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Programvezető: Dr. Antal István, egyetemi tanár

Témavezető: Dr. Kéry Ágnes, c. egyetemi tanár

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COMPARATIVE STUDY OF ANTIOXIDANT FLAVONOIDS IN *LYSIMACHIA* SPECIES

PhD thesis

Anita Zempléni-Tóth

Pharmaceutical Sciences Doctoral School

Semmelweis University

Supervisor: Ágnes Kéry, Ph.D.

Official reviewers: Márta Mazákné Kraszni, Ph.D.
Kamilla Ács, Ph.D.

Head of the Final Examination Committee: Imre Klebovich, D.Sc.

Members of the Final Examination Committee: Huba Kalász, D.Sc.
Zsuzsanna Hajdú, Ph.D.

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)
AE	antiradical efficiency
Agly	aglycone
AH	antioxidant
amu	atomic mass unit
$ArOH^{+\bullet}$	flavonoid cation radical
ARP	antiradical power
BDE	bound dissociation enthalpy
BPC	base peak cromatogram
CAT	catalase
CID	collision-induced dissociation
COX	cyclooxygenase
CUPRAC	cupric reducing antioxidant assay
CVD	cardiovascular disease
DAD	diode array detector/detection
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC_{50}	effective concentration that reduces activity by 50%
ESI	electrospray ionisation
ET	electron transfer mechanism
F-C	Folin-ciocalteu reagent
FRAP	ferric ion reducing antioxidant power assay
GAE	gallic acid equivalent
HAT	hydrogen atom transfer
HPLC	high-performance liquid chromatography
HPLC-MS	high- performance liquid chromatography coupled with mass spectrometry
IC_{50}	half maximal inhibitory concentration
IHB	intramolecular H-bond
IP	ionization potential
LOD	limit of detection

LOQ	limit of quantitation
LOX	lipoxygenase
MS	mass spectrometry
MS/MS	tandem mass spectrometry
m/z	mass-to-charge-ratio
NMR	nuclear magnetic resonance spectroscopy
NOS	nitric oxide synthase
$^1O_2$	singlet oxygen
$O_2^{\bullet-}$	superoxide anion
$OH^{\bullet}$	hydroxyl radical
ORAC	oxygen radical absorbance capacity
PA	proton affinity enthalpy
PDE	proton dissociation enthalpy
Ph. Hg.	Pharmacopoea Hungarica
QSAR	quantitative structure–activity relationship
QMS	single quadrupole mass spectrometer
$RO^{\bullet}$	alkoxyl radical
$ROO^{\bullet}$	peroxyl radical
ROS	reactive oxygen sepcies
SAR	structure-activity realtionship
SD	standard deviation
SET-PT	single electron transfer (– proton transfer mechanism)
SOD	superoxide dismuthase
SPLET	sequential proton loss electron transfer
TEAC	trolox equivalence antioxidant capacity
TEC_{50}	the time which needed to reach the steady state
TFA	trifluoroacetic acid
TPC	total phenolic content
FXOR	xanthin oxidoreductase
UPLC	ultrahigh performance liquid chromatography

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1. INTRODUCTION

Humans have been using natural products to treat various diseases for thousands of years. Assuming, that a wide range of plants, and their active ingredients found on Earth, are still unexplored, phytochemical studies focus on exploring potential drug candidate molecules. In today's busy world, the increasing incidence of diseases associated with aging and oxidative (endogenous and environmental) stress is affecting more and more people. Thus, there is an increasing need to prevent and treat these conditions.

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.

The plant phenolics are structurally diverse, basic secondary plant metabolites, 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. 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 may contribute to the authentication of herbal extracts.

High performance liquid chromatography (HPLC) coupled with diode array detection (DAD) or mass spectrometry (MS) provides a great analytical tool for the phytochemical characterisation of plant extracts. Furthermore, separation techniques coupled with *in vitro* antioxidant assays promote rapid and effective identification of biologically active substances. It also makes possible to examine the contribution of the individual compounds to the free radical scavenging activity.

In light of the above, species of *Lysimachia* genus can serve as a good example for investigation, since their known pharmacological effect is partly attributed to their

flavonoid content. Literature data on their phenolic composition and antioxidant activity is incomplete. Thus, 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 ethnomedicinal use, their phytochemical exploration is insufficient. Furthermore, investigation of cultivated species: *L. clethroides* Duby, L. *ciliata* L. *var*. 'Firecracker' and the traditional Chinese medicinal herb *L. christinae* Hance was also planned. There are no reports 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.

Presence of phenolics in *Lysimachia* genus is reported, and pharmacological effects can be traced back to their flavonoid content. Therefore 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. Further aim was the development of a semi-quantitative HPLC-DPPH method, in order to investigate the contribution of individual compounds to the total antioxidant activity.

The first chapters (**1.1**-**1.3.**) of the literature overview summarise the most important knowledge about oxidative stress and antioxidants, antioxidant structure activity relationship of flavonoids, and the most widely used antioxidant activity assays. The following sections present the botanical, phytochemical and pharmacological properties of the selected *Lysimachia* (Primulaceae) species (**1.4**-**1.10**).

1.1. Redox-homeostasis

The oxidation-reduction processes are fundamental in biological systems for life functions. In these essential metabolic reactions electron transfer occurs from one species to another. Maintenance and regulation of this oxidation-reduction homeostasis via a prooxidant and antioxidant defence mechanism is central to life. Under physiological conditions, different physical, chemical or biological challenges upset this steady state condition, which trigger a response that restore the homeostasis. The defensive mechanisms of the cells allow free radical reactions to occur without damage under physiological conditions (Ursini et al., 2016, He et al., 2017).

1.1.1. Oxidative stress

The redox homeostasis in aerobic organisms is ensured by a complex and sensitive system. Oxygen is essential for efficient energy production. Paradoxically, an overwhelming part of the cellular energy metabolism is coupled with ROS (reactive oxygen species) formation. As a result of imbalance, the increasing ROS concentrations lead to chronic toxic stress in cells, which goes hand in hand with the inability to deal with challenges. Thus, the harmful oxidative stress is the consequence of the failure to maintain the physiological redox steady state (Sies et al., 2017). Several factors may play a role in the induction. These factors are for example cigarette smoke, alcohol, ozone exposure, ionizing radiation, heavy metal ions, malnutrition, physical overload, xenobiotics, heat exposure, air pollution and other environmental damages (Birben et al., 2012, Aseervatham et al., 2013). Oxidative stress is a harmful process that can cause tissue injury due to inhibition of normal function of cellular lipids, proteins, and nucleic acids. As a result of these, lipid peroxidation, DNA damage, protein polymerisation, and aggregation, loss of enzyme function, receptor and cofactor damages, and impairment of neurotransmitter function may occur (Fig. 1) (Avery 2011, Avery 2014, Gülcin 2020). Oxidative stress may induce several chronic and degenerative diseases (Pizzino et al., 2017). Its role is proven in various cancer (Gao & Schöttker 2017, Chio & Tuveson, 2017), CVDs (Hopps et al., 2010, Maulik & Kumar 2012, Siti et al., 2015), neurological disorders (Rodrigo et al., 2013, Pohanka 2014, Gaki & Papavassiliou 2014, Patel 2016), respiratory diseases (Domej et al., 2014, Kleniewska & Pawliczak 2017), and rheumatoid arthritis (Quinonez-Flores et al., 2016).

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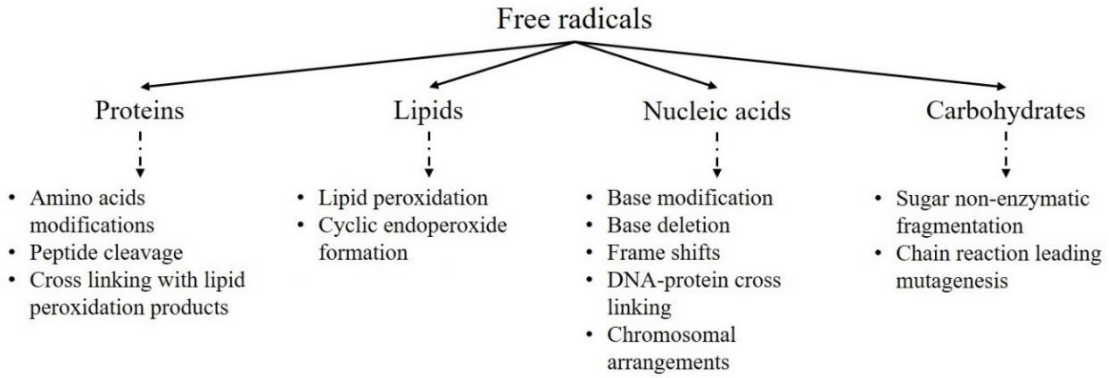

Figure 1. Biological targets of free radicals (Gülcin, 2020)

1.1.2. Free radicals

Free radicals are atoms, molecules or ions that have unpaired electrons. They are highly unstable and react easily with other molecules. Endogenous ROS are continuously produced in the oxidative reaction process of mitochondrial respiratory chain as by-products of normal cellular mechanisms. This catalytic process is the Fenton reaction, (eq. 1-2), in which formed and effluent hydroxyl radicals could cause significant biological damage (Thomas et al., 2009, Kivrak et al., 2017).

$$R\text{-}Fe^{2+} + H_2O_2 \rightarrow R\text{-}Fe^{3+} + OH^- + OH^{\bullet} \quad \text{(eq. 1)}$$

$$R\text{-}Fe^{3+} + O_2^{\bullet -} \rightarrow R\text{-}Fe^{2+} + O_2 \quad \text{(eq. 2)}$$

The free radical generation is an autocatalytic chain reaction, with three primary steps: initiation, propagation and termination, where RH is a substrate (e.g.: a lipid), $R^{\bullet}$ is allyl radical. The peroxyl radical ($ROO^{\bullet}$) is the chain carrier of the reaction. In the termination step, radicals and antioxidants are combined to form non-radical products (eq. 3-8) (Huang et al., 2005, Yoo et al., 2007).

1, Initiation

$$RH + O_2 \rightarrow R^{\bullet} + HOO^{\bullet} \quad \text{(eq. 3)}$$

2, Propagation

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad \text{(eq. 4)}$$

$$ROO^{\bullet} + R_1H \rightarrow ROOH + R_1^{\bullet} \quad \text{(eq. 5)}$$

3, Termination

$$R^{\bullet} + R^{\bullet} \rightarrow R \quad \text{(eq. 6)}$$

$$R^{\bullet} + ROO^{\bullet} \rightarrow ROOR \quad \text{(eq. 7)}$$

$$ROO^{\bullet} + ROO^{\bullet} \rightarrow ROOR + O_2 \quad \text{(eq. 8)}$$

Well known oxygen centred free radicals are superoxide anion ($O_2^{\bullet -}$) hydroxyl radical ($OH^{\bullet}$) peroxyl radical ($ROO^{\bullet}$), alkoxyl radical ($RO^{\bullet}$) and nitric oxide. There are other nonradical ROS, such as the singlet oxygen ($^1O_2$), hydrogen peroxide and hypochlorous acid (Phaniendra et al., 2015).

1.1.3. Antioxidants

Antioxidants play a crucial role in living systems to reduce the oxidative process. They react in low concentration by decreasing the local amount of free radicals and prooxidative metal ions. There are several classifiactions of antioxidants. Primary antioxidants delay or inhibit the initiation and the propagations step of radical chain reaction by reacting with radicals. Thereby, they can terminate the chain reaction (eq. 9-10). The natural polyphenolic antioxidants exert their effect partly in this way (Yoo et al., 2007).

$$ROO\cdot + AH \rightarrow ROOH + A\cdot \quad \text{(eq. 9)}$$

$$A\cdot + ROO\cdot \rightarrow ROOA \quad \text{(eq. 10)}$$

The secondary antioxidants prevent Fenton-type oxidation reaction by decomposing peroxides or chelating transition metal ions. They act indirectly via upregulating enzymes of antioxidant defence system or interfering with cellular signal transduction processes through enzyme gene expression. These are for example the antioxidant enzyme cofactors, such as selenium or coenzyme Q10 (Yoo et al., 2007, Apak 2019).

Endogenous and exogenous antioxidants are distinguished. Among endogenous antioxidant, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, thiols, uric acid, bilirubin, melatonin, metal-binding proteins, such as transferrin, ferritin, lactoferrin, ceruloplasmin, and even albumin, reduced coenzyme Q, and alpha lipoic acid are the most important (Pisoschi & Pop 2015).

It has been suggested by epidemiologic studies that diets rich in antioxidants can bring health benefits (Jayedi et al., 2018, Mentella et al., 2019, Aune 2019). The most prominent representatives of dietary or exogen antioxidants are vitamin A, C and E, carotenoids, phenolic compounds: stilbenes, phenolic acids, and flavonoids (Pisochi & Pop 2015, Neha et al., 2019, Gülcin 2020). From those listed, antioxidant activity of flavonoids will be discussed in detail below.

1.2. Flavonoids

Flavonoids are polyphenolic natural compounds, and more than 8000 of them have been described and identified until now (Wang et al., 2011, Alseekh et al., 2020). They are synthetized in cytosol, and derived from phenylalanine and malonyl-CoA on the shikimic acid pathway (Tohge et al., 2017). Flavonoids are characterised by diphenyl-propane (C_6-C_3-C_6) backbone, in which two aromatic rings are linked via a three carbon chain. The linked benzene (A) and pyrone (C) ring is substituted with a phenyl ring (B) at the 2 or 3 position (Fig. 2). Flavonoids are sub-classified by oxidation degree, and connection position of B ring into several groups, namely calchones, flavans, flavan-3-ols, anthocyanidins, flavanones, flavones, flavonols, isoflavans, and isoflavones (Fig. 2). Further modification, such as hydroxylation, glycosylation and methylation or acylation increase the variability and number of molecules (Wang et al., 2018a).

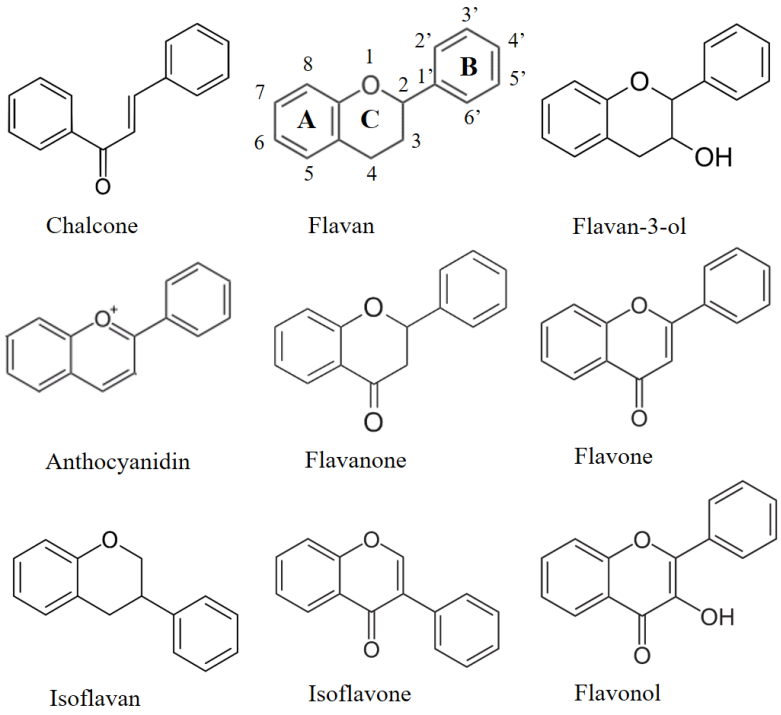

Figure 2. Structure of flavonoids

The biological effects of flavonoids are being intensively researched. Recent studies exhibit various positive effects on the human body, including antioxidant activity (section **1.2.1.-1.2.2.**), anti-cancer (Romagnolo & Selmin 2012, Abotaleb et al., 2019, Alfa & Arroo 2019), anti-inflammatory (Pan et al., 2010, Maleki et al., 2019), antimicrobial

(Gorniak et al., 2018, Farhadi et al., 2018, Biharee et al., 2020, Ninfali et al., 2020, Jin 2019), anti-atherogenic (Mulvihill & Huff 2010, Fardoun et al., 2020), anti-thrombotic and vasodilatory (Agrawal 2011, Lopez et al., 2019) effects. They have estrogenic (Vitale et al., 2013, Krizova et al., 2019) and capillary fragility and permeability decreasing (Maggioli 2016, Casili et al., 2020), anti-allergic (Tanaka 2014, Shi et al., 2018) actions, too. Flavonoids are beneficial also in diabetes mellitus (Ghorbani 2017, Jaitak 2019), and various cardiovascular diseases (Kim & Je 2017, Maaliki et al., 2019, Parmenter et al., 2020), furthermore, a role in autoimmune (Rengasamy et al., 2019) and neurological disease prevention and treatment (Airoldi et al., 2018, Putteeraj et al., 2018, Maan et al., 2020) has been reported.

1.2.1. Antioxidative effects – Structure – activity relationship

Flavonoids can act as antioxidants by four mechanism. They are able to scavenge free radicals or ROS, chelate metal ions, inhibit prooxidant enzymes and activate antioxidant enzymes (Heim et al., 2002, Procházková et al., 2011). Flavonoids can prevent injuries caused be ROS by direct free radical scavenging effect and are able to reduce radicals, while they are oxidizing into a stable hydroquinone and o-quinone form (Fig. 3) (Havsteen 2002, Procházkova et al., 2011, Panche et al., 2016).

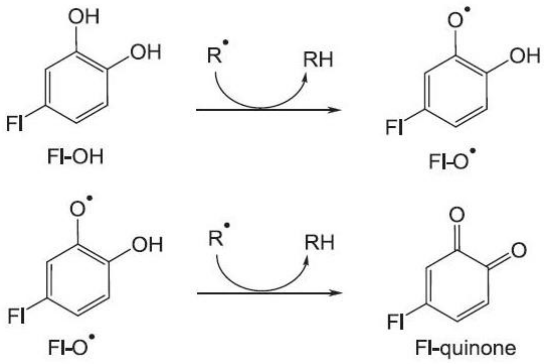

Figure 3. Free radical scavenging reaction of flavonoids. Abbreviation: Fl: chromone structure

Another antioxidant mechanism is the ability to chelate prooxidant metal ions such as Cu, Fe and other trace metals, inactivating them and preventing the free radical generating mechanism. Plant flavonoids are capable of forming stable metal complexes through their ortho- diOH groups on ring B, as well as C_3 and/or C_5 hydroxyl group in conjugation with

the C_4 carbonyl group (Symonowicz & Kolanek 2012, Eghbaliferiz & Iranshahi 2016, Cherrak et al., 2016).

Flavonoids are able to inhibit prooxidant enzymes which are responsible for ROS generation. Generally, the planar structure, the existence of C_2=C_3 double bond and C_4 carbonyl group is also required for this effect. Activity is enhanced by the catechol structure on ring A (Amic et al., 2007, Galleano et al., 2010). They inhibit nitric oxide synthase (NOS) (Duarte et al., 2014. Kim et al., 2015, Chen et al., 2017), xanthin oxidoreductase (XOR) (Lespade & Bercion 2010, Lin et al., 2015), lipoxygenase (LOX) (Ribeiro et al., 2014, Sroka et al., 2017) and cyclooxygenase (COX) (Ribeiro et al., 2015, Chandel et al., 2018).

Finally, flavonoids are able to modulate antioxidant enzyme expression, resulting in an increase of activities of SOD, CAT, and glutathione peroxidase (Zhang et al., 2020), and may interfere with cell interaction pathways (Mansuri et al., 2014).

