical characterisation of Hungarian native species, HPLC-DAD quantitative analysis of the main phenolic compounds in *L. vulgaris*, *L. nummularia* and *L. punctata* was performed and validated. Quantitative determination of chlorogenic acid, myricitrin, rutin, kaempferol and quercetin and their derivatives were performed in the methanolic extracts by using external standard method.

The two main compounds of *Lysimachia vulgaris* are rutin and quercetin-3-*O*-hexosyl-desoxyhexoside-7-*O*-desoxyhexoside which accumulated in the herb nearly the same amount. The two flavonoid aglycones, kaempferol and quercetin were measured in very low amount. In contrast, *L. punctata* herb is containing quercetin nearly the same amount but kaempferol aglycones represents twice higher in the sample. The mass of main compound myricitrin is much lower than in *L. nummularia* herb. Quantitative data of flavonoids in *L. punctata* herb are reported first time.

Quantitative analysis of hydrolysed *Lysimachia* extracts by UPLC-DAD

According to the results of the characterisation of phenolic compounds in six *Lysimachia* species, the presence of more than 50 distinctive flavonoid components was described, half of them has flavonol structure. The most abundant aglycones were kaempferol, quercetin and myricetin, especially in the Hungarian native species. In view of the required effort in the separation of flavonols, quantification on the basis of a reduced number of sample components may pose a feasible alternative. Therefore, mild hydrolysis were performed. Under the used conditions, *C*-glycosidic bonds are unimpaired. For the quantitation of flavonoid aglycones in *L. vulgaris*, *L. nummularia*, *L. punctata*, *L. christinae*, *L. ciliata* and *L. clethroides* extracts, this hydrolysis step was applied prior to the analysis. UPLC method was developed in order to shorten the analysis time. The calculated total flavonol aglycone content (sum quantity of kaempferol, quercetin and myricetin) was the highest in the *L. clethroides* and the lowest in *L. christinae* samples. The *L. clethroides* extract was found to be the most abundant in kaempferol and quercetin, while the *L. nummularia* sample contained the highest amount of myricetin. The amount of myricetin was the lowest in both *Lysimachia clethroides* and *L. christinae*. The lower quantity of flavonol aglycones, however, other type of flavonoids:

flavone-, flavanone- and methylated flavonoid derivatives as well as *C*-glycosides are cumulated in *Lysimachia* species herb. Presence of kaempferol-, quercetin- and myricetin aglycones in the hydrolysed *Lysimachia* extract and their quantity were described for the first time.

Antioxidant activity assays

$DPPH^{\bullet}$ and $ABTS^{\bullet+}$ systems were used for screening the antiradical activities of *Lysimachia* methanolic and hydrolysed extracts and reference compounds. The results demonstrate that the various samples investigated have a significant concentration dependent antioxidant activity in the presence of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical. All the plant extracts were capable of scavenging the radicals in a concentration-dependent manner. The IC_{50} values are 43.3-229.6 μg/ml in DPPH system and 21.3-70.80 μg/ml in ABTS system. Among the crude extracts *L. punctata* proved to have the strongest $DPPH^{\bullet}$ scavenging capacity, followed by *L. nummularia*. Flavonoid content of these species is the highest however the order is reversed between them. The presence and high number of flavonoids with myricetin aglycone can contribute to the good values among the investigated species. The lowest scavenging activity of *L. ciliata* can be explained by its low content of polyphenols and flavonoid. In $ABTS^{\bullet+}$ system nearly the same trend was observed for 50% inhibition values, with the highest activity in *L. punctata* and *L. nummularia* and lowest in *L. ciliata*.

Hydrolysed *Lysimachia* extracts showed stronger radical scavenging activity than crude methanol extracts (IC_{50} values 6.4-59.6 μg/ml in DPPH and 5.2-21.8 μg/ml in ABTS). The acidic treatment significantly increased the radical scavenging activity owing to the increase in the number of phenolic groups due to the decomposition of the *O*-glycosidic bonds. In $DPPH^{\bullet}$ system *L. nummularia* hydrolysed extract showed the highest antioxidant activities which can be explained by the high amount of myricetin. Besides *L. nummularia*, *L. vulgaris* and *L. christinae* hydrolysed extracts have higher scavenging activity than kaempferol. These contain quercetin and myricetin in relatively high amounts. It is important to note that *L. clethroides* hydrolysed extract showed low antioxidant capacity despite the high amount of flavonol aglycones. Besides flavonol-*O*-glycosides, *C*-glycosides also are accumulated. Thus, these and other currently not quantified components may impair the free radical scavenging effect.