However, it should be noted that at the concentration that flavonoids can reach the cell, their capability to directly scavenge ROS is marginal if compared with the whole detoxifying system. Therefore, their scavenging effect is probably not very relevant *in vivo*. Nevertheless, flavonoids contribute to the maintenance of redox homeostasis *in vivo* by indirectly modulating antioxidant cell response (Galleano et al., 2010, Gibellini et al., 2015).

1.2.2. Bors' structural criteria for antioxidant action

Over the past thirsty years, numerous studies have been published on the SAR of flavonoids (Rice-Evans et al., 1996, Van Acker et al., 1996, Amic et al., 2007, Chen et al., 2018, Zheng et al., 2019a, Zheng et al., 2019b, Alseekh et al., 2020, Spiegel et al., 2020). Based on these, the structure of flavonoids strongly determines the intensity of their antioxidant activity. It is well accepted, that this is mainly due to the position and number of the hydroxyl groups on the rings A and B, as well as the extensive conjugations between the rings B and C. According to these findings, the basics of general structural criteria for antioxidant activity of flavonoids is summarised by Bors and co-workers (1990) have not changed since then.

These are as follows (Fig. 4):

- The orto 3',4'-dihydroxy or catechol structure on the ring B, which allows high stability to the flavonoid phenoxy radical due to the formation of expanded electron delocalization or hydrogen bonding
- The C_2=C_3 double bond in conjugation with the 4-oxo group on ring C determines the co-planarity of the ring and enhance radical stabilization due to the extensive electron delocalization all over the three ring system
- Presence of 3-OH group on ring C, and 5-OH group on ring A increases antioxidant activity.

According to aforementioned papers, an additional criteria could be added:

- If the catechol structure is not realized on ring B, OH- substituents on ring A are able to compensate, and result in larger radical scavenging activity (Bors et al., 1990, Bors et al., 1995 Amic et al., 2007).

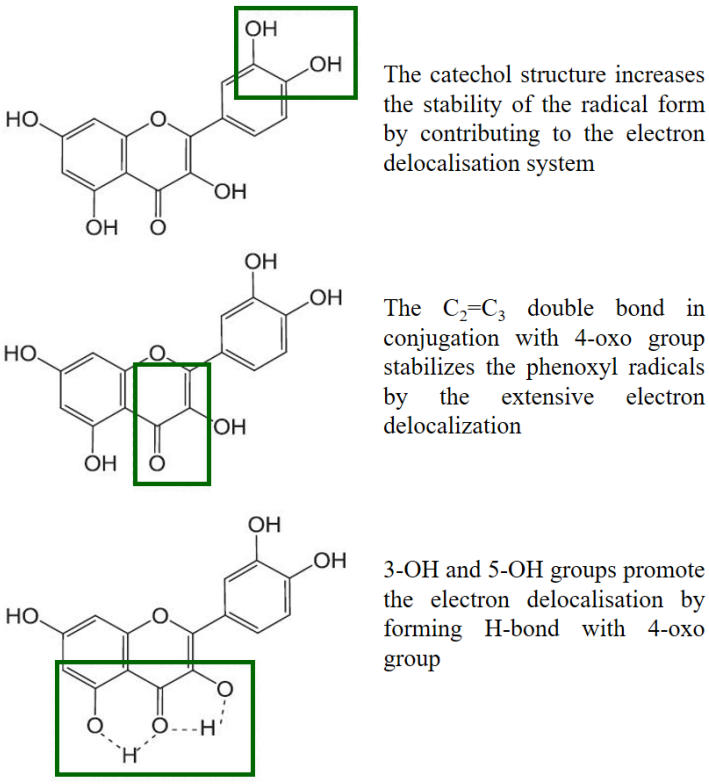

Figure 4. Structure-Activity Relationship of antioxidant flavonoids (Bors et al., 1990)

1.2.3. Radical Scavenging Mechanism

Reaction pathways of the radical scavenging mechanism for flavonoid have been investigated intensively in the last decade. The computation methods for studying SAR and QSAR may provide valuable information about the antioxidant character of flavonoids. Amongst computational methods, density functional theory (DFT) have been successfully used to evaluate the structural-chemical properties related with antioxidant activity (Zheng et al., 2019a, Zheng et al., 2019b, Spiegel et al., 2020). DFT method is suitable for detailed investigations based on three prevalently accepted radical scavenging mechanism of flavonoids: HAT, SET-PT and SPLET, and summarises as follows (Wright et al., 2001, Leopoldini et al., 2011):

HAT – Hydrogen atom transfer mechanism

This is the simplest mechanism, in which flavonoids undergo a homolytic bond dissociation between the H and O atom during free radical trapping (eq. 11). It is characterised by bound dissociation enthalpy (BDE). The lower BDE, the stronger antiradical activity of the flavonoid (Wright et al., 2001, Leopoldini et al., 2011).

$$R^{\bullet} + ArOH \rightarrow RH + ArO^{\bullet} \qquad \text{(eq. 11)}$$

SET -PT – Single electron transfer – proton transfer mechanism

In this two-step mechanism, the first step is an electron release from the molecule forming a flavonoid cation radical (eq. 12), and characterised by ionization potential (IP). In the second step, deprotonation from the cation-radical happens (eq. 13), described by the proton dissociation enthalpy (PDE) (Wright et al., 2001, Leopoldini et al., 2011).

$$R^{\bullet} + ArOH \rightarrow R^{-} + ArOH^{+\bullet} \qquad \text{(eq. 12)}$$

$$R^{-} + ArOH^{+\bullet} \rightarrow RH + ArO^{\bullet} \qquad \text{(eq. 13)}$$

SPLET - Sequential proton loss – electron transfer mechanism

In this two-step mechanism, the first step is the deprotonation of the flavonoid (eq. 14) resulting in a flavonoid phenoxide anion, and characterised by proton affinity enthalpy (PA) followed by an electron transfer from the flavonoid phenoxide anion to the radical (eq. 15), and related to the electron transfer enthalpy (ETE). The antioxidant activity is inversely proportional to the enthalpy required the first step (Wright et al., 2001, Leopoldini et al., 2011).

$$ArOH \rightarrow ArO^{-} + H^{+} \qquad \text{(eq. 14)}$$

$$ArO^{-} + R^{\bullet} \rightarrow ArO^{\bullet} + R^{-} \qquad \text{(eq. 15)}$$

Spiegel and co-workers (2020) concluded that flavonols are more reactive than flavones, and higher the number of hydroxyl groups is higher the antiradical properties of compounds, according to their investigation (Table 1).

Table 1. Relative activity of investigated compounds. Flavonoids have been sorted in decreasing order of the enthalpy for the first step of given mechanism (Spiegel et al., 2020)

Activity	HAT	SET-PT	SPLET
	myricetin	quercetin	quercetin
	quercetin	myricetin	luteolin
	luteolin	kaempferol	morin
	morin	morin	myricetin
	kaempferol	diosmetin	kaempferol
	galangin	galangin	galangin
	diosmetin	luteolin	diosmetin
	apigenin	apigenin	apigenin

1.2.4. Influence of structure modification on antioxidant activity of flavonoids

Flavonoids are one of the most diverse group of plant secondary metabolites. Given the large number of these compounds, it is clear, that many structural modifications occur during biosynthesis, contributing markedly to their effect. According to the Bors' criteria (section **1.2.2.**), these structural modifications significantly affect their antioxidant activity. In the following, the possible modifications are summarised.

Existence of C_2=C_3 double bond and C_4 carbonyl group on ring C

Bors and co-workers (1990) have already described, that the existence of a C_2=C_3 double bond in conjugation with the C_4-carbonyl group on ring C plays important role in antioxidant activity. The electron donating groups are able to induce electron shifts via resonance effect thereby delocalizing ring B electron, which stabilises the phenoxy radical and the planar structure, contributing to the extension of conjugation between ring C and ring A and B (Wang et al., 2018a). The C_2=C_3 double bond saturation limits the electron delocalization, indirectly decreasing reduction potential. Therefore, flavones have stronger radical scavenging activity than flavanones (Burda & Oleszek, 2001, Martino et al., 2012, Spiegel et al., 2020). However, Zheng and co-workers (2019b) reported, that for compounds that only differ with the C_2=C_3 double bond, the antioxidant effect of flavonol and flavone is not always stronger than that of flavanone. In apolar phase, apigenin and luteolin showed weaker effect than naringenin and eryodictiol respectively, but morin was stronger than dihydromorin. In polar medium, the double

bond weaken the scavenging activity via enlarging the proton affinity, consequently, antioxidant capacity of flavonols or flavones are weaker than that of flavanones (Zheng et al., 2019b).

Effect of intramolecular H-bond

The polyhydroxy structure of flavonoids provides to form intramolecular H-bond (IHB). At least two hydroxyl groups, located close to each other is necessary. It most commonly occurs between C_3 or C_5 hydroxyl and C_4 carbonyl groups, or between the hydroxyl groups of ring B. The IHB increases the stability of both the molecule and the radical, due to decrease the energy required to form the radical (Amic et al., 2007, Spiegel et al., 2020). All of the OH groups are affected by IHB originating by C_4 carbonyl group. The hydroxyl group at C_5 is the most influenced and its antiradical activity is the most decreased. In addition, the C_2=C_3 double bond has strong effect on the H-donating ability of OH groups– enhance at C_3, reduce at C_5 (Zheng et al., 2019a, Zheng et al., 2019b). In apolar medium, the IHB reduce the antiradical capacity of OH groups, while in polar phase, C_5-OH proton donating ability was weakened, while that of the other OH groups on ring B and C were strengthened by H5--O=C_4 IHB (Zheng et al., 2019a).

Hydroxylation pattern

The ability of flavonoids to scavenge free radicals correlates with the presence, position and number of OH groups on ring A and B (Bors et al., 1990, Mierziak et al., 2014, Wang et al., 2018a). Only one OH group at position 4' has been deduced to have antioxidant activity. Besides, ortho-dihidroxy group on ring B possess stronger effects, than those with monohydroxy group. Sroka and co-workers (2015) reported, that luteolin with 3',4'-diOH groups is nearly a hundred times stronger antioxidant, than that of apigenin, with mono 4'-OH group on ring B. Flavonoids with at least two hydroxyl group on ring B prefer to donate H atom from the ring B instead of A or C ring. Each additional OH group reduces the system's total enthalpy due to the stabilisation effect. Therfore, di-catechol structure on ring B is preferred, to form ortho-quinone via diradical intermediate state, which is thermodynamically favourable (Amic et al., 2007, Spiegel et al., 2020). However, presence of meta-diOH groups on ring B resulted reduced activity compared to the ortho configuration (Zhang et al., 2020). Hydroxylation pattern of ring A and C affect radical scavenging capacity less than that of ring B, because ortho-dihydroxyl groups on benzopyrane backbone occur less frequently. Generally, meta-dihydroxy groups more

often appears on ring A, especially at position 5 and 7, which may interfere with antioxidant property (Chen et al., 2012).

Glycosylation

Position and number of sugar moiety affects the activity. Although, flavonoid glycosides have notable radical scavenging capacity, the glycosides are weaker than corresponding aglycones (Burda & Oleszek, 2001, Wang et al., 2018a, Zhang et al., 2020). The decreasing activity can be explained by the increasing steric hindrance due to the glycosidic blockage of 3-OH group. Furthermore, co-planarity and electron delocalization are influenced by the sugar chains in any position, which may also decreases the activity (Chen et al., 2012).

Methylation

The methyl substitution of hydroxyl groups has influence not only on hydrophobicity, but on electron donation ability and planarity of the molecule. The steric hindrance of methyl groups on ring B may alter co-planarity which may decrease antioxidant activity (Choe & Min 2009, Wang et al., 2018a).

1.2.5. Prooxidant properties

The prooxidant activity of flavonoids is related to their metal chelating ability. They are capable of autoxidation in the presence of transition metals to produce ROS. The reaction is highly pH and concentration dependent. The higher the pH or the concentration of flavonoids and metal ions, is higher the autooxidation. The number of OH groups, the C_2=C_3 double bond, steric hindrance are also important factors (Fig. 5). Flavonoids with lower reduction potential have higher prooxidant activity (Cao et al., 1997, Prochazkova et al., 2011, Eghbaliferiz & Iranshahi 2016).

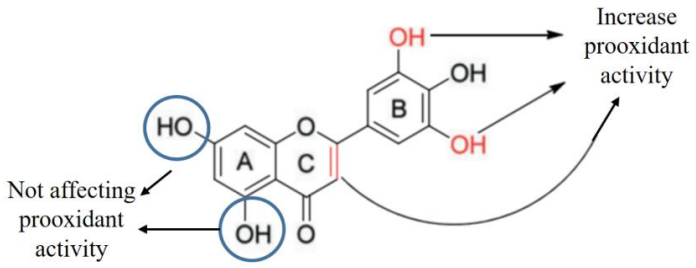

Figure 5. Structur - Activity Relationship of prooxidant effects of flavonoids (Eghbaliferiz & Iranshahi 2016)

1.2.6. Flavonoid-flavonoid interaction

Flavonoids are present in plants as complex mixture, they can interact, which may affect the total antioxidant capacity. Existence of synergistic or antagonistic effects between the various compounds is probable. Hidalgo and co-workers (2010) studied antioxidant activity of different flavonoid mixture with DPPH and FRAP assay. In DPPH system there was statistically significant antagonism between weak antioxidant compounds, e.g.: between pellargonidin-3-glucosid and kaempferol. The occurring H-bonding between flavonoids decreases the availability of OH groups, consequently reduces the DPPH radical scavenging ability. However, synergistic effect was obtained, if anthocyanin was combined with stronger antioxidant flavonoids, such as myricetin. In contrast, combination of flavonoids revealed increased activity using FRAP assay (Hidalgo et al., 2010). Interaction of red grape flavonoids was studied by Iacopini and co-workers (2008). Their results indicated antagonism between flavonoids in DPPH assay, in contrast, synergism was observed between quercetin, rutin and resveratrol in peroxynitrite assay. Moreover, the effect was additive for catechin and epicatechin. So, interaction between flavonoids depends on various factors and requires more detailed research.

It should be mentioned, that due to the weak absorption and low plasma concentration of flavonoids, the significance of *in vivo* interaction between them is limited (Galleano et al., 2010, Gibellini et al., 2015).

1.3. Methods used to evaluate the antioxidant activity

There is a growing interest in the antioxidant activity of natural plant extracts, due to the possible application in prevention and treatment of various diseases associated with oxidative stress (section **1.1.**). Thus, antioxidant capacity is a leading parameter for the determination of biological activities of herbal extracts. For this purpose many different methods have been developed, although, there is still a strong demand for assays that are fast, inexpensive, validated and standardised. According to Apak and co-workers (2016) and Prior and co-workers (2005), antioxidant activity assays should meet the following requirements:

- Simple, fast and available
- Stable and reproducible
- Have a defined endpoint and mechanism

- Adaptable both lipophilic and hydrophilic phase
- Working pH should be around physiological pH
- Utilizes a biologically relevant radical source
- Colorimetric assay should meet Beer's law, and comply with linearity of responses
- Preferential absorption of the chromophore should be characteristic to discriminate analytes from others
- Optimal redox potential of reagent to oxidise the studied antioxidant is required, but should be low enough to leave out non-antioxidants
- Exclusion of strong chelators and reductants when use of transition metal ion coordination complexes is needed.

Therefore, antioxidant activity should not be concluded based on a single test model. Several *in vitro* assays based on different mechanism should be carried out to reveal the real antioxidant capability (Gülcin 2020).

Antioxidant methods can be divided into two groups according to their reaction mechanism: HAT and SET-PT (Apak 2019). HAT based methods measure the ability of quenching free radicals by hydrogen donation. These rapid methods are generally pH and solvent independent, although, presence of metal ions may cause apparently high activity. HAT methods are for example: oxygen radical absorbance capacity (ORAC) and chemiluminescent assays (Prior et al., 2005, Gülcin 2020).

SET-PT base methods measure the ability of potential antioxidant to transfer one electron to reduce compounds such as metal ions, carbonyl groups and radicals. Trace metals may interfere with this method, and these reactions are pH dependent. SET-PT based methods are ferric ion reducing antioxidant power assay (FRAP), cupric reducing antioxidant assay (CUPRAC), 1,1-diphenyl-2-picrylhydrazyl assay (DPPH), trolox equivalence antioxidant capacity or 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) assay (ABTS-TEAC). The total phenolic content assay is also based on SET-PT mechanism (Wright et al., 2001, Prior et al., 2005, Gülcin 2020). Antioxidants can scavenge free radicals by HAT and SET-PT leading to the same results. Although DPPH and ABTS methods are originally classified as SET-PT reactions, these radicals may be also neutralised via H atom transfer (Prior et al., 2005).

The next section presents the widely used radical scavenging methods. These methods are based on scavenging capacity against specific radical species. These species are mostly artificial or biological irrelevant, therefore they are criticised for not reflecting *in vivo* situation. However, results obtained from these methods provide important information about their antioxidant potential. The activities are expressed generally as equivalents of a selected reference compound, such as trolox (Shahidi & Zhong 2015).
ABTS-TEAC, DPPH and HPLC coupled DPPH methods are discussed in detail. In addition, measurement of total phenolic content is also mentioned, due to the correlation between phenolic content and antioxidant activity.

1.3.1. ABTS –TEAC

The ABTS assay was originally developed by Miller and co-workers (1993) and improved by Rice-Evans and Miller (1994) and Re's worker group (1999). The assay measures the ability of an antioxidant to scavenge the stable radical cation $ABTS^{\bullet+}$ (2,2'-azinobis(3-ethylbenzothiazoline-6-sulphoric acid). The $ABTS^{\bullet+}$ should be generated before the assay from ABTS in the presence of a strong oxidative agent. The radical cation can be generated by chemical reaction with manganese dioxide, potassium persulfate or enzyme reactions by horseradish peroxidase. The chemical generation requires a long time or high temperature. For the mechanism of $ABTS^{\bullet+}$ generation see equation 16-17 below (Prior et al., 2005, Gülcin 2020, Ilyasov et al., 2020):

$$ABTS + S_2O_8^{2-} \rightarrow ABTS^{\bullet+} + SO_4^{\bullet-} + SO_4^{2-} \quad \text{(eq. 16)}$$

$$ABTS + SO_4^{\bullet-} \rightarrow ABTS^{\bullet+} + SO_4^{2-} \quad \text{(eq. 17)}$$

The bluish-green $ABTS^{\bullet+}$ has absorption maximum at 415, 645, 734 and 815 nm. Among them 734 nm was adopted usually for spectrophotometric investigations to eliminate the possible interference of other compounds. The intensity of absorption of radical solution decreases in the presence of an antioxidant (Cano et al., 2000). The neutralisation reaction (Fig. 6) may occur by quenching the radical via hydrogen atom donation or by direct reduction via electron donation. The mechanism depends on the structure of the antioxidant and the medium pH. However, polyphenols in aqueous or methanolic medium imply the SPLET mechanism (Prior et al., 2005, Ilyasov et al., 2020).
The reaction times range from 1 to 30 minutes, however commonly used end-points are 4 or 6 minutes (Gülcin 2020, Ilyasov et al., 2020). Usually, the result are expressed by

comparison with standard antioxidant such as trolox, hence, the name trolox equivalent antioxidant capacity (TEAC). Results can also be expressed as IC_{50}, which is the effective concentration of the antioxidant, which is necessary to decrease the initial ABTS concentration by 50% (Miller et al., 1993, Prior et al., 2005).

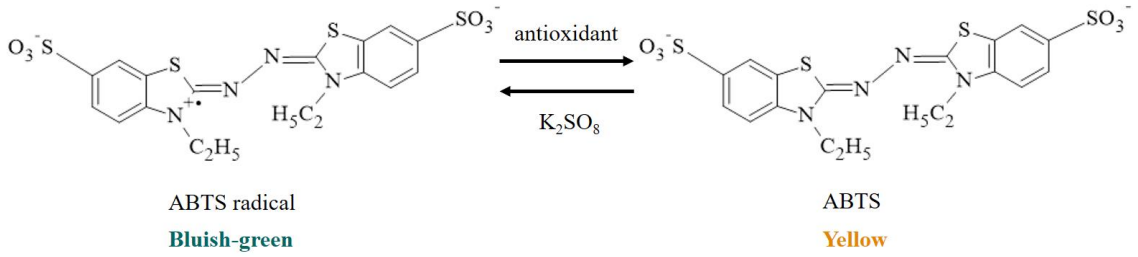

Figure 6. Neutralisation reaction of ABTS assay

ABTS assay is simple, fast, and can be done as a routine analysis. It can be performed in water and organic solvents enabling the measurement of both lipophilic and hydrophilic compounds, and is not affected by ionic strength. The method can be evaluated over a wide pH range (Shahidi & Zhong 2015, Gülcin 2020). It can be automated and coupled with HPLC for post column on-line antioxidant detection (Stalmach et al., 2006, Shi et al., 2009, Grandois et al., 2017).

The method has disadvantages. ABTS is a non-physiological radical source. In storage, due to temperature and light exposition, the $ABTS^{\bullet+}$ may decompose, or partly turn back to ABTS which can affect the measured antioxidant capacity. Even for some standard antioxidants, different TEAC values can be observed between laboratories due to incomplete scavenging reaction, or concentration independent antioxidant activity (Ilyasov et al., 2018. Ilyasov et al., 2020). The maximal absorption wavelengths of $ABTS^{\bullet+}$ is solvent dependent (Dong et al., 2015). The number of phenolic hydroxyl groups generally correlated with the ABTS scavenging capacity, however, some results are out of this line due to steric effect. It makes it difficult to compare antioxidant activity of different classes of compounds, or compare natural extracts with very different composition (Nenadis et al., 2004, Tian & Schaich 2013).

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1.3.2. DPPH

DPPH radical scavenging assay is one of the most frequently used methods, developed by Blois (1958). The widely used modified assay was reported by Brand-Williams and co-workers (1995). DPPH$^{\bullet}$ (2,2-diphenyl-picrylhydrazyl) is a stable radical due to the electron delocalisation spread over the whole molecule. It has deep purple colour characterised by an absorption maximum around 517 nm, and which is vanishing in presence of antioxidant samples, therefore can be measured by spectrophotometry. The method is based on reduction of the DPPH by a H-donating antioxidant, results the yellow, non-radical hydrazine form (Fig. 7) (Blois 1958).

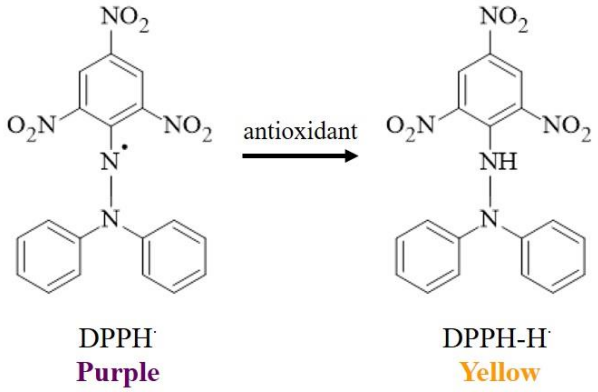

Figure 7. Reduction reaction of DPPH assay

DPPH reacts with polyphenols via two mechanisms. The first is the direct abstraction of H atom from a phenolic compound (HAT), the second is an electron transfer process from ArOH or its phenoxide anion to DPPH (SET-PT), see the following equations 18-21 (Foti et al., 2004, Litwinienko & Ingold 2004, Apak et al., 2016):

$$ArOH + DPPH^{\bullet} \rightarrow ArOH^{\bullet} + DPPH_2 \text{ (HAT)} \quad \text{(eq. 18)}$$

$$ArOH \leftrightarrow ArO^{-} + H^{+} \text{ (SET-PT)} \quad \text{(eq. 19)}$$

$$ArO^{-} + DPPH^{\bullet} \rightarrow ArO^{\bullet} + DPPH^{-} \quad \text{(eq. 20)}$$

$$DPPH^{-} + H^{+} \rightarrow DPPH_2 \quad \text{(eq. 21)}$$

Results are expressed as EC_{50} or IC_{50}, which is the effective concentration of the antioxidant necessary to decrease the initial DPPH concentration by 50%, which values were calculated from the plot of inhibition percentages in function of concentration. The lower is the value of EC_{50} is the higher antioxidant activity. Term of ARP (Antiradical power) is also used, which is the invers of EC_{50}, hence larger the ARP, the more efficient

is the antioxidant (Brand-Williams et al., 1995). TEC_{50}, the time which is needed to reach the steady state is also reported and Antiradical efficiency (AE) is also used (eq. 22) (Sánchez-Moreno et al., 1998, Villano et al., 2007 Shahidi & Zhong 2015).

$$AE= (1/EC_{50})*TEC_{50} \quad \text{(eq. 22)}$$

The DPPH method is simple, inexpensive, available, accurate, reproducible, and it does not require special sample treatment. DPPH is commercially available and does not have to be generated before the assay. The method may examine both lipophilic and hydrophilic compounds (Prior et al., 2005). Automation, as well coupling with chromatographic methods are solved, and complex biological systems, solid or liquid samples can be measured (Kedare & Singh 2011, Zhang et al., 2012).

The method also has disadvantages. Interference with for example anthocyanins and carotenoids with maximum absorption around 500 nm may lead to underestimation of antioxidant activity (Prior et al., 2005). Sensitivity may be affected by the type and amount of solvent used, presence and concentration of metal ions. The sensitivity depends on pH and freshness of DPPH, too. It does not imitate the radical scavenging mechanism of biological systems (Shahidi & Zhong 2015, Magalhaes et al., 2008). Absorbance of methanolic or acetonic solution of DPPH is decreased under light, therefore, measurement must be performed protected from light (Ozcelik et al., 2003). If DPPH scavenging reaction follows HAT mechanism, it will be strongly influenced by kinetic solvent effect, and differences in the strength of H-bonding of the solvent to phenolic hydroxyl groups. Furthermore, steric accessibility may influence the applicability, large compounds may react slowly or may be even inert (Litwinienko & Ingold 2004, Prior et al., 2005, Xie & Schaich 2014, Amorati & Valgimigli 2014, Apak et al., 2016). Over 60% water content DPPH may precipitates (Stasko et al., 2007).

HPLC-coupled DPPH methods

The HPLC based hyphenated techniques coupled with DPPH radical scavenging assay have been successfully utilized for rapid screening and identification of antioxidant compounds from complex mixtures such as plant extracts. These methods enable complete separations and activity tests without prior isolation, thereby saving time and money. Two main methods based on HPLC separation combined with DPPH assay have been developed.

The first one is off-line DPPH spiking HPLC analysis developed by Yamaguchi and co-workers (1998) and it is still widely used (Chandrasekar et al., 2006, Tang et al., 2008, Könczöl et al., 2012, Zhang et al., 2012, Riethmüller et al., 2016, Ayanlowo et al., 2020). In this method the peak areas of antioxidant compounds are decreased after spiking with DPPH, due to the oxido-reduction reaction of the DPPH (Fig. 8) while peak areas of non-active compounds are not changed. Consequently, peak area of DPPH must decrease when it is reacted with antioxidants (Fig. 8) (Ayanlowo et al., 2020).

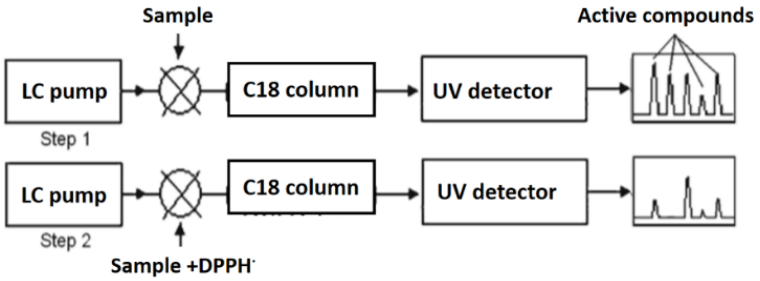

Figure 8. Schematic view of DPPH spiking HPLC assay (Zhang et al., 2012)

Therefore, this method is suitable to screen individual antioxidants as well as determining the total antioxidant activity of plant extracts. Advantages are that the interference caused by coloured pigments are removed and that the method is more sensitive and more rapid than *in vitro* spectrophotometric methods (Chandrasekar et al., 2006, Kedare &Singh 2011, Zhang et al., 2012).

The second one is the on-line post column HPLC-DPPH analysis, which was first developed by Koleva and co-workers (2000). In this method compounds are separated by HPLC method and then are reacted post-column with DPPH. It requires more complex instrumental set up (Fig. 9). The method optimisation is more difficult than other coupled techniques. The on-line HPLC-DPPH method is widely applicable, hydrophilic and lipophilic constituents also can be evaluated, mobile phase and pH can be changed on a wide range. The major advantage is that it provides immediately clear results on which compounds possesses antioxidant activity (Bandoniene 2002, Li et al., 2012, Zhang et al., 2012, Pedan et al., 2016, Dang et al., 2020).

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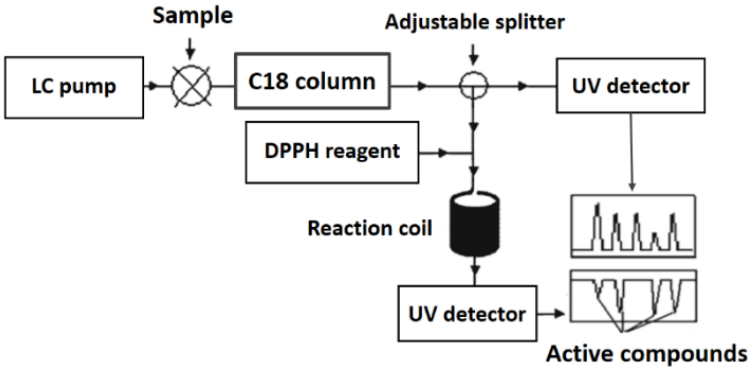

Figure 9. Schematic view of on-line post column HPLC-DPPH assay (Zhang et al., 2012)

1.3.2. Total Phenolic Content (TPC) - Folin Ciocalteu assay

Although total phenolic content is actually not a free radical neutralisation based antioxidant method, but shows linear correlation with antioxidant ability. The original method was developed by Folin and Ciocalteu to determine a phenolic amino acid tyrosine in proteins in 1927. Singleton and Rossi (1965) improved the method, and their method with some modification is official in Pharmacopoeia Hungarica (2003). Generally, total phenolic content is expressed in gallic acid equivalent (GAE) g/100g herbal drug.

The assay is based on a reduction via electron transfer mechanism of the Folin-Ciocalteu (F-C) reagent by phenolic compounds. The F-C reagent is mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$) that are reduced to yield a blue reaction product in alkaline medium with maximum absorption at 765 nm. The improved method uses molybdotungstophosphoric heteropolyanion reagent (Singleton & Rossi 1965). The exact mechanism is not known, however probably the molybden centre of the complex is the reduction site (eq. 23-24) (Singleton 1999, Prior et al., 2005).

$$Na_2WO_4 / Na_2MoO_4 + \text{phenol} \rightarrow (\text{Phenol-}MoW_{11}O_{40})^{4-} \quad \text{(eq. 23)}$$

$$Mo^{5+}\ (\text{yellow}) + e^- \rightarrow Mo^{4+}\ (\text{blue}) \quad \text{(eq. 24)}$$

The advantages are the sensitivity, simplicity, reproducibility and robustness. The results correlate with those obtained with other antioxidant assays such as ORAC, ABTS, DPPH, and FRAP (Prior et al., 2005, Piluzza & Bullita 2011, Büyüktuncel et al., 2014, Ulewicz-Magulska & Wesolowski 2019). The absorption of the product at 765 nm minimizes interferences from sample matrix and other constituents (Gülcin 2020).

The TPC method is sensitive to pH, temperature and reaction time. There are a lot of interfering compounds such as non-phenolic organic compounds, sugars, ascorbic acid, inorganic substances, metal ions etc. It has to be performed in aqueous medium, thus not suitable to determine lipophilic compounds (Prior et al., 2005, Gülcin 2020).

1.3.3. Comparison of various methods for antioxidant activity

Nowadays, there is a growing interest in naturally occurring antioxidant compounds, which are becoming an essential part of preventive healthcare. Their measurement requires reliable methods. Since plant extracts are complex mixtures, it must be understood, that there is no single, universal method, by which antioxidant activity can be measured accurately and quantitatively. Many variables associated with the free radicals and various reaction mechanism of individual antioxidants, and other factors must be considered. To have a reliable result of antioxidant measurement, at least two assays should be used. However, differences in the implementation of antioxidant activity measurement, and in terms of expression of the obtained results, make the comparison difficult between antioxidant data of different laboratories, worker groups and publications, even if the same sample was investigated. The comparison of methods evaluating antioxidant activity are summarised in Table 2.

Due to the rapidity, simplicity and most widespread use of DPPH and ABTS assays in analysis of natural plant compounds, these two methods were chosen to determine the antioxidant activity of *Lysimachia* herbal extracts. Furhtermore, the HPLC-coupled DPPH technique allows to screen individual antioxidants to get a more accurate comparison of the six *Lysimachia* sepcies studied.

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Table 2. Comparison of the main antioxidant activity assays

Assay	Mecha-nism	Oxidant	Probe	Detection	Unit	Advantages	Disadvantages	Reference
ORAC	HAT	$ROO^{\cdot}$ generated by AAPH	Fluores-cein	Fluoro-metry	Trolox equivalent	biological relevant free radical integrates both degree of inhibition and time of antioxidant reaction	pH and temperature sensitive fluorimeter is not routinely available long analysis time	Ou et al., 2001, Ou et al., 2002
Chemi-luminescence	HAT	H_2O_2	Luminol	Fluoro-metry	EC_{50}	fast, sensitive, inexpensive, reproducible both lipo- and hydrophilic measurement	the redox active may impair the measured antioxidant activity fluorimeter is not routinely available	Lind et al., 1983, Shahidi & Zhong 2015
ABTS-TEAC	HAT and SET-PT	$ABTS^{\cdot 2+}$ generated by $K_2S_2O_8$	ABTS radical cation	Spectro-fotometry	IC_{50} / Trolox or ascorbic acid equivalent	applicable in both aqueous and organic phase stable to pH	sensitive with temperature and light hard to compare values across laboratories	Miller et al., 1993, Re et al., 1999
DPPH	HAT and SET-PT	$DPPH^{\cdot}$	DPPH radical	Spectro-fotometry	IC_{50}	simple, inexpensive and widely used good repeatability, fast	dissolved in organic solvent sensitive to pH and light colour interference	Blois 1958
FRAP	SET-PT	Fe^{3+}	Ferri-cyanide	Spectro-fotometry	Fe^{2+} or Trolox / ascorbic acid equivalent	easy inexpensive	SH containing antioxidant are not detected uses low pH, colour interference reaction times varies	Benzie & Strain 1996, Benzie & Strain 1999
CUPRAC	SET-PT	Cu^{2+}	Neo-cuproin	Spectro-fotometry	Trolox equivalent	inexpensive, fast, stable, and selective and measure both lipophilic and hydrophilic compounds	reaction time varies	Apak et al., 2004, Apak et al., 2006
TPC – Folin-Ciocalteu	SET-PT	Mo^{5+}	Phospho-molibdo-tungstic acid	Spectro-fotometry	gallic acid equivalent	sensitive, simple, reproducible, robust correlate with results of ORAC, ABTS, DPPH, FRAP assays	Sensitive to pH, temperature and reaction time colour interference not suitable for lipophilic compounds	Folin & Ciocalteu 1927

1.4. *Lysimachia* genus

1.4.1. Taxonomy and spreading

The *Lysimachia* genus contains more than 190 species, and traditionally belongs to Spermatophyta superdivision, Magnoliophyta division, Magnoliopsida class, Dilleniidae subclass, Primulales order and Primulaceae family. However, according to former morphological studies and molecular phylogenetic analyses the genus was moved to the Myrsinaceae family (Anderberg and Ståhl, 1995, Anderberg et al., 1998, Anderberg et al., 2002, Anderberg et al., 2007, Hao et al., 2004, Källersjö et al., 2000), and then transferred back to Primulaceae again (Rose et al., 2018).

They are perennial, rarely evergreen herbaceous plants and commonly found in the temperate zone of the Northern Hemisphere. The most known species are native to China. Various species occur in swamps, wet meadows, riverside and waterfront associations, groves and bog forests. *Lysimachia* species mostly prefer moist, weakly acidic soils (Wagner 1903, Hänsel et al., 1976).

The *Lysimachia* genus comprises five species native to Hungary: *L. vulgaris* L. (közönséges lizinka), *L. nummularia* L. (pénzlevelű lizinka), *L. punctata* L. (pettyegetett lizinka), *L. thyrsiflora* L. (fürtös lizinka), *L. nemorum* L. (berki lizinka). The last two are extremely rare and the latter one is protected plant (Wagner 1903, Hänsel et al., 1976).

1.4.2. Etymology

The name Lysimachia has Greek origin. It derives from “lysis” meaning loose or released from, and “machia” meaning fight or strife, given the loosestrife name in English. Another possibility is that the name was given in honour of Lysimachus, the ancient king of Thrace and Macedonia. It was said, that Lysimachus was being chased by an angry bull in a battle, and in desperation he picked up a plant of loosestrife and waved it in the bull’s face to calm it down (Runkel & Bull 2009). The name of Lysimachia is still preserved by a village and a see in Greece. In Hungarian, Lizinka, is an officially registered female name (Magyar Tudományos Akadémia 2020).

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1.5. *Lysimachia vulgaris* L.

The yellow or garden loosestrife (*Lysimachia vulgaris* L.) is a perennial herbaceous plant with few elongated rhizomes (Fig. 10). The barely square and woolly stem is usually 40-120 cm tall, simple or paniculately branched at the top. The short stemmed, almost sessile leaves are located opposite or verticillate. The fluffy lanceolate or oblong-ovate leaves are 5-10 cm long and dotted with yellow. The flowers forms terminal panicles or sit one by one in the axils of the upper leaves. The sepals have reddish margin. The five-petalled corolla is yellow, with red glandulars. The unequal anthers are long and hairy. It has approximately 5 mm subglobes capsules are usually reddened above. It contains white, trigonal 15-25 seeds. Flowering time is from June to late August (Wagner 1903, Ray 1956).

Figure 10. *Lysimachia vulgaris* L. (https://inflora.ch, 02.02.2021.)

The juice of the leaves was used to relieve gastrointestinal diseases such as diarrhoea and dysentery. It is used externally to stop nasal and oral mucosal haemorrhage and to treat purulent inflammatory wounds due to astringent properties (Zelenyák 1908, Szabó 2010). Although *L. vulgaris* is used as traditional remedy, only few studies have been published on its biological effects. It has antioxidant (Hanganu et al., 2016, Yildirim et al., 2017), antiproliferative, cytotoxic (Podolak et al., 1998, Polodak et al., 2013a), antifungal (Podolak et al., 1998), antimicrobial (Yildirim et al., 2017), and hepatoptrotecitve (Kim et al., 2019) effects.

1.5.1. Phytochemical characterisation

Phytochemical characterisation of *L. vulgaris* has been under investigation since the seventies. Presence of various phenolic compounds, including flavonoids, especially flavonols, such as kaempferol quercetin and myricetin and their glycosidic form, as well as flavones, luteolin and apigenin are described (Rzadkowska-Bodalska & Olechnowicz-Stepień 1975, Karakas et al., 2015, Hanganu et al., 2016, Yildirim et al., 2017).

Plant acids, for example chlorogenic-, gallic- and caffeic acid derivatives are also reported (Luczak et al., 1989, Karakas et al., 2015, Hanganu et al., 2016, Yildirim et al., 2017).