HPLC-based DPPH scavenging assay

The hydrolysed *Lysimachia* extracts possessed notable DPPH free radical quenching ability. However, no correlation regarding the antioxidant activity has been found neither with the

individual aglycone content nor with the calculated total aglycone content (sum of the kaempferol quercetin and myricetin content). Thus, examination of the contribution of these compounds to total antioxidant activity was found to be reasonable. Coupling the DPPH assay to HPLC separation was considered the most appropriate method of choice for this purpose.
After spiking the *Lysimachia* samples with the DPPH radical solution, the decrease in the chromatographic peak areas of the main three flavonoid compounds was examined. The following trend was observed regarding the decrease in the peak area: myricetin > quercetin > kaempferol, which is in good accordance with the flavonoids' structure –activity relationship. In *L. punctata* extract relatively high amounts of quercetin and myricetin were determined. The contribution of the two compounds to the DPPH scavenger activity was nearly 75% that resulted in moderately low DPPH activity of the extract. Similar results were obtained regarding the cultivated *L. ciliate* var. Firecracker herb extracts; medium high level of kaempferol and quercetin with approximately 60% contribution to the scavenger capacity determined the lowest DPPH scavenging activity for the whole extract. This let us suppose impaired free radical neutralization reaction by the presence of currently not identified phenolic compounds. The strongest DPPH scavenging activity was detected in *L. nummularia* sample. Myricetin was found to be predominant in the antioxidant effect, which was present in the highest amounts among the investigated samples. The contribution of the mentioned flavonoid was also the highest (73.5%). The *L. vulgaris* sample revealed higher amounts of quercetin and kaempferol than the former discussed species. Although the myricetin content was lower, the extract showed the second highest DPPH scavenging activity. The nearly 76% contribution of quercetin to the scavenger capacity may determine the relatively high total antioxidant activity. Besides, the participation of the three investigated flavonoids to the radical quenching activity was the highest (91.2%) in the *L. vulgaris*. The lowest quantities of the three measured flavonoid aglycones, were cumulated in the *L. christinae* herb. Neither the contribution to the DPPH scavenger capacity was explicit. In contrast to these results, the hydrolysed extract possessed moderate scavenging activity. Therefore, synergistic interaction between the antioxidant compounds were supposed. Furthermore, according to our results and literature data besides flavonol-*O*-glycosides, *C*-glycosides may also be accumulated in the plant. The latter ones are unmodified during the hydrolysis and are present in the investigated samples. They may also influence the DPPH scavenger activity. In *L. clethroides*, the highest kaempferol and quercetin content was measured. Although, the contribution of the three flavonoid compounds to the antioxidant activity was high (90%), only moderate scavenger capacity was measured. These results indicate that other compounds may act as antagonists. However, the total amount

of three flavonoid aglycones (sum of the kaempferol, quercetin and myricetin amount) was the highest among the investigated species, and the contribution of these flavonoids to the total radical scavenging activity was almost the highest (90%).

Conclusion

1. Total phenolic, flavonoid, hydroxycinnamic and tannin contents of the *Lysimachia* herbal drugs were determined, and it could be concluded that all the crude drugs contained notable amounts of flavonoid compounds, with *Lysimachia nummularia* herb being the richest in these constituents.
2. HPLC-ESI-MS methods were applied for the characterisation of phenolics in the *Lysimachia* species. UV spectral data, obtained by LC-DAD and fragmentation pattern, given by LC-ESI-MS/MS analyses were compared to those of authentic standards and to literature data. In the six *Lysimachia* species altogether eighty six compounds were tentatively identified, amongst them seventeen organic plant acid, six catechin derivatives, two flavonol aglycones and seventeen flavonol-*O*-glycosides, twenty seven flavone-*C*-derivatives and seventeen other flavonoids were probable. The applied HPLC-DAD-ESI-MS methods can be successfully utilised for the identification and differentiation of the *Lysimachia* extracts obtained from six species. The phytochemical investigation of *Lysimachia ciliata* and the characterisation of its phenolic compound have been reported in this study first time. Further studies, including isolation and identification of these constituents by NMR spectroscopy, would be worthy to perform. Results of the detailed investigation of the phenolic profile of the *Lysimachia* species might explain their previously reported antioxidant, anti-inflammatory, antithrombotic and hepatoprotective effects. Besides, comparison of the phenolic profile of the investigated *Lysimachia* species to other members of the Primulaceae family would be interesting from the chemotaxonomic point of view as well.
3. Our work is the first to describe a validated quantitative HPLC-DAD method for analysis of phenolic metabolites in three Hungarian native species. The method was successfully utilised for the quantitative determination of main the phenolic compounds, namely, chlorogenic acid, myricitrin, rutin, quercetin and kaempferol. Quantitative data of flavonoids in *L. punctata* herb are reported first time- Our results contribute to the more detailed description of these members of the domestic flora. In addition, this quantitative method would be applicable for the investigation of the influence of geographical and seasonal variations on the yield of the compounds with relevant antioxidant activity.