Benzoquinone pigment, embelin and rapanone in the underground part (Janik et al., 1994, Podolak et al., 1998, Podolak & Strzalka 2008), benzofuran loliolide and a lignin, pinoresinol in the aerial part of the plant have been revealed (Kim et al., 2019). Compounds responsible for scent were characterised with benzyl alcohol as the most abundant component (Schäffler et al., 2012). The presence of genus specific triterpene saponins, among them 13,28-epoxy-3,16-oleananediol derivative lysikokianoside 1 were confirmed (Podolak et la., 1998, Podolak et al., 2013a).

1.6. *Lysimachia nummularia* L.

Creeping jenny or moneywort (*L. nummularia* L.) is a perennial, evergreen herbaceous plant and has 10-50 cm long branched repent stems with mostly bald or rarely hairy shoots (Fig. 11). It rises slightly only in dense stocks. The leaves are opposite arranged in plane, mostly suborbicular or rounded-heart shaped 1.5-3 cm wide, with entire margins, dark green and mottled with red glandular. The five-petalled flowers develop solitary in the axils of the middle leaves. The corolla is crateriform, yellow, the lobes are ovate or oblanceolate and dotted with dark red glands inside. The staminal tube densely glandular and slightly fused at the base. The filaments are unequal and glandular. It has spherical capsules with black-brown trigonal warts seeds. The flowering period is from May to August (Wagner 1903, Ray 1956).

Figure 11. *Lysimachia nummularia* L. (https://fs.fed.us, 02.02.2021.)

It is used in the treatment of rheumatic pain, bruises and fractures. It is useful for chronic skin infections, erythrasma, stomatitis, gingivitis, and paradontosis due to its astringent and anti-inflammatory properties. It was also used to treat wrinkles and varicose veins. Alcoholic or wine extracts and tea were used to cure stomach ailments, dysentery, haemorrhoids and lung diseases (Zelenyák 1908, Rácz 1984, Papp 2011, Makai 2005).

Although *L. nummularia* is used as traditional medicine, its biological effects are less researched. Antioxidant effect (Hanganu et al., 2016, Csepregi et al., 2020) and cytotoxicity is reported (Podolak et al., 2013a, 2013b). Csepregi and co-workers (2020)

publicated, that ethanolic extract of *L. nummularia* showed moderate antimicrobial activity on *B. subtilis* and *S. pyogenes*.

1.6.1. Phytochemical characterisation

Flavonoids of *L. nummularia* have been getting to the forefront of interest in the last decade. Presence of various flavonols and their glycosides, such as kaempferol, quercetin and myricetin derivatives are reported (Yasukawa et al., 1990, Hanganu et al., 2016, Csepregi et al., 2020). Yasukawa and co-workers (1990) mentioned methylated compounds, mearnsitrin and syringetin-3-*O*-galactoside. However, Hanganu's research group (2016) does not confirm the accumulation of methylated flavonoids, but mention flavone type luteolin and apigenin.

Plant acids are also characteristic compounds of the species, among them caffeoyl-coumaroyl- and feruloyl derivatives are the most common (Luczak et al., 1989, Csepregi et al., 2020). The root, stem and leaves contains benzoquinone type embelin (Podolak & Strzalka 2008). Volatile constituents were summarised by Schäffler and co-workers (2012), with 1-phenyl-1,2-propanedione was identified as a major component. Triterpene saponins are also genus specific in *L. nummularia*, among them nummularoside was isolated and reported as a new compound (Podolak et al., 2013a, 2013b).

1.7. *Lysimachia punctata* L.

The morphological appearance of spotted loosestrife (*Lysimachia punctata* L.) closely resembles *L. vulgaris*, but it usually has more hair on vegetative and reproductive organs and its inflorescence is simpler (Fig. 12). The *L. punctata* is a perennial herbaceous plant with few slender and cord-like rhizomes. The erect stem is usually 40-120 cm tall, simple or branched, glabrescent below and villous above. The lower leaves are scale-like, the medials are verticillate or opposite, 5-10 cm long and has ovate to lanceolate shape, and being glandular punctated or puncticulate near the apex. The five

Figure 12. *Lysimachia punctata* L. (http://brainmanpictures.piwigo.com, 02.02.2021.)

petalled, yellow flowers located axillary in the upper leaves. The calix is imbricate, the corolla is craterifrom with yellow glandular. It has globose capsules and few trigonal seeds. Flowering time is from June to late August (Wagner 1903, Ray 1956).

There is no data on its medicinal use, but *L. punctata* is preferred to be planted as an ornamental plant. The biological effects of the species have been incompletely explored. As far as we know, only a benzoquinone pigment, embelin, isolated from the root was studied. It has cytotoxic activity against mouse melanoma and rat sarcoma cell lines (Podolak et al., 2005).

1.7.1. Phytochemical characterisation

Although *L. punctata* is one of the most common ornamental plant species in the genus, its phytochemical description is strongly incomplete. Myricetin -rhamnoside, -arabinoside and -glucoside are reported by Mendez (1970). The root contains benzoquinone type embelin and rapanone (Podolak et al., 2003, Podolak et al., 2005, Podolak & Strzalka 2008). Triterpene saponin ardisicrispin A was also isolated from the root (Podolak et al., 2013a). Volatile compounds of floral stem were characterised and fatty acid derivatives, aromatics, mono- and sesquiterpenoids were found. The main compound of floral essential oil was 1-phenyl-1,2-propanedione (Dötterl & Schäffler 2007, Schäffler et al., 2012).

1.8. *Lysimachia christinae* Hance

Jin Qian Cao or gold coin grass (*L. christinae* Hance) is a perennial rhizomatous creeping plant (Fig. 13). The procumbent stems are 20-60 cm long, and glabrous or rust-coloured pilose, young parts can be glandular. The 2-6 cm long leaves are opposite and has ovate to suborbicular or reniform shape, mostly glabrous and densely covered transparent glandular stripes. The five parted yellow flowers are located solitary and axillary. Capsules are round. The flowering lasts May to July (Flora of China, 1996).

Figure 13. *Lysimachia christinae* Hance (http://plantjdx.com, 02.02.2021.)

Lysimachia christinae or Jin Qin Cao is a very popular traditional Chines medicinal herb. It is used internally to treat urinary retention, painful urination, kidney stones, jaundice, bloating, loss of appetite, fatigue and gallstones. It is considered to have hepatoprotective properties, therefore it is used in fungal and drug poisoning, and infections. Externally, it is used to treat ulcers, infected wounds, snake bites, eczema and burns, because it reduces swelling and oedema. It is often combined with other medicinal plants (Hempen & Fischer 2009, Hou & Jin 2005).

L. christinae is the most widely used one among the mentioned species, and it is official in the Pharmacopoeia of the People's Republic of China (2005), therefore, numerous pharmacological studies have been published in the literature, especially in the Far East. According to recent publications from the last five year, the most researched effects are its cholagogue, serum cholesterol level decreasing, hepatoprotective (Deng et al., 2015, Wang et al., 2018b), aldose reductase inhibitory (Wang et al., 2017) and neuroprotective effect (Ryu & Ma 2020). Furthermore, it has beneficial effect on kidney and endothelial function (Meng-Yan et al., 2018, Wang et al., 2020, Wu et al., 2018). Cytotoxic and apoptosis promoting activity have also been reported (Cui et al., 2017, Kim et al., 2020a). Alcoholic extracts function as antioxidant (Wu et al., 2018) and protect the skin from exposition of UV radiation (Kim et al., 2018), moreover, they effectively prevent wrinkles and skin aging by activating reduced collagen synthesis (Kim et al., 2020b). It has beneficial effects on postmenopausal osteoporosis by inhibiting osteoclast differentiation and obesity by suppressing fat accumulation in mice (Shim et al., 2020).

1.8.1. Phytochemical characterisation

The results of phytochemical investigations are published in detail. The main constituents are phenolics, and the pharmacological effects are attributed to the flavonoid content of the plant. The main compounds are flavonols as kaempferol, quercetin and myricetin derivatives, apigenin and luteolin flavones and their C-glycosidic derivatives as vitexin and orientin. Flavanone type metabolites as naringenin, methylated flavonoids as diosmetin and flavan-3-ols, like catechin and its derivatives are also reported (Gao et al., 2013, Sun et al., 2013, Kim et al., 2018, Liu et al., 2019, Shim et al., 2020).

Beside flavonoids, it contains plant acids as quinic acid, coumaric acid, ferulic acid and chlorogenic acids (Sun et al., 2013, Kim et al., 2018, Shim et al., 2020). The genus-specific triterpene saponins (Tian et al., 2008) have been less researched in this species.

1.9. *Lysimachia clethroides* Duby

Gooseneck loosestrife (*L. clethroides* Duby) is frequently cultivated in gardens (Fig. 14). It is a perennial herbaceous and rhizomatous plant with upright, 50-100 cm tall stem which is villous above and glabrescent below. The leaves are alternate, scattered, and have an elliptic to lanceolate shape with glandular dots. The white, five petalled flowers are grouped in terminal spikes. The corolla is contorted, without glandular dots. The capsules are turbinate shape and 5-valvate. It flowers throughout summer (Ray 1956). *Lysimachia clethroides* is a favored ornamental plant, and a traditional Chinese herbal medicine. It has been widely used to treat dysmenorrhea, throat ache and oedema (Lee et al., 2010, Liang et al., 2011). Pharmacological research showed that *L. clethroides* have many beneficial activities. Among others, it has antitumor (Liu et al., 2010, Liang et al., 2011, Xu et al., 2012, Podolak et al., 2013a), antioxidant (Wei et al., 2012a, Wei et al., 2012c, Wei et al., 2017), antidiabetic (Wei et al., 2012a, Liang et al., 2013b), hepatoprotective (Wei et al., 2012c Zhang et al., 2016), vasorelaxant (Lee et al., 2010), antimicrobial (Han & Shin 2001, Joung et al., 2018), anti-inflammatory (Shim et al., 2013) effects. Polyphenols including flavonoids, enriched in alcoholic extracts, have been shown to play role in the development of the mentioned effects, however, the antitumor effect is due to its triterpene saponin content.

Figure 14. *Lysimachia clethroides* Duby (http://calphotos.berkeley.edu, 02.02.2021. (V.Kurzenko))

1.9.1. Phytochemical characterisation

Flavonoid pattern of *L. clethroides* is reported in detail. Flavonol derivatives such as astragalin, isoquercitrin, quercetin, kaempferol and their various glycosides and methylated derivatives (e.g.: isorhamnetin-rutinoside) (Liu et al., 2010, Wei et al., 2017),

flavanone, such as naringenin and eriodictiol and flavan-3-ol type catechin derivatives are described (Liu et al., 2010). Furthermore, acylated flavonol glycosides and δ-truxinate derivatives (Liang et al., 2015), benzoquinone type embelin (Podolak & Strzalka 2008) are published.

Presence of triterpene saponins are widely reported, among them betulinic acid, hederagenin, α- and β amyrin, lysilactones A-E, clethroidosides A-H are structurally characterised (Liang et al., 2011, Wei et al., 2012a, Xu et al., 2012, Podolak et al., 2013a, Liang et al., 2013a). Volatile constituents of the stem, leaves and flowers are summarised by Wei's worker group (2012b), with limonene identified as a major component. However, in the flower benzyl alcohol, and in the aerial parts, (Z)-3-hexenyl acetate were reported as main compounds by Schäffler and co-workers (2012). Presence of carboxylic acid derivatives (Liang et al., 2013b) and glycerolipids (Liang et al., 2012) was also mentioned.

1.10. *Lysimachia ciliata* L. var. 'Firecracker'

Fringed loosestrife (*L. ciliata* L.) is an upright, 50-100 cm tall plant (Fig. 15) with few elongate rhizomes. The stem is apparently bare, but in the upper region glandular. The 5-15 cm long leaves are opposite, thin, glabrous, and has ovate to lanceolate shape. They are purple but at the end of summer become more or less green. The five petalled, star-shape yellow flowers grows solitary in the upper leaves axils, sometimes with red blotches at the base of each petals The blooming time is from early to late summer. *L. ciliata* is a popular garden plant (Ray 1956).

Figure 15. *Lysimachia ciliata* L. *var.* 'firecracker' (http://tuja.hu, 02.02.2021.)

Although, *L. ciliata* is widespread on the Northern – American continent (Simpson & Neff 1983, Coffey & Jones 1980), no data has been reported about its traditional medical application. Only a few research deals with the biological effects. Desglucoanagalloside B and anagallosaponin IV isolated from the root possessed synergistic cytostatic and proapoptotic effect with mitoxantron on prostate cancer cell lines (Koczurkiewicz et al.,

2013. Koczurkiewicz et al., 2016). Significant cytotoxic and antiproliferative effect of anagallisin C and anagallosaponin IV against high metastatic melanoma cell line (BLM) have been described (Podolak et al., 2013a).

1.10.1. Phytochemical characterisation

Results of phytochemical investigation of *L. ciliata* are insufficient. Presence of the genus specific triterpene saponins has been described. Anagallisin C and anagallosaponin IV were isolated underground and aerial part of *L. ciliata* (Podolak et al., 2013a). The most abundant compound in the floral essential oil of *L. ciliata* is benzaldehyde (Schäffler et al., 2012). Benzoquinone type embelin is reported (Podolak & Strzalka 2008). No previous literature data has been found dealing with the phenolic composition.

The ethnomedicinal use, phytochemical characteristics and pharmacological effects of the six *Lysimachia* species were summarised in Table 3.

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Table 3. Summary of ethnomedicinal use, phytochemichal characteristics and pharmacological effects of *Lysimachia* species

Species	Spread	Traditional medicinal use	Phytochemistry	Pharmacology
Lysimachia vulgaris	Europe	Diarrhoea and dysentery Nasal and oral mucosal haemorrhage Purulent inflammatory wounds	Flavonoids: flavonols and flavones Plant acids: chlorogenic-, gallic- and caffeic acid derivatives Benzoquinone pigment: embelin and rapanone Benzofuran derivative: loliolide, lignin: pinoresinol Volatile compounds: benzyl alcohol Triterpene saponins: Lysikokianoside 1	Antioxidant Antiproliferative, Cytotoxic Antifungal Antimicrobial Hepatoptrotecitve
Lysimachia nummularia	Europe	Rheumatic pain, bruises, fractures Chronic skin infections, stomatitis, gingivitis, Wrinkles and varicose veins Dysentery, haemorrhoids Lung diseases	Flavonoids: flavonols, flavones, methylated derivatives Plant acids: caffeoyl- coumaroyl- and feruloyl derivatives Benzoquinone: embelin Volatile compounds Triterpene saponins: nummularoside	Antioxidant Antimicrobial Cytotoxic
Lysimachia punctata	Europe	No data	Flavonoids: flavonols Benzoquinone: embelin, rapanone Volatile compounds: aromatics, mono- and sesquiterpenoids Triterpene saponins: ardisicrispin A	Cytotoxic
Lysimachia christinae	Far-East	Urinary retention, Painful urination, kidney stones, Bloating, fatigue Loss of appetite, Gallstones, jaundice, Fungal and drug poisoning, Ulcers, infected wounds, Snake bites, Eczema and burns,	Flavonoids: flavonols, flavones, flavone-C-glycosides, flavaonones, methylated derivatives, flavan-3-ols Plant acids as quinic acid, coumaric acid, ferulic acid and chlorogenic acids Triterpene saponins	Cholagogue, hepatoprotective Serum cholesterol level decreasing Aldose reductase inhibitor Neuroprotective Renal protection Cytotoxic, Antioxidant Prevent winkles and skin aging Inhibiting osteoclast differentiation
Lysimachia clethroides	Far-East	Dysmenorrhea, Throat ache Oedema	Flavonoids: flavonols, flavaones, methylated derivatives, acylated flavonol glycosides, flavan-3-ols Benzoquinone: embelin Volatile compounds Triterpene saponins: lysilactones A-E, clethroidosides A-H	Antitumor, antioxidant Antidiabteic Hepatoprotective Vasorelaxant Antimicrobial, antiinflamatory
Lysimachia ciliata	Northern America	No data	Benzoquinone type embelin Volatile compounds Triterpene saponin:	Cytotoxic

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2. 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'. As *Lysimachia christinae* Hance is an official herbal drug in the Pharmacopoeia of People's Republic of China, and has various uses by Chinese medicine, partly due to radical scavenging phenolic content, we aimed to compare their phenolic metabolic pattern of the six *Lysimachia* species.

Literature data on the phenolic composition and antioxidant activity of the *Lysimachia* species mentioned above is limited. The phenolic composition of two species: *L. ciliata* and *L. punctata* have 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 compound 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 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. 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.

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3. MATERIALS AND METHODS

3.1. Plant material

Aerial parts of *Lysimachia vulgaris* L. were obtained from Bükk National Park, Hungary (June-July, 2011).

Aerial parts of *Lysimachia nummularia* L. were obtained from Bükk National Park, Hungary (June-July in 2011).

Aerial parts of *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 were obtained from a controlled cultivation of the Garden of Medicinal Plants, Jagiellonian University, Cracow, Poland, (2013).

Aerial part of *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 (aerial parts) 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 (LN/201106, LP/201106, LV/2001106) are deposited. Voucher specimens of polish samples (KFg/2010036 and KFg/2010016) are deposited in the Department of Pharmacognosy, Pharmaceutical Faculty, Medical College, Jagiellonian University, Cracow, Poland. The fresh plant materials were dried at room temperature. The final moisture content was less than 14%.

3.2. Solvents and chemicals

Kaempferol, quercetin, rutin, myricetin, myricitrin, chlorogenic acid, caffeic acid standards, DPPH and ABTS free radicals, trifluoroacetic acid, Folin - Ciocalteu's reagent, potassium peroxydisulfate, aluminium chloride and hexamethylenetetramine were acquired from Sigma-Aldrich (St. Louis, USA). Hide powder was supplied by Filk GmbH (Freiburg, Germany). HPLC grade and super gradient grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Chloroform, acetone, ethyl acetate,

methanol, acetic acid, formic acid, hydrochloric acid of reagent grade, spectroscopic grade ethanol and anhydrous sodium sulphate and sodium carbonate were purchased from Reanal-Ker (Budapest, Hungary) and Molar Chemicals Kft. (Budapest, Hungary). The deionised HPLC grade water was prepared with a Milli-Q Direct 8 Millipore water purification system (Bedford, MA, USA). All aqueous eluents for HPLC were filtered through MF-Millipore membrane filters (0.45 μm, mixed cellulose esters) (Billerica, MA, USA) and degassed in an ultrasonic bath before use.

3.3. 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 ml of chloroform for 6 h at 60 ºC, followed by methanol extraction for 6 h at 90 ºC. The methanolic 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 (Gen-Lab Ltd, Budapest, Hungary), and subjected to the following phytochemical and bioassay studies.

Hydrolysis

Hydrolysis was performed according to the method of the Ph. Hg. VIII. (2003) with minor modifications. Briefly, 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. The samples were heated in water bath at 85 °C for 1 h. After the acidic hydrolysis were completed, each mixture was cooled down to room temperature and was transferred to a 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. The hydrolysates were evaporated to dryness under reduced pressure in a rotary evaporator at 40 °C, redissolved in HPLC grade methanol and filtered through Phenex-RC 15 mm 0.2 μm syringe filters (Gen-Lab Ltd, Budapest, Hungary).

3.4. Quantitative phytochemical analysis

Determination of total phenolic, tannin, flavonoid and hydroxycinnamic acid contents of the *Lysimachia* herb samples was performed according to the guidance of Ph. Hg. VIII. (2003). Measurements were carried out in three parallels. Total polyphenol and tannin contents are expressed as pyrogallol, flavonoid content as hyperoside, and hydroxycinnamic acid content as rosmarinic acid (mg/g dry plant sample).

Contents of polyphenols, tannins, flavonoids and hydroxycinnamic acids in the methanolic extracts were determined by applying the mentioned method, with some modification. Briefly, 1 ml of herbal extract was mixed with 10 ml distilled water, and added to the appropriate reagents described in the original prescription, in the following, measurements were continued in accordance with the Pharmacopoeia related articles. The results were expressed in mg/g dried extract. All analyses were run in triplicate and averaged. The results are expressed as mean values and standard deviation (SD).

3.5. Antioxidant activity assays

Antioxidant activity of *Lysimachia* methanolic extracts, their hydrolysates and myricetin, quercetin, kaempferol, myricitrin, rutin, caffeic acid and chlorogenic acid standards was determined by spectrophotometric *in vitro* decolourization assay using $DPPH^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl) and $ABTS^{\bullet+}$ (2,2'azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) as free radicals. Theoretical background of DPPH and ABTS method, as well as advantages and disadvantages were described in section **1.3.1.-1.3.2.**, respectively. Both methods were applied with some modification. DPPH was dissolved in HPLC grade methanol (0.25 g/l). 10 mg ABTS was dissolved in 2.6 ml HPLC grade water and reacted with 1.72 mg potassium persulfate to produce ABTS radical cation. The $DPPH^{\bullet}$ stock solution was diluted with HPLC grade methanol and the $ABTS^{\bullet+}$ solution with spectroscopic grade ethanol to absorbances of 0.900±0.05 at 515 nm and 734 nm, respectively, immediately before measurement. 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 absorbance was recorded against a blank sample (methanol and ethanol for DPPH and ABTS, respectively). The decrease of the absorbance values were measured after 6 min by Hitachi U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan)

at the characteristic wavelengths. The inhibition percentage produced by a given sample concentration was calculated from the following equation:

$$\frac{A_0 - A_t}{A_t} \times 100 \qquad \text{(eq. 25)}$$

A_t = the extrapolated final absorbance,

A_0 = the absorbance of the blank solvent

The antioxidant activity was characterised by plotting the inhibition percentage of the samples as a function of sample concentration followed by linear regression. The concentrations belonging to the half maximal inhibition (IC_{50} value as μg/ml) were determined by linear regression analyses. All analyses were run in triplicates and averaged. The results are expressed as mean values and standard deviation (SD).

3.6. HPLC-DAD-ESI-MS/MS evaluation of *Lysimachia* methanolic extracts

For chromatographic separation an Agilent 1100 HPLC system (G1379A degasser, G1312A binary gradient pump, G1329A autosampler, G1316A column thermostat and G1315C diode array detector) was used (Agilent Technologies, Waldbronn, Germany). The *Lysimachia* methanolic extracts were separated on a Zorbax SB C18 column (3.0 x 150 mm, 3.5 μm; Agilent Technologies, Waldbronn, Germany) 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: 100% (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. Triple quadrupole mass spectrometric parameters were as follows:

- Ion source: ESI, negative
- Drying gas (N_2) temperature: 350 °C
- Drying gas (N_2) flow rate: 9 l/min

- Nebuliser gas (N_2) pressure: 40 psi
- Fragmentor voltage: 135 V
- Capillary voltage: 3500 V

Full-scan mass spectra were acquired over the *m/z* range of 50-1000 (1 scan/sec). Collision energy was changed between 15–50 eV, according to differences in molecule structures (high purity nitrogen was used as collision gas). During LC-MS analyses ESI was operated in the negative ionization mode, which provided better sensitivity due to the phenolate group of the investigated compounds. For structural characterisation of the compounds retention times, UV and mass spectral data were compared to literature data and to those of authentic standards, where available.

3.7. Quantitative analysis of Hungarian *Lysimachia* extracts by HPLC-DAD

3.7.1. HPLC-DAD condition for quantitative analysis

Quantification of kaempferol, quercetin, myricetin-3-*O*-rhamnoside (myricitrin), rutin and chlorogenic acid in *L. vulgaris, L. nummularia,* and *L. punctata* methanolic 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 (G1312B binary gradient pump, G1367E autosampler, G1315C diode array detector) was used (Agilent Technologies, Waldbronn, Germany). The *Lysimachia* samples were separated on a Zorbax Eclipse Plus C18 column (4.6 x 100 mm, 3.6 µm; Agilent Technologies, Waldbronn, Germany) 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 350 nm. UV spectra were recorded between 200 and 400 nm.

3.7.2. Determination of flavonoids

Standard solutions for the calibration were prepared at concentrations of 1, 10, 50, 150, and 300 μg/ml, using kaempferol, quercetin, rutin, myricetin-3-*O*-rhamnoside and chlorogenic acid standards in 80% v/v methanol. Quality control samples were prepared at concentrations of 1, 50 and 300 μg/ml from the standard solutions.

3.7.3. Method validation

Calibration, precision, accuracy and quality control samples

Standard solutions for the calibration were prepared in triplicate and injected once. Calibration plot was constructed by plotting peak areas against corresponding concentrations. Linearity was determined by analysing the standards at five concentrations, each in triplicate. Slope, intercept and correlation coefficient were determined by the 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 *L. vulgaris, L. nummularia*, and *L. punctata* 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).

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

3.8.1. UPLC-DAD condition for quantitative analysis

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 ultra-performance liquid chromatography system managed by Acquity console software was used (Waters MA, USA). The *Lysimachia* samples were separated on an Acquity UPLC HSS C18 column (2.1 × 100 mm, 1.8 μm; Waters, MA, USA) 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.

3.8.2. Determination of flavonoids

Standard solutions for the calibration were prepared at concentrations of 1, 10, 50, 150, 300 and 500 µg/ml, using kaempferol, quercetin and myricetin standards in gradient grade methanol. Quality control samples were prepared at concentrations of 1, 50 and 500 µg/ml for the standard solutions.

3.8.3. Method validation

Calibration, precision, accuracy and quality control samples

Standard solutions for the calibration were prepared in triplicate and injected once. Calibration curve was constructed by plotting peak areas against corresponding concentrations. 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 *L. vulgaris*, *L. nummularia*, *L. punctata, L. christinae*, *L. ciliata* and *L. clethroides* hydrolysed 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).

3.9. HPLC-based DPPH scavenging assay of *Lysimachia* hydrolysed extracts

3.9.1. Sample preparation

The HPLC method coupled with off-line DPPH scavenging assay was developed and described previously (Riethmüller et al., 2016). 100 µl of the *Lysimachia* hydrolysed

extracts of known concentrations (Table 4) 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. The chromatograms of the control and sample solutions were compared with regard to the changes in the peak areas of certain phenolic compounds. Table 4. summarises the sample preparation for the HPLC-based DPPH measurement.

Table 4. Sample preparation for the HPLC-based DPPH measurements

Sample name	Sample concentration (mg/ml)	DPPH concentration (mg/ml)
Lysimachia vulgaris	5.00	1.50
Lysimachia punctata	5.30	1.50
Lysimachia nummularia	4.70	1.50
Lysimachia clethroides	4.75	1.50
Lysimachia ciliata	4.85	1.50
Lysimachia christinae	2.30	0.75

3.9.2. HPLC-DAD-QMS condition for HPLC-based DPPH scavenging assay

For chromatographic separation an Agilent 1100 HPLC system (G1379A degasser, G1312A binary gradient pump, G1329A autosampler, G1316A column thermostat and G1315C diode array detector) was used (Agilent Technologies, Waldbronn, Germany). The *Lysimachia* samples were separated on a Kinetex-XB C18 column (150 × 4.6 mm, 2.6 µm; Agilent Technologies, Waldbronn, Germany) 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). The Agilent MassHunter B.01.03 software was used for data acquisition, and for qualitative analyses. Mass spectrometric parameters were as follows:

- Ion source: ESI, positive,
- Drying gas (N_2) temperature: 350°C,
- Drying gas (N_2) flow rate: 12 l/min,
- Nebuliser gas (N_2) pressure: 10 psi,
- Fragmentor voltage: 120 V,
- Capillary voltage: 4000 V

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4. RESULTS

4.1. Quantitative phytochemical analysis

In order to gain preliminary information about the phenolic constitution of the different *Lysimachia* herbal drugs and methanolic extracts as well, total phenolic, tannin, flavonoid and hydroxycinnamic acid contents were determined according to the methods of Ph. Hg. VIII. (2003). Results are presented in Table 5-6 as mean ± standard deviation of three parallel measurements.

Table 5. Quantitative determination of phenolics in the *Lysimachia* herbal drugs

Quantitative determination of phenolics in the *Lysimachia* herbal drugs expressed in mg/g herbal drug				
	Total phenolic content*	**Tannin content***	**Hydroxycinnamic acid content****	**Flavonoid content*****
L. vulgaris	34.2±1.5	18.2±1.1	24.3±0.1	5.5±0.1
L. nummularia	30.0±1.2	19.5±1.0	12.5±0.3	10.2±0.2
L. punctata	40.5±1.3	28.1±1.4	17.8±0.8	8.7±0.7
L. christinae	7.9±0.4	4.1±0.2	7.3±0.3	2.8±0.1
L. clethroides	51.8±2.3	35.8±1.8	62.2±2.9	8.8±0.4
L. ciliata	10.1±0.4	4.9±0.2	3.8±0.2	2.2±0.1

*expressed as pyrogallol
**expressed as rosmarinic acid
***expressed as hyperoside

Table 6. Quantitative determination of phenolics in the *Lysimachia* methanolic extracts

Quantitative determination of phenolics in the *Lysimachia* methanolic extracts expressed in mg/g dried extract				
	Total phenolic content*	**Tannin content***	**Hydroxycinnamic acid content****	**Flavonoid content*****
L. vulgaris	110.7±2.5	25.5±1.2	39.3±2.1	62.2±2.5
L. nummularia	75.3±2.8	24.8±8.9	3.5±0.2	26.1±1.3
L. punctata	108.1±4.2	28.1±1.3	42.5±2.3	30.2±1.4
L. christinae	66.4±2.6	41.0±1.9	34.8±1.9	7.8±0.4
L. clethroides	192.1±10.0	105.7±4.6	171.5±4.6	33.9±1.4
L. ciliata	15.5±0.8	5.9±0.3	3.6±0.2	8.4±0.4

*expressed as pyrogallol
**expressed as rosmarinic acid
***expressed as hyperoside

4.2. Evaluation of phenolics in *Lysimachia* methanolic extracts by HPLC-MS

RP-HPLC method was utilised for the separation of phenolics in the six *Lysimachia* methanolic extracts, which provided appropriate platform for the investigation of flavonoid-type compounds (Figures 16-21). The efficiency of the applied chromatographic method (section **3.6.**) proved to be appropriate, most of the chromatographic peaks could have been separated in 35 minutes. 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 (Ma et al., 1997, Fabre et al., 2001, Ferreres et al., 2003., Ferreres et al., 2004, Marin et al., 2004, Ablajan et al., 2006, Ferreres et al., 2007, Nandutu et al., 2007, Ablajan et al., 2013, Guo et al., 2013, Cao et al., 2014, Singh et al., 2015). UV chromatograms of methanolic herbal extracts are shown in Fig.16-21. The studies on the extracts of *Lysimachia* species herbs revealed that the different types of flavonoids were the major constituents of the samples, but various phenolic acid derivatives were also detected (Table 7). Altogether 63 flavonoids 16 caffeic acids, and 6 catechin derivatives and quinic acid were characterised in the *Lysimachia* methanolic extracts. The different *Lysimachia* species contain these components in various combinations and ratios.

L. vulgaris methanolic extracts comprised twelve tentatively identified compounds. Presence of two phenolic acids (compounds **1**, **8**), altogether with eight flavonol- mono- di- and tri–*O*–glycosides (compounds **28**, **31**, **40**, **47**, **57**, **60**, **62, 63**), and two flavonol aglycones: quercetin (**79**) and kaempferol (**82**) were described. Based on chromatographic data (Fig. 16) the main compounds of yellow loosestrife were quercetin-3-*O*-hexosyl-desoxyhexoside-7-*O*-desoxyhexoside (**28**) and rutin (**47**).

Seven phenolic compounds were identified in *L. nummularia* herbal extract. It contained one caffeic acid derivative (compound **1**), one kampferol- (compound **63**), three quercetin- (compounds **47, 57** and **65**) and two myricetin-O-glycosides (compounds **36** and **52**). According to the UV chromatographic data (Fig. 17) the main compound of moneywort was myricitrin (**52**).

In the *L. punctata* herbal extract nine phenolic compounds were detected. Presence of a caffeic acid derivative (compound **1**), six flavonol-*O*-glycosides (compounds **36**, **52**, **57**, **63**-**65**) and two flavonol aglycones, quercetin (**79**) and quercetin (**82**) were proven. Based on chromatographic data (Fig. 18) the main compound was myricitrin (**52**).

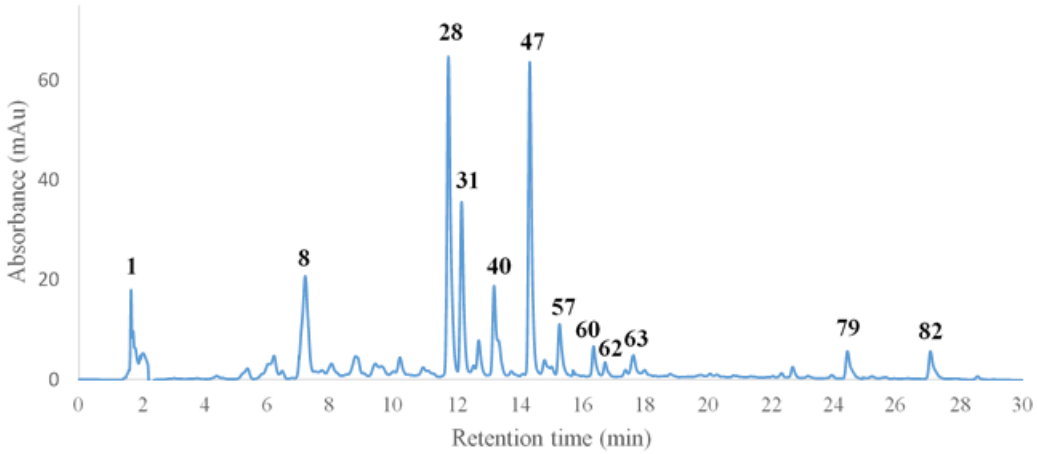

Figure 16. HPLC-UV chromatogram of *L. vulgaris* methanolic extract (280 nm)

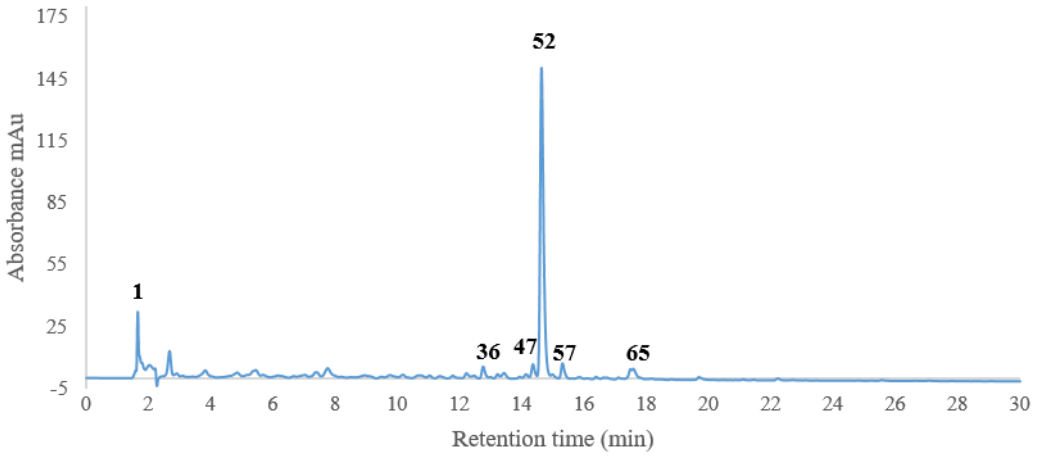

Figure 17. HPLC-UV chromatogram of *L. nummularia* methanolic extract (280 nm)

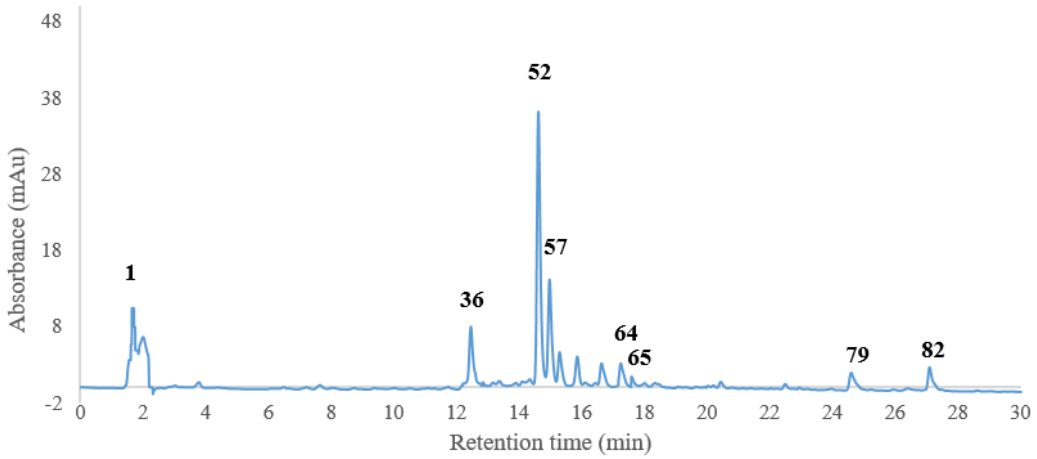

Figure 18. HPLC-UV chromatogram of *L. punctata* methanolic extract (280 nm)

In the *L. christinae* extract sixty seven phenolic compounds were detected, among them fourteen hydroxycinnamic acid derivatives (compounds **1**, **2**, **9**, **10**, **14**, **15**, **38**, **61**, **66**, **68**, **69**, **72**, **73**, **75**), nine flavonol-*O*-glycosides (compounds **32**, **47**, **52-54**, **57**, **63**, **65**, **76**), one flavonol aglycone (**79**) twenty six flavone-*C*-glycosides (compounds **11**, **12**, **16**, **17**, **20**, **22-25**, **27**, **29**, **30**, **34**, **41-46**, **48-50**, **55**, **56**, **58, 67**), nine methylated flavone derivatives (compound **70**, **71**, **74**, **78**, **80**, **81**, **84-86**), further six other flavonoid components (compounds **5**, **33**, **37**, **59**, **77**, **83**), quinic acid (**3**) and gallic acid-hexoside derivative (**4**) were described. According to chromatographic data (Fig. 19) the main compound of the extracts was apigenin-6-*C*-hexosyl-8-*C*-pentoside (**27**).

In the *L. clethroides* extract nineteen phenolic compounds were detected, among them a caffeic acid derivative (**1**), and caffeic acid-hexoside (**2**), six catechin derivatives (compounds **6**, **7**, **13**, **18**, **19**, **26**), dihydrokaempferol-hexoside (**21**), and ten flavonol-*O*-glycosides (compounds **28**, **32**, **39**, **40**, **47**, **51**, **53**, **57**, **60**, **63**) were tentatively characterised. According to chromatographic data (Fig. 20) the main compounds of the gooseneck loosestrife extract was quercetin-3-*O*-hexoside (**57**).

Twelve phenolic compounds were tentatively identified in the *L. ciliata* methanolic extract. The presence of a caffeic acid derivative (compound **1**) and caffeic acid-hexoside **(2**), three flavone-*C*-glycosides, (compounds **29**, **35**, **48**) and seven flavonol-*O*-glycosides (compounds **28**, **32**, **36**, **39**, **40**, **51**, **53**) were described. Based on chromatographic data (Fig. 21) the main compound of the fringed loosestrife extract was quercetin-3-*O*-hexoside (**53**).