4. In view of the required effort in the separation of flavonoids, quantification and comparison on the basis of reduced number of sample components may pose a feasible alternative. Thus, for the quantitation of flavonoid aglycones, hydrolysis step was applied prior to the analysis. This is the first study presents a fast, selective and validated UPLC-DAD quantitation method for three most abundant flavonoid aglycones, namely kaempferol, quercetin and myricetin in six *Lysimachia* species.
5. Since plant flavonoids are widely reported to act as antioxidants, and the *Lysimachia* herbal drugs were proved to be rich in these constituents, determination of the *in vitro* radical scavenging activity of methanolic extracts was found to be reasonable. Two *in vitro* tests using DPPH and ABTS as free radicals were utilised. Our results indicated that all the extracts possessed concentration-dependent activity in both *in vitro* tests compared to well-known antioxidant standards. The data also suggest that the acidic treatment increased the free radical scavenging potential of the crude extracts.
6. Our qualitative and quantitative results supported the presumption that the phenolic compounds played significant role in the antioxidant activity of the *Lysimachia* extracts. Comparison of the antiradical power of different hydrolysed extracts to the amount of each flavonoids and to the calculated total flavonoid content revealed that no trend occurred regarding the scavenger activity. These observations may be explained by the differences in the phenolic profile of the *Lysimachia* extracts studied. Therefore, a high-performance liquid chromatographic-mass spectrometric method coupled with DPPH free radical scavenging assay was applied to reveal the contribution of certain flavonoid aglycones of the *Lysimachia* extracts to the total scavenger capacity for the first time. Based on our results, we could concluded that quantity of the main flavonol aglycones in some extracts strongly affected the antioxidant activity, but in other extracts some other compounds, especially flavones and flavone-*C*-derivatives played role in the radical scavenging activity. Thus, additional studies aiming the clarification of the interactions between the antioxidant compounds are needed.

Publications

Publications related to the thesis

Tóth A, Riethmüller E, Végh K, Alberti Á, Béni Sz, Kéry Á. (2018) *Lysimachia* fajok flavonoid összetételének és antioxidáns aktivitásának összehasonlító vizsgálata. Acta Pharm Hung, 88: 75-83.

Tóth A, Riethmüller E, Végh K, Alberti Á, Béni Sz, Kéry Á. (2018) Contribution of individual flavonoids in *Lysimachia* species to the antioxidant capacity based on HPLC-DPPH assay. Nat Prod Res, 32: 2058-2061.

Tóth A, Végh K, Alberti Á, Béni Sz, Kéry Á. (2016) A new ultra-high pressure liquid chromatography method for the determination of antioxidant flavonol aglycones in six *Lysimachia* species. Nat Prod Res, 30: 2372-2377.

Tóth A, Tóth G, Kéry A. (2014) Polyphenol composition and antioxidant capacity of three *Lysimachia* species. Nat Prod Commun, 9: 473-1478.

Tóth A, Riethmüller E, Alberti Á, Végh K, Kéry Á. (2012) Comparative phytochemical screening of phenoloids in *Lysimachia* species. Eur Chem Bull, 1: 27-30.

Further scientific publications

Varga E, Barabás C, **Tóth A**, Boldizsár I, Noszál B, Tóth G. (2019) Phenolic composition, antioxidant and antinociceptive activities of *Syringa vulgaris* L. bark and leaf extracts. Nat Prod Res, 33: 1664-1669.

Végh K, Riethmüller E, Hosszú L, Darcsi A, Müller J, Alberti Á, **Tóth A**, Béni S, Könczöl Á, Balogh GT, Kéry Á. (2018) Three newly identified lipophilic flavonoids in *Tanacetum parthenium* supercritical fluid extract penetrating the Blood-Brain Barrier. J Pharm Biomed Anal, 149: 488-493.

Varga E, Schmidt I, Szövérfi B, Pop MD, Kelemen H, **Tóth A**. (2018) Phenolic content from medicinal plants and their products used in veterinary medicine. Acta Medica Marisiensis, 64: 77-82.

Pénzes R, Mészáros A, **Tóth A**, Darcsi A, Béni Sz. (2017) Separation and identification of diarylheptanoids from *Morus alba* L. Reviews On Clinical Pharmacology And Drug Therapy 15: 51-52.

Végh K, Riethmüller E, Alberti Á, **Tóth A**, Béni Sz, Kéry Á. (2017) A *Tanacetum parthenium* L. partenolid tartalmának szuperkritikus fluid extrakciója: kísérlettervezés, modellezés, optimalizálás. Acta Pharmaceutica Hungarica 87: 77-83.

Tóth G, Barabás C, **Tóth A**, Kéry Á, Béni S, Boldizsár I, Varga E, Noszál B. (2016) Characterization of antioxidant phenolics in *Syringa vulgaris* L. flowers and fruits by HPLC-DAD-ESI-MS. Biomed Chromatogr, 30: 923-932.

Végh K, Riethmüller E, **Tóth A**, Alberti Á, Béni S, Balla J, Kéry Á. (2016) Convergence chromatographic determination of camphor in the essential oil of *Tanacetum parthenium* L. Biomed Chromatogr, 30: 2031-2037.

Végh K, Alberti Á, Riethmüller E, **Tóth A**, Béni S, Kéry Á. (2014) Supercritical fluid extraction and convergence chromatographic determination of parthenolide in *Tanacetum parthenium* L.: Experimental design, modeling and optimization. J. Supercrit Fluids, 95: 84-91.