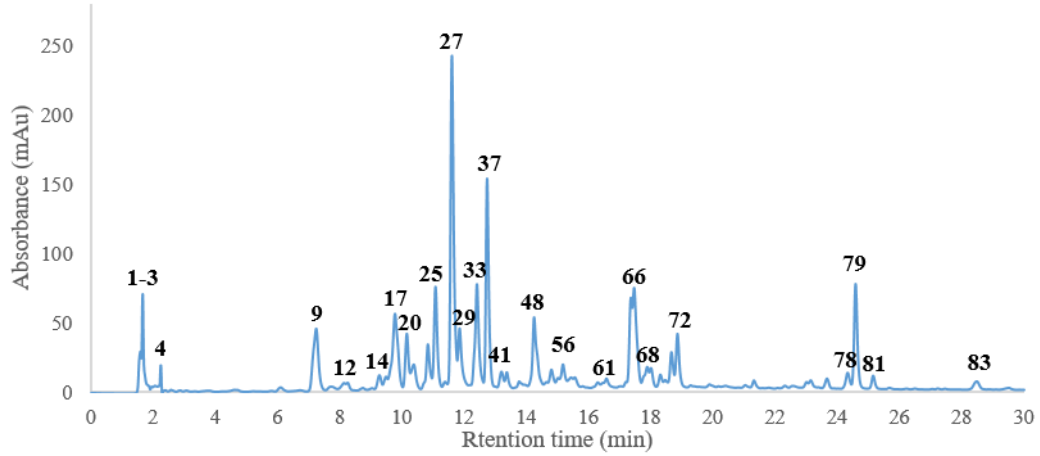

Figure 19. HPLC-UV chromatogram of *L. christinae* methanolic extract (280 nm)

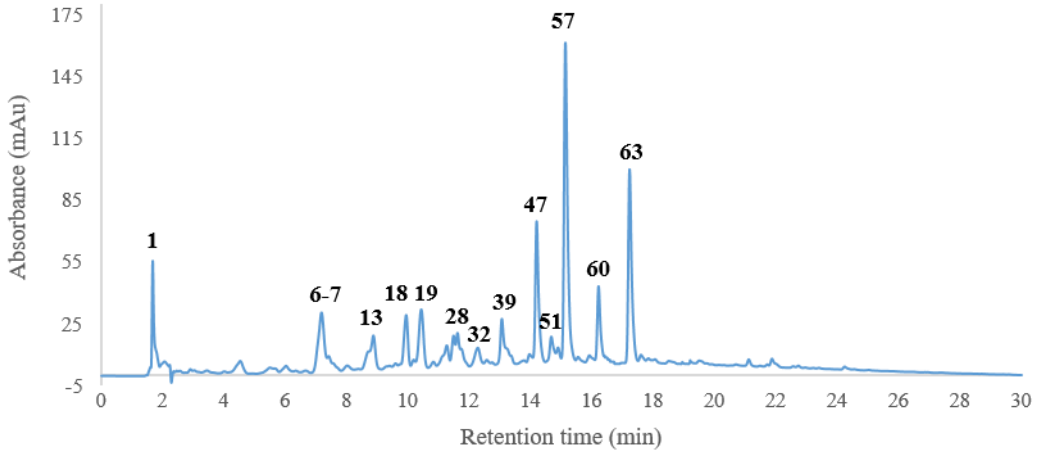

Figure 20. HPLC-UV chromatogram of *L. clethroides* methanolic extract (280 nm)

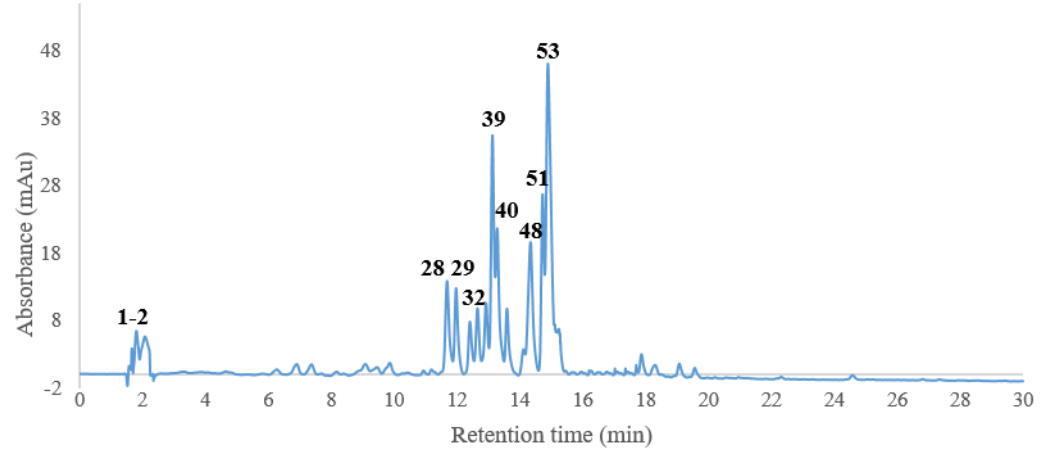

Figure 21. HPLC-UV chromatogram of *L. ciliata* methanolic extract (280 nm)

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Table 7. Tentative identification of phenolic compounds in *Lysimachia* spcies methanolic extract

No	t_R (min)	$[M-H]^-$ (*m/z*)	Fragment ions (*m/z*)	Compound name	LV	LN	LP	LC	LK	LF
1	1.6	377	341, 179, 161, 113, 89	Caffeic acid derivative	X	X	X	X	X	X
2	1.6	341	179, 161, 89	Caffeic acid hexoside				X	X	X
3	1.6	191	171, 127, 111, 85	Quinic acid				X		
4	2.6	419	257, 239, 195, 169, 125	Gallic acid hexoside derivative				X		
5	6.3	449	287, 269, 259, 179	Dihydrokaempferol-hexoside (1)				X		
6	6.7	289	245, 205, 161, 137,	+/- catechin / epicatechin					X	
7	7.3	289	245, 205, 161, 137, 109	+/- catechin / epicatechin					X	
8	7.3	353	191, 161, 135	Chlorogenic acid	X					
9	7.3	707	353, 191	5-Caffeoyl-quinic acid dimer				X		
10	7.8	353	191,	5-Caffeoyl-quinic acid				X		
11	8.3	609	519, 489, 429, 399, 369, 339,	Luteolin-6,8-di-*C*-hexoside (1)				X		
12	8.8	579	519, 489, 471, 459, 441, 429, 411, 399, 369, 339, 297	Luteolin-6-*C*-pentosyl-8-*C*-hexoside (1)				X		
13	9.0	577	451, 407, 289, 245, 161, 125	Procyanidin dimer					X	
14	9.1	177	163, 149, 119, 117, 105	Methyl-4-hydroxycinnamate				X		
15	9.5	353	191, 179	3-Caffeoyl-quinic acid				X		
16	9.7	579	519, 489, 471, 459, 441, 429, 411, 399, 369, 339, 297	Luteolin-6-*C*-pentosyl-8-*C*-hexoside (2)				X		
17	9.8	593	575, 503, 473, 413, 383, 353, 325, 297	Apigenin-6,8-di-*C*-hexoside (1)				X		
18	9.8	289	245, 205, 167, 119	+/- catechin / epicatechin					X	
19	10.0	289	245, 205, 125, 119	+/- catechin / epicatechin					X	
20	10.2	579	519, 489, 471, 459, 441, 429, 411, 399, 369, 339, 297	Luteolin-6-*C*-pentosyl-8-*C*-hexoside (3)				X		
21	10.3	449	287, 269, 259, 179, 151, 125	Dihydrokaempferol-hexoside (2)					X	
22	10.4	563	545, 503, 473, 443, 413, 383, 353, 325, 297	Apigenin-6-*C*-hexosyl-8-*C*-pentoside (1)				X		
23	10.5	579	519, 489, 471, 459, 441, 429, 411, 399, 369, 339, 297	Luteolin-6-*C*-pentosyl-8-*C*-hexoside (4)				X		
24	10.9	579	519, 489, 471, 459, 441, 429, 411, 399, 369, 339, 297	Luteolin-6-*C*-pentosyl-8-*C*-hexoside (5)				X		
25	11.2	563	545, 503, 473, 443, 413, 383, 353, 325, 297	Apigenin-6-*C*-hexosyl-8-*C*-pentoside (2)				X		
26	11.2	865	695, 575, 407, 125	Procyanidin trimer					X	
27	11.7	563	545, 503, 473, 443, 413, 383, 353, 325, 297	Apigenin-6-*C*-hexoside-8-*C*-pentoside (3)				X		
28	11.8	755	609, 301, 300, 271, 179, 151	Quercetin-3-*O*-hexosyl-desoxyhexoside-7-*O*-desoxyhexoside	X				X	X
29	11.9	447	429, 357, 327, 311, 297, 285, 269	Luteolin-6-C-hexoside (isoorientin)				X		X
30	12.1	593	575, 503, 473, 413, 383, 353, 325, 297	Apigenin-6,8-di-*C*-hexoside (2)				X		
31	12.2	625	317, 316, 179	Myricetin-3-*O*-hexosyl-desoxyhexoside	X					
32	12.4	479	317, 316, 291	Myricetin-3-*O*-hexoside (1)				X	X	X
33	12.5	563	269	Trihydroxiflavone-pentosyl-hexoside				X		
34	12.7	475	431, 341, 311	Vitexin derivative				X		
35	12.7	447	429, 357, 327, 297, 285	Luteolin-6-*C*-hexoside						X
36	12.7	479	317, 316, 291	Myricetin-3-*O*-hexoside (2)		X	X			X
37	12.8	563	269	Trihydroxiflavone-pentosyl-hexoside				X		
38	13.0	367	179, 161, 135, 89	Caffeic acid derivative				X		
39	13.1	609	301,300, 255, 179, 151, 121	Quercetin-3-*O*-hexosyl-desoxyhexoside (1)					X	X
40	13.2	739	285, 284, 227, 151, 133, 107	Kaempferol-3-*O*-hexosyl-desoxyhexoside-7-*O*-desoxyhexoside	X				X	X
41	13.3	593	575, 503, 473, 413, 383, 353, 325, 297	Apigenin-6,8-di-*C*-hexoside (3)				X		
42	13.4	533	473, 443, 413, 383, 353, 283	Apigenin-6,8-di-*C*-pentoside (1)				X		
43	13.8	433	343, 313, 183	Flavone-*C*-glycoside (1)				X		
44	13.8	577	549, 487, 457	Flavone-*C*-glycoside				X		
45	14.1	533	473, 443, 413, 383, 353, 283	Apigenin-6,8-di-*C*-pentoside (2)				X		

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No	t_R (min)	$[M-H]^-$ (*m/z*)	Fragment ions (*m/z*)	Compound name	LV	LN	LP	LC	LK	LF
46	14.2	533	473, 443, 413, 383, 353, 283	Apigenin-6,8-di-*C*-pentoside (3)				X		
47	14.3	609	301, 300, 255	Quercetin-3-*O*-hexosyl-desoxyhexoside (rutin)	X	X		X	X	
48	14.3	431	413, 341, 311, 285, 269	Apigenin-6-*C*-hexoside (isovitexin)				X		X
49	14.3	433	343, 313, 183	Flavone-*C*-glycoside (2)				X		
50	14.4	533	473, 443, 413, 383, 353, 283	Apigenin-6,8-di-*C*-pentoside (4)				X		
51	14.6	593	429, 285, 284 ,255, 227, 179, 151, 133, 107	Kaempferol-3-*O*-hexosyl-desoxyhexoside (1)					X	X
52	14.7	463	317, 316, 287, 271, 179, 151	Myricetin-3-*O*-desoxyhexoside (myricitrin)		X	X	X		
53	14.8	463	301, 300, 255, 179	Quercetin-3-*O*-hexoside (1)				X	X	X
54	14.9	609	301, 300, 179, 151, 121	Quercetin-3-*O*-hexosyl-desoxyhexoside (2)				X		
55	15.1	461	371, 341, 298, 271, 227, 179	Flavone-*C*-glycoside (1)				X		
56	15.2	447	357, 327, 311, 297, 285, 269	Luteolin-8-*C*-hexoside (orientin)				X		
57	15.4	463	301, 300, 255, 179, 151, 121	Quercetin-3-*O*-hexoside (2)	X	X	X	X	X	
58	15.5	461	371, 341, 298, 271, 227, 179	Flavone-*C*-glycoside (2)				X		
59	16.4	449	287, 269, 259, 179	Dihydrokaempferol-hexoside (3)				X		
60	16.4	593	285, 284, 256, 227, 151, 133, 107	Kaempferol-3-*O*-hexosyl-desoxyhexoside (2)	X				X	
61	16.7	515	353, 191, 179, 173, 161	Caffeoyl-quinic acid-hexoside (1)				X		
62	16.8	623	315, 271, 243	Isorhamnetin-hexosyl desoxyhexside	X					
63	17.3	447	285, 284, 255, 239227, 151, 133, 107	Kaempferol-3-*O*-hexoside	X	X	X	X	X	
64	17.3	433	301, 300, 271, 255, 179, 151, 121	Quercetin-3-*O*-pentoside			X			
65	17.4	447	300, 271, 243, 227, 179, 151, 121	Quercetin-3-*O*-desoxyhexoside		X	X	X		
66	17.4	515	353, 191, 179, 173, 161	Caffeoyl-quinic acid-hexoside (2)				X		
67	17.8	431	341, 323, 311, 295, 283, 269	Apigenin-8-*C*-hexoside (vitexin)				X		
68	18.0	515	353, 191, 179, 173, 161	Caffeoyl-quinic acid-hexoside (3)				X		
69	18.1	515	353, 191, 179, 173, 161	Caffeoyl-quinic acid-hexoside (4)				X		
70	18.4	445	283, 269, 255	Dihydroxy-methoxy-flavone-hexoside				X		
71	18.4	461	446, 299, 284	Kaempferol-methyl ether-hexoside (1)				X		
72	18.5	515	353, 191, 179, 173, 161	Caffeoyl-quinic acid-hexoside (5)				X		
73	18.7	515	353, 191, 179, 173, 161	Caffeoyl-quinic acid-hexoside (6)				X		
74	18.9	461	446, 283, 255,	Kaempferol-methyl ether-hexoside (2)				X		
75	18.9	515	353, 191, 179, 173, 161	Caffeoyl-quinic acid-hexoside (7)				X		
76	20.0	431	285, 284, 255, 227	Kaempferol-3-*O*-desoxyhexoside				X		
77	22.4	253	223, 208, 196, 180, 168, 133, 117	Dihydroxyflavone				X		
78	24.4	285	270, 199	Dihydroxy-methoxy-flavanone				X		
79	24.4	301	179, 151, 121	Quercetin	X		X	X		
80	24.6	283	268, 239, 211	Dihydroxy-methoxy-flavone				X		
81	24.7	491	283, 268	Dihydroxy-methoxy-flavone-glycoside				X		
82	24.8	285	257, 227, 151, 133, 107	Kaempferol	X		X			
83	28.5	269	225, 197, 183, 159, 151, 107	Trihydroxyflavone				X		
84	29.6	299	284, 255, 227	Kaempferol-methyl ether				X		
85	30.0	329	314, 299, 271, 229, 211, 193, 171	Trihydroxy-dimethoxy-flavone (1)				X		
86	30.3	329	314, 299, 271, 229, 211, 193, 171	Trihydroxy-dimethoxy-flavone (2)				X		
				Number of characterised compounds	**12**	**7**	**9**	**67**	**19**	**12**

Peak numbers and retention times (t_R) refer to EIC chromatograms. Abbreviations: LV: *L. vulgairs*, LN: *L. nummularia*, LP: *L. punctata*, LC: *L. christinae*, LK: *L. clethroides*, LF: *L. ciliata*. Flavonoids, phenolic acids and catechin derivatives marked with black, blue and green, respectively.

4.2. Quantitative analysis of Hungarian native species by HPLC-DAD

Due to the incomplete scientific investigations and the more deteailed phytochemical characterisation of Hungarian native species, quantitative analysis of the main phenolic copounds in *L. vulgaris*, *L. nummularia* and *L. punctata* methanolic extract was conducted. According to the HPLC-DAD-ESI-MS results, the main flavonoids of the Hungarian species extracts were kaempferol, quercetin and myricetin derivatives. Furthermore, chlorogenic acid is also present in *L.vulgaris*. Therefore, quantitative determination of chlorogenic acid, myricitrin, rutin, kaempferol and quercetin and their derivatives were performed in the methanolic extracts by using external standard method. For the quantitative analysis of these constituents a HPLC-DAD method was utilised and validated. The performed gradient elution method was completely appropriate for the determination of these constituents, base-line separation could be achieved in the case of all the five compounds. The methods were validated for linearity, intra- and inter-day precision and accuracy (see section **3.7.**).

4.3.1. Method validation

The quantitative methods provided linear responses for all standards within the investigated range (Table 8). Retention time repeatability was checked with six successive runs of the *L. vulgaris*, *L. nummularia*, *L. punctata* methanolic extracts. Retention time repeatability was suitable for all compounds, relative standard deviation was <0.15% (n = 6).

Precision of the methods was tested by performing intra- and inter-day evaluation of solutions containing the standards in three concentrations (low, mid and high values of the calibration range), precision and accuracy tests were performed in triplicate. The relative standard deviation for intra- and inter-day precision was <8% for all quantitative methods, while intra- and inter-day accuracy ranged from 90.0% to 104.2% (Table 9).

Table 8. Parameters of the analytical curves, LOD and LOQ for chlorogenic acid and flavonoids quantification by HPLC-UV/DAD

Compound	regression equation	r^2	Regression range(µg/ml)	LOD (µg/ml)	LOQ (µg/ml)
chlorogenic acid	y=3.8236x+5.3796	0.9997	1-300	0.09	0.20
rutin	y=6.4203x+2.0045	0.9996	1-300	0.07	0.19
myricitrin	y=13.4251x+6.3910	0.9999	1-300	0.12	0.34
quercetin	y=6.2159x+2.4319	0.9999	1-300	0.06	0.19
kaempferol	y=6.4014x+8.003	0.9998	1-300	0.10	0.33

Table 9. Method validation: precision and accuracy of the quantitative methods

Nominal concentration	Precision RSD%		Accuracy %	
ug/ml	Intra day	Inter day	Intra day	Inter day
Chlorogenic acid				
1	3.56	7.79	92.8	90.0
50	1.12	3.72	98.7	95.1
300	1.85	4.46	95.1	93.5
Rutin				
1	3.91	4.75	97.6	94.9
50	2.41	3.56	99.5	103.1
300	4.12	4.79	100.2	96.3
Myricitrin				
1	4.39	5.32	95.3	91.3
50	1.87	3.01	97.4	94.6
300	2.56	4.56	92.1	91.2
Quercetin				
1	3.88	4.44	94.3	97.5
50	1.05	3.06	99.8	94.2
300	1.96	3.75	96.4	104.2
Kaempferol				
1	2.81	7.56	94.3	91.3
50	1.65	4.14	95.6	90.4
300	4.41	5.16	94.8	92.2

4.3.2. Quantitative results

Quantity of chlorogenic acid, myricitrin, rutin, quercetin and kaempferol were determined by HPLC-DAD experiments using external standard calibration. Amounts of quercetin-3-*O*-neohesperoside-7-*O*-rhamnoside, myricetin-3-*O*-hexoside, myricetin-3-*O*-rutinoside and quercetin-3-*O*-hexoside were inferred from the calibration data of standard

compound with the corresponding aglycone. Results of the quantitative analyses are presented in Table 10 as mean and standard deviation of three parallel measurements.

Table 10. Flavonoid contents of the *Lysimachia* extracts, expressed in mg/g dried extract.

	L. vulgaris	***L. nummularia***	***L. punctata***
Chlorogenic acid	5.0±0.2	-	-
Quercetin-3-*O*-neohesperoside-7-*O*-rhamnoside	12.7±0.3	-	-
Myricetin -3-*O*-hexoside	-	1.3±0.1	2.5±0.1
Myricetin-3-*O*-rutinoside	8.2±0.2	-	-
Myricitrin	-	26.2±0.6	8.3±0.2
Quercetin-3-*O*-hexoside	-	-	4.0±0.1
Rutin	14.2±0.3	3.8±0.2	-
Quercetin	0.4±0.0	-	0.4±0.0
Kaempferol	0.1±0.0	-	0.8±0.0

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

According to the HPLC-DAD-ESI-MS results, more than 80 phenolic components were described for six *Lysimachia* species. The most abundant aglycones were kaempferol, quercetin and myricetin especially in Hungarian native species. In view of the required effort in separation of flavonols, quantification on the basis of a reduced number of sample components may pose a feasible alternative. Given that aglycones originating from flavonol-*O*-glycosides were planned to be quantified, mild hydrolysis method (25 v/v% hydrochloric acid 85 °C and 1 hour) was considered according to the Ph. Hg. VIII. (2003). Under these conditions, *C*-glycosidic bonds are unimpaired.

For the quantitative analysis of the three most abundant flavonol aglycone, UPLC-DAD method was developed. The performed gradient elution method was completely appropriate for the determination of kaempferol, quercetin and myricetin, base-line separation could be achieved in the case of all the three compounds. The methods were validated for linearity, intra- and inter-day precision and accuracy.

The UV chromatograms of standards and *Lysimachia* hydrolysed extracts are shown in Figure 22.

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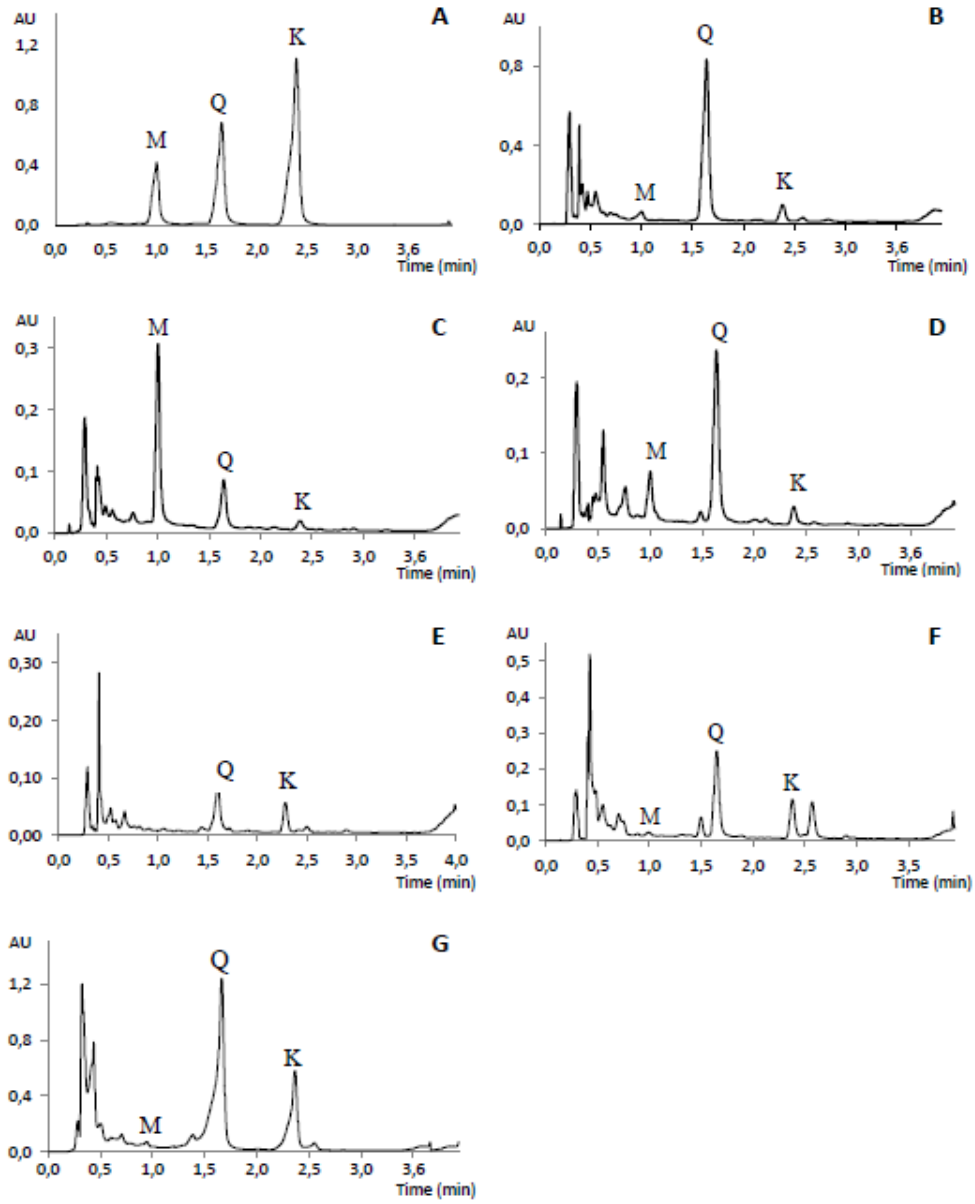

Figure 22. UPLC-DAD chromatograms (254 nm) of flavonoid standard mixtures: myricetin, quercetin, kaempferol (A) and hydrolysed flavonoid extracts of six *Lysimachia* species: *L. vulgaris* (B), *L. nummularia* (C), *L. punctata* (D), *L. christinae* (E), *L. ciliate* (F), *L. clethroides* (G). Abbreviations: K: kaempferol, M: myricetin, Q: quercetin

4.4.1. Method validation

The quantitative methods provided linear responses for all standards within the investigated range, their coefficient of determination (r^2) were above 0.9998 (Table 11). Retention time repeatability was checked with six successive runs of the *L. nummularia*, *L. vulgaris*, *L. punctata*, *L. christinae*, *L. ciliata* and *L. clethroides* hydrolysed extracts.

Retention time repeatability was suitable, relative standard deviations of retention time of kaempferol, quercetin and myricetin were 0.022 %, 0.021% and 0.024% (n=6), respectively.

Table 11. Regression, LOQ and LOD of the quantitative methods

Standard	Regression equation	r^2	regression range (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)
kaempferol	y=14403x-4380.2	0.9999	1-500	0.10	0.31
quercetin	y=13710x-2568	1.0000	1-500	0.13	0.42
myricetin	y=9994.4x-9143	0.9999	1-500	0.15	0.50

Precision of the methods was tested by performing intra- and inter-day evaluation of solutions containing the standards in three concentrations (low, mid and high values of the calibration range), precision and accuracy tests were performed in triplicate. The relative standard deviation for intra- and inter-day precision was <4% for all quantitative methods, while intra- and inter-day accuracy ranged from 93.1% to 107.8% (Table 12) indicating that the analysis is precise and stable in the present method.

Table 12. Method validation: Precision and accuracy of the quantitative methods

Nominal concentration (µg/ml)	Precision (RSD%)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
Kaempferol				
1	2.60	1.88	93.7	93.1
50	1.31	3.69	99.6	95.7
500	1.24	3.28	100.2	96.7
Quercetin				
1	3.44	2.86	107.6	107.8
50	3.58	2.29	101.7	100.9
500	1.19	1.37	100.7	99.6
Myricetin				
1	0.71	0.67	102.2	102.1
50	1.34	3.75	105.3	104.2
100	0.27	1.00	100.0	100.1

4.4.2. Quantitative results

Quantity of myricetin, quercetin and kaempferol were determined by UPLC-DAD experiments. Linear regression analyses were performed by using external calibration (Table 11). Results of the quantitative analyses are presented in Table 13, as mean and standard deviation of three parallel measurements.

Table 13. Quantity of kaempferol, quercetin and myricetin in the *Lysimachia* extracts expressed in mg/g dried extract.

	Kaempferol	**Quercetin**	**Myricetin**
Lysimachia vulgaris	4.53±0.23	34.88±1.67	2.73±0.13
Lysimachia nummularia	0.71±0.04	4.87±0.23	20.79±1.00
Lysimachia punctata	0.79±0.04	12.15±0.58	5.44±0.26
Lysimachia christinae	0.83±0.04	1.53±0.07	0.76±0.03
Lysimachia ciliata	3.48±0.17	13.66±0.65	1.37±0.06
Lysimachia clethroides	25.77±1.29	97.67±4.61	0.90±0.04

4.4. Antioxidant activity assays

The antioxidant activity of the *Lysimachia* methanolic and hydrolysed methanolic extracts was determined by *in vitro* tests using DPPH (2,2-Diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)), as free radicals (conditions see in text, section **3.5.**).

Scavenging activity of the samples of interest was compared to that of well-known antioxidant standards, three flavonol aglycones: myricetin, quercetin and kaempferol, two flavonol-glycoside: myricitrin and rutin, and two plant acids: caffeic acid and ascorbic acid (Tables 14 and 15).

4.5.1. Scavenging activity on DPPH free radical

Results of the determination of scavenging activity of the *Lysimachia* extracts and the standards on DPPH free radical are presented in Table 14 as mean±SD of three parallel experiments.

Table 14. Scavenging activity of the *Lysimachia* methanolic and their hydrolysed extracts and the standard phenolics on DPPH free radical (mean IC_{50}±SD of three parallel experiments). Abbreviations: MeOH: methanolic extract, H: hydrolysed methanolic extract

Sample	IC_{50} (μg/ml)	Sample	IC_{50} (μg/ml)	Sample	IC_{50} (μg/ml)
Myricetin	1.42±0.07	*L. punctata* MeOH	43.33±2.64	*L. nummularia* H	6.42±0.30
Quercetin	4.86±0.16	*L. nummularia* MeOH	52.42±3.20	*L. vulgaris* H	8.27±0.39
Myricitrin	4.83±0.21	*L. clethroides* MeOH	62.30±2.96	*L. christinae* H	17.29±0.81
Caffeic acid	5.10±0.29	*L. christinae* MeOH	77.27±4.79	*L. clethroides* H	21.75±1.02
Rutin	7.30±0.40	*L. vulgaris* MeOH	78.70±4.80	*L. punctata* H	31.47±1.94
Kaempferol	19.10±0.90	*L. ciliata* MeOH	229.61±12.88	*L. ciliata* H	59.59±2.79

4.5.2. Scavenging activity on ABTS free radical

Results of the determination of scavenging activity of the *Lysimachia* extracts and the standards on ABTS free radical are presented in Table 15 as mean±SD of three parallel experiments.

Table 15. Scavenging activity of the *Lysimachia* methanolic and their hydrolysed extracts and the standard phenolics on ABTS free radical (mean IC_{50}±SD of three parallel experiments). Abbreviations: MeOH: methanolic extract, H: hydrolysed methanolic extract

Sample	IC_{50} (μg/ml)	Sample	IC_{50} (μg/ml)	Sample	IC_{50} (μg/ml)
Myricetin	0.47±0.03	*L. punctata* MeOH	21.29±1.79	*L. nummularia* H	5.21±0.27
Quercetin	1.45±0.06	*L. nummularia* MeOH	24.80±2.08	*L. vulgaris* H	7.61±0.40
Caffeic acid	1.93±0.19	*L. vulgaris* MeOH	36.66±3.08	*L. christinae* H	8.34±0.44
Myricitrin	4.70±0.39	*L. clethroides* MeOH	50.09±0.43	*L. clethroides* H	9.22±0.48
Rutin	3.26±0.27	*L. christinae* MeOH	61.16±5.14	*L. punctata* H	14.51±0.76
Kaempferol	8.56±0.45	*L. ciliata* MeOH	70.80±5.94	*L. ciliata* H	21.82±1.14

4.6. HPLC-based DPPH scavenging assay

The HPLC method coupled with off-line DPPH scavenging assay was developed to get a semi-quantitative method in order to investigate the contribution of individual compounds to the total antioxidant activity. After spiking the *Lysimachia* samples with the DPPH radical solution, the decrease in the chromatographic peak areas of the three main flavonoid compounds were examined. 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. 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 methanol to the extracts. The chromatograms of the control and sample solutions were compared with regard to the changes in the peak areas of certain phenolic compounds.

The enlarged chromatograms of *L. nummularia* hydrolysed methanolic extract are presented in Figure 23 as a representative example. The following trend was observed regarding the decrease in the peak area: myricetin (88.7±7.1%) > quercetin (76.4±8.7%) > kaempferol (65.3+8.0%).

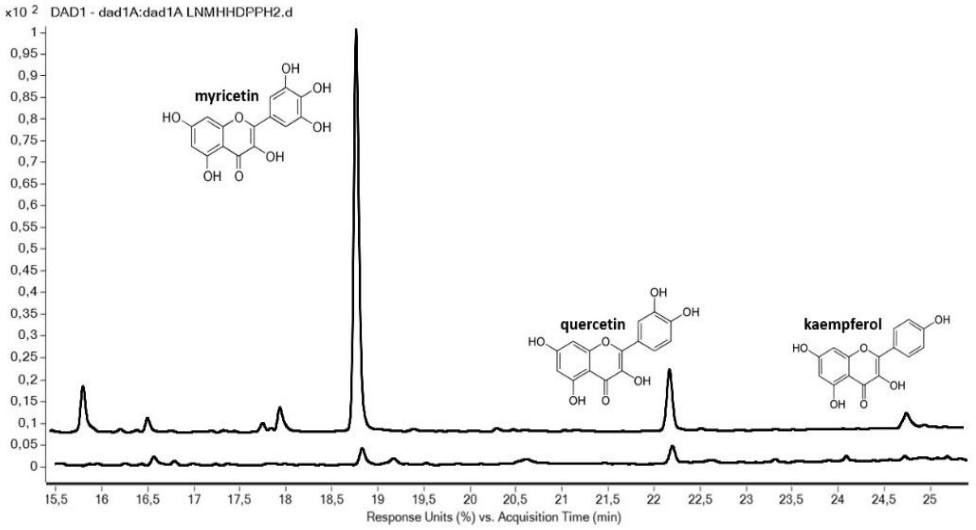

Figure 23. HPLC-UV chromatograms of the control sample and the sample after spiking with DPPH of *Lysimachia nummularia* hydrolysed methanolic extract (see section **3.9.**)

The contribution of certain compounds to the total antioxidant capacity of the *Lysimachia* extracts has also been investigated (Table 16). It could be estimated by calculating the ratio of the decrease in the compounds' peak area and the sum of total peak area decrement.

ΔArea was calculated according to eq. 26 and 27 and the ΔArea ratio according to eq 28.

$$\Delta\text{Area} = \text{peak area}_1 - \text{peak area}_2 \quad \text{(eq. 26)}$$

$$\text{Decrease in the peak area (\%)} = (\Delta\text{Area}/\ \text{peak area}_1)*100\% \quad \text{(eq. 27)}$$

$$\Delta\text{Area ratio} = (\Delta\text{Area}/\Sigma\ \Delta\text{Area})*100\% \quad \text{(eq. 28)}$$

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where ΔArea (mAu*s) is the change in the peak area; peak area1 (mAu*s) is the area of the compound's chromatographic peak in the control sample; peak area2 (mAu*s) is the area of the compound's chromatographic peak in the DPPH spiked sample.

Table 16. Contribution percentages of the main compounds detected in the *Lysimachia* extracts to the DPPH scavenging activity

Contribution (%) of the three flavonoid aglycone to the DPPH scavenging activity				
Sample name	**kaempferol**	**quercetin**	**myricetin**	**other**
Lysimachia vulgaris	10.4	76.3	4.5	8.8
Lysimachia nummularia	2.9	10.2	73.5	13.4
Lysimachia punctata	5.9	44.9	29.7	19.5
Lysimachia christinae	3.3	23.0	3.3	70.4
Lysimachia clethroides	18.8	68.0	3.2	10.0
Lysimachia ciliata	17.6	41.9	2.8	37.7

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5. DISCUSSION

5.1. Quantitative phytochemical analyses

Quantitative spectrometric methods described in Ph. Hg. VIII. (2003) were used to determine phenolic compounds in the herbal drugs and methanolic extracts of six *Lysimachia* species (Table 5-6, Figure 24-25). Based on the results, it could be concluded, that all the Hungarian native species contained notable amounts of polyphenol compounds, while in cultivated species large variance was observed, with *L. clethroides* herb being the richest in these constituents (51.8±2.3 mg total phenolics in 1 g herbal drug). Furthermore, *L. clethroides* aerial part was found to contain the highest quantities of tannins and hydroxycinnamic derivatives. Tannin content of gooseneck loosestrife was 35.8±1.8 mg/g herbal drug, while hydroxycinnamic content was 62.2±2.9 mg/g herbal drug. However, the flavonoids were not presented in such outstanding quantities compared to the other species.

The total polyphenol and tannin content expressed in pyrogallol found to be considerably lower (around 10 mg/g herbal drug) in herb of *L. christinae* and *L. ciliata*, than other cultivars (30-50 mg/g herbal drug). The flavonoid and hydroxycinnamic acid content was lower in these herb samples, too. The lowest flavonoid content (2.2±0.1 mg total flavonoid in 1 g herbal drug) was measured in *L. ciliata* herb among all the herbal drugs, while these kind of phenolic compounds were accumulated in *L. nummularia* in the highest amount (10.2±0.2 mg/g herbal drug).

The total flavonoid content expressed in hyperoside found to be considerably lower in aerial part of *L. vulgaris* than in *L. nummularia* and *L. punctata*. More similar accumulations were observed in the total polyphenol and tannin content of the samples. The total polyphenol content was around 3-4%, while the flavonoid content was around 0.5-1% in the Hungarian native *Lysimachia* species (Toth et al., 2014). Hanganu and co-workers (2016) measured twice as much polyphenol and five times higher flavonoid content (76.12±0.35 mg/g and 26.42±1.3 mg/g, respectively) in yellow loosestrife collected in Romania. Nevertheless, nearly the same amount of these compounds were reported in creeping jenny (35.51 ±0.21 mg/g polyphenol and 11.26±0.40 mg/g flavonoid, respectively) (Hanganu et al., 2016). The different environmental effects probably play a role in the accumulation properties of secondary metabolites.

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In most cases, the methanolic extract of the herbs were richer in these metabolites, up to 3-4 times higher concentration were achieved.

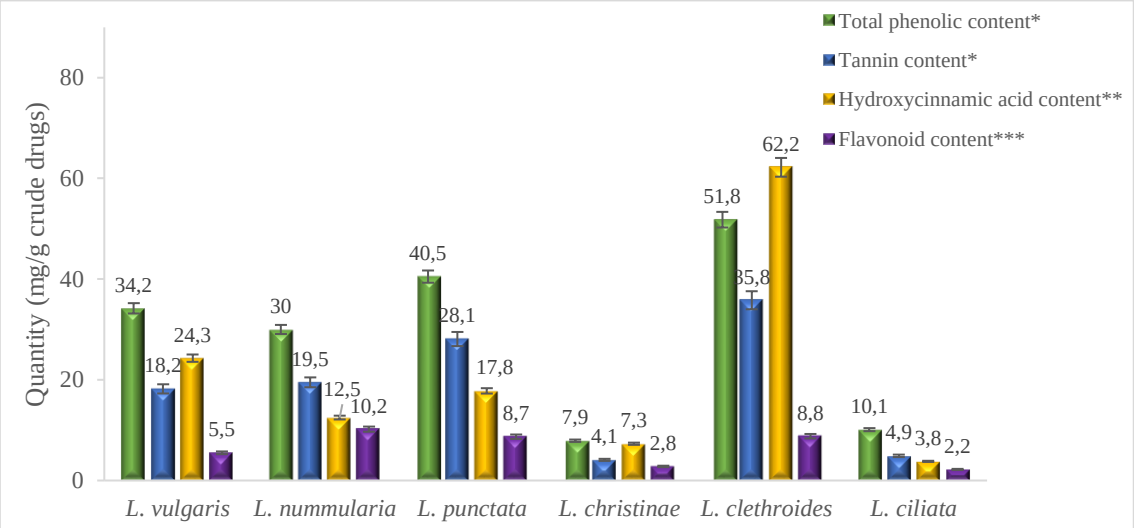


Figure 24. Quantitative determination of phenolics in the *Lysimachia* herbal drugs
* expressed in pyrogallol
**expressed in hydroxycinnamic acid
***expressed in hyperoside

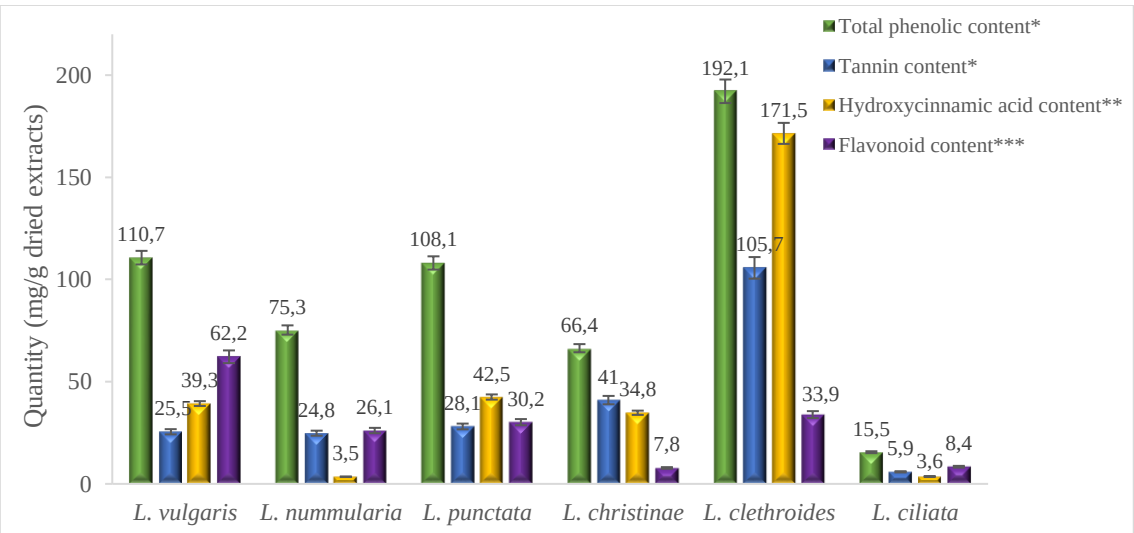


Figure 25. Quantitative determination of phenolics in the methanolic extracts of *Lysimachi*a herbal drugs
* expressed in pyrogallol
** expressed in hydroxycinnamic acid
***expressed in hyperoside

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

Since negative ion ESI-MS/MS characterisation of phenolic acids, and flavonoids (Ma et al., 1997, Fabre et al., 2001, Ferreres et al., 2003, Ferreres et al., 2004, Marin et al., 2004, Ablajan et al., 2006, Ferreres et al., 2007, Nandutu et al., 2007, Ablajan et al., 2013, Guo et al., 2013, Cao et al., 2014, Singh et al., 2015) is well reported, explanation of fragmentation behaviour is not discussed here in full details. Furthermore, due to the large number of compounds detected, the mass spectrometric identification is illustrated on only a few representative examples.

The qualitative LC-MS/MS analyses of six *Lysimachia* species resulted in identification of 86 various compounds. *Lysimachia* species contain these components in varied combination and ratios. Altogether seventeen phenolic acids, six catechin derivatives, seventeen flavonol-*O*-glycosides, two flavonol aglycones, twenty-seven flavone-*C*-glycosides and further seventeen flavonoids were detected in the samples. UV chromatograms of methanolic herbal extracts are shown in Fig. 16-21 in section **4.2**. Characterisation was based on results presented in Table 7. Discussion of the characterisation of phenolics detected in *Lysimachia* herbal extracts is based on structural groups not on plant species or retention time.

Phenolic acid derivatives

Compounds **1** and **38** exhibited molecular ions $[M-H]^-$ at *m/z* 377 and 367, respectively, and characteristic fragment ions at *m/z* 179, 161 and 89. They were tentatively identified as two different caffeic acid derivatives based on comparison of their (-) ESI-MS/MS spectra to that of caffeic acid authentic standard. Product ions at *m/z* 179 and 161 were presented in both MS spectra, and compound **1** gave neutral loss of 162 amu between *m/z* 341 and 179, which pointed to a hexose moiety but no further conclusions could have been drawn about the accurate structure of either compounds **1** and **38**. However, compound **1** was presented in all six investigated species.

Compound **2** and **4** provided molecular ions $[M-H]^-$ at *m/z* 341 and 419 respectively. The neutral loss of 162 amu between *m/z* 341 and 179 and *m/z* 419 and 257 respectively, indicated a hexose sugar residue. Fragment ions at *m/z* 179, 161, and 89 are characteristic for caffeic acid moiety, while *m/z* 169 and 125 refer to gallic acid. Therefore, compound

2 and **4** were tentatively identified as caffeic acid hexoside and gallic acid hexoside derivative, respectively.

Although, quinic acid does not have phenolic structure, its characterisation is discussed here. Compound **3** exhibited molecular ion $[M-H]^-$ at *m/z* 191 and fragment ions at *m/z* 171, 127, 111, and 85 respectively, that might indicate a quinic acid. Compound **8** was detected only in *L. vulgaris* methanolic extract and identified as chlorogenic acid, with molecular ion at *m/z* 353 and characteristic product ions at *m/z* 191, 161 and 135, which was confirmed with comparison to reference standard.

Compound **9** was identified as caffeoyl-quinic acid dimer, compound **10** and **15** were described as 5-caffeoyl-quinic acid and 3-caffeoyl-quinic acid, respectively. Compounds **61**, **66**, **68**, **69**, **72**, **73** and **75** were tentatively identified as isomers of caffeoyl-quinic acid-hexoside. Compound **10** and **15** provided molecular ion $[M-H]^-$ at *m/z* 353. The peak at *m/z* 191 was found to be the base peak in both (-) ESI-MS/MS spectrum, while peaks at *m/z* 179, relatively weaker in spectrum of compound **10** than that of compound **15**. By comparison of these results to literature data (Nandutu et al., 2007) compound **10** and **15** were tentatively described as 5-caffeoyl-quinic acid and 3- caffeoyl-quinic acid. Molecular ion $[M-H]^-$ at *m/z* 707 of compound **9** is assumed to be a dimer of compound **10** ($[M-H]^-$ at *m/z* 353). Neutral loss of 162 amu and the same fragment ions characteristic to caffeoyl-quinic acid indicates caffeoyl-quinic acid hexoside isomers for compounds **61**, **66**, **68**, **69**, **72**, **73**, and **75**. It was not possible to draw any further conclusions about the connection and accurate structure of sugar moiety by the current method.

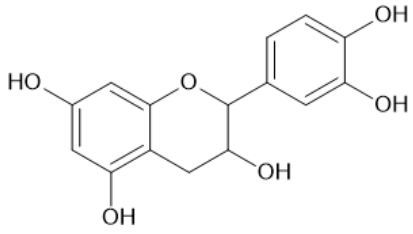
Compound **14** provided molecular ion at *m/z* 177. Fragment ion at *m/z* 163 $[M-CH3]^{\bullet-}$ indicates the loss of methyl group and the one at *m/z* 119 [coumaric acid-H-CO_2]$^-$ is characteristic (Sánchez-Rabaneda et al., 2004) therefore, compound **14** tentatively identified as methyl-4-hydroxycinnamate.

Unlike our results, Hanganu and co-workers (2016) reported the presence of coumaric acid in *L. vulgaris* and *L. nummularia* herb collected in Transylvania, however no mention is made of caffeic acid derivatives. According to literature data, presence of plant acids as ferulic acid and chlorogenic acid are described in *L. christinae* (Sun et al., 2013, Shim et al., 2020), however based on our study, mainly caffeic acid derivatives (compounds **1**, **2**, **9**, **10**, **15**, **38**, **61**, **66**, **68**, **69**, **72**, **73** and **75**) were formed in plant metabolism. Accumulation of caffeic acid in *L. clethroides* has been published earlier

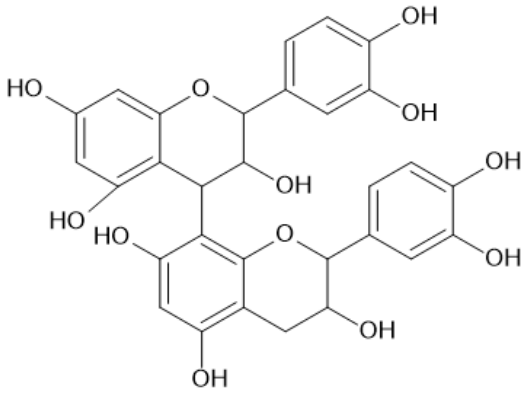
(Liang et al., 2013), although, its derivatives (compounds **1** and **2**) were detected in the present study. As far as we know, caffeic acid derivative (**1**) and caffeic acid-hexoside (**2**) are described in *L. ciliata* for the first time.

Catechin derivatives

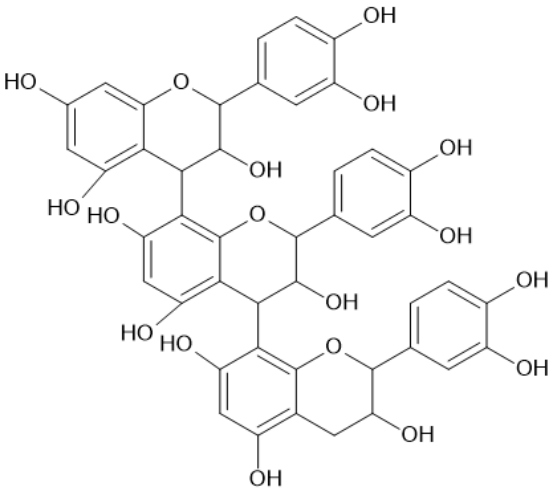
Compounds **6, 7, 18,** and **19** gave deprotonated molecular ions $[M-H]^-$ and characteristic fragment ions at *m/z* 289 and at *m/z* 245, 205, 179, 161 and 137, respectively. These compounds were characterised as catechin or epicatechin based on their UV, and (-) ESI-MS/MS spectra, although by the current method it was not possible to draw any further conclusions about their accurate structure. Compounds **13** and **26** exhibited molecular ions $[M-H]^-$ at *m/z* 577 and 865 respectively, and the same fragment ions were detected at *m/z* 451, 407, 339, 289, 161, and 125. Based on the comparison of UV and MS spectra to literature data (Nandutu et al., 2007) we presumably identified compound **13** as a procyanidin dimer, and compound **26** as procyanidin trimer. Chemical structures of the identified catechin derivatives are depicted in Fig. 26.



catechin/epicatechin (**6, 7, 18, 19**)



procyanidin dimer (**13**)



procyanidin trimer (**26**)

Figure 26. Catechin derivatives detected in *Lysimachia clethroides* extract

Presence of (+) catechin and (-) epicatechin have been formerly described in *Lysimachia clethroides* (Shin-Kim et al., 1993, Wan et al., 2011). Catechin derivatives are reported in *L. christinae* (Shim et al., 2020), however compounds composed of catechin units as procyanidins have not been detected previously.

Flavonoids

Flavonol-*O*-glycosides

UV spectra with absorption maxima at 240-280 nm and 300-380 nm and characteristic mass spectra indicated flavonoid structures in the case of 13 compounds of the investigated Hungarian *Lysimachia* extracts (Fig. 27). From the fragmentation patterns acquired by collision-induced dissociation (CID) in ESI-MS/MS analyses compared to authentic standards and to literature data (Ma et al., 1997, Fabre et al., 2001, Ablajan et al., 2006, Ablajan et al., 2013,) it was possible to characterise the structures of the flavonoids.

Although ESI-MS/MS is not capable for the accurate structural identification of flavonoid glycosides, it provides appropriate information of the aglycone structure and the glycan sequence. The glycosides attached were identified according to the neutral losses of sugar units: difference of 162 amu indicated a hexose (e.g. glucose, galactose), 146 amu presumably a desoxyhexose (e.g. rhamnose), and 132 amu pointed to a pentose (e.g. xylose, arabinose) moiety. The loss of the sugar gives deprotonated flavonol aglycones Y_0^- at *m/z* 285, 301 and 317, corresponding to kaempferol, quercetin and myricetin, respectively. Furthermore, the following observed fragment ions, $[Y_0\text{-H}]^{-\bullet}$ at *m/z* 284, 300, 316, $[Y_0\text{-H-CO-H}]^-$ at *m/z* 255, 271 and 287 are characteristic to the listed aglycones, kaempferol, quercetin and myricetin (Ma et al., 1997, Fabre et al., 2001). The sugar chain may be connected to the flavonoid aglycone through oxygen (*O*-glycoside) or carbon atom (*C*-glycoside). Moreover the sugar molecules may be attached to the aglycone at different positions, or at the same ranged in a row. The *C*-glycosides can be distinguished from the *O*-glycosides, as well as the di-*O*-glycosides from *O*-diglycoside flavonoids by the absence / presence and the relative intensities of characteristic product ions. The cleavage of the glycosidic bond in deprotonated flavonol-*O*-glycosides provide both radical aglycone ion ($[Y_0\text{-H}]^{-\bullet}$) and aglycone ion $[Y_0^-]$ products. Where the relative abundance of $[Y_0\text{–H}]^{-\bullet}$ ion was higher in the product ion spectra than $[Y_0]^-$ ion, 3-*O*-

glycosylated flavonols were presumed. In the mass spectra of di-*O*-glycosylic flavonoids, further observed $[Y_0–2H]^-$ ion with high abundance can be explained by the elimination of two glycosyl radicals at the 3-*O* and 7-*O* positions successively (Ferreres et al., 2004, Ablajan et al., 2006, Ablajan et al., 2013).

Compound **82** was identified as kaempferol in the extracts by comparison of chromatographic and mass spectrometric behaviour to those of an authentic standard. The molecular ion of kaempferol was detected at *m/z* 285 and the characteristic product ions at *m/z* 255 and 227.

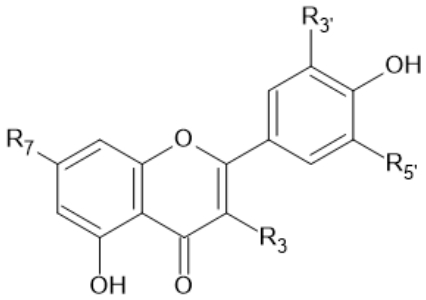
Compound **40** exhibited molecular ion $[M\text{-}H]^-$ at *m/z* 739. The product ion spectra showed higher abundance of characteristic $[Y^7_0]^-$ ion at *m/z* 593 with neutral loss of 146 amu, that of $[Y^3_0]^-$ ion at *m/z* 431, which suggest the 7-*O*-desoxyhexose residue. $[Y^3_0\text{-}H]^{-\bullet}$ radical ion at *m/z* 430 formed through elimination of 3-*O*-glycosyl unit, which indicates a loss of hexosyl-desoxyhexosyl moiety. The subsequently generated $[Y_0\text{-}2H]^-$ ion by cleavage of the 7-*O*-glycosidic bond at *m/z* 283 and $[Y_0]^-$ ion at *m/z* 285 corresponds to the kaempferol aglycone. Thus, compound **40** was tentatively described as kaempferol-3-*O*-hexosyl-desoxyhexoside-7-*O*-desoxyhexoside. Compound **51** and **60** gave molecular ion $[M\text{-}H]^-$ at *m/z* 593, while compound **63** at *m/z* 447. Loss of 162 amu between *m/z* 447 and 285, and loss of 308 amu between *m/z* 593 and 285 indicated hexose and hexosyl-desoxyhexose sugar residues respectively, while the other characteristic product ions at *m/z* 285, 284, 255 and 179 refer to a kaempferol moiety, therefore compounds **51** and **60** assumed as isomers of kamepferol-3-*O*-hexosyl-desoxyhexoside, and compound **63** as kaempferol-3-*O*-hexoside. Compound **76** presented a $[M–H]^-$ ion at *m/z* 431 and fragments at *m/z* 285, with losses of 146 amu, hence, it was identified as kaempferol-3-*O*-desoxyhexoside.

Compounds **71** and **74** provided molecular ions at *m/z* 461. Loss of 162 amu and further 15 amu indicates a hexose and a methyl group. A definitive assignment of the methoxy groups in the aglycone could not be carried out without standards, thus these compounds were tentatively characterised as kaempferol-methyl ether-hexoside isomers. Compound **84** exhibited molecular ion $[M–H]^-$ at *m/z* 299 and $[M–H–CH_3]^{-\bullet}$ as a base peak in the product ion spectrum due to the loss of 15 mass units at m/z 284, thus identified as kaempferol-methyl-ether.

Compound **79** was identified as quercetin by comparing its retention time and product ion spectra with those of authentic standard, showing the characteristic molecular ion at *m/z* 301 and product ions at 271, 255, and 151. Compound **65** provided molecular and fragment ions at *m/z* 447, 300, 271, 243, and 151 indicating quercetin-3-*O*-desoxyhexoside, while compounds **53** and **57** gave molecular ions at *m/z* 463, and neutral loss of 162 amu with further characteristic fragment ions that refer to quercetin-3-*O*-hexoside isomers. Compounds **39**, **47**, **54** were identified as quercetin-3-*O*-hexosyl-desoxyhexosides, according to the same fragment pathway of quercetin and molecular ions at *m/z* 609 with a neutral loss of 308 amu. Compound **47** assigned as rutin according to fragmentation pattern of authentic standard. Compound **28** has probably three sugar units on the aglycone. Based on literature data (Ablajan et al., 2006), the molecular ion at *m/z* 755, and the characteristic fragment ions at *m/z* $[Y^7_0]^-$ 609, $[Y^3_0\text{-H}]^{-\bullet}$ 446, $[Y_0\text{-H}]^{-\bullet}$ 300, and $[Y_0\text{–2H}]^-$ 299, and the higher abundance of $[Y^7_0]^-$ than that of $[Y^3_0]^-$ indicates a desoxyhexose unit at 7-*O*- position and a hexosyl-desoxyhexoside moiety at 3-*O*- position on quercetin aglycone. Therefore compound **28** was presumed as quercetin-3-*O*-hexosyl-desoxyhexoside-7-*O*-desoxyhexoside. Compound **64** with molecular ion at *m/z* 433 is characterised as quercetin-3-*O*-pentoside according to the neutral loss of 132 amu and product ions at m/z 301, 300 and 275, respectively. Compound **62** exhibited deprotonated molecular ion at *m/z* 623, aglycone fragment at *m/z* 315 ($[Y_0\text{-H}]^{-\bullet}$) and other characteristic fragment ion at *m/z* 271. The neutral loss of 308 amu and the relatively high intensity of the product ion ($[Y_0\text{-H}]^{-\bullet}$) suggested the presence of a hexosyl-desoxyhexoside in the 3-*O* position. According to literature data and our previous study compound **62** was tentatively identified as isorhamnetin-3-*O*-desoxyhexosyl-hexoside (Yasukawa & Takido 1988, Toth et al., 2014).

Compounds **31**, **32**, **36** and **52** exhibited radical aglycone ion ($[Y_0\text{-H}]^{-\bullet}$) and aglycone ion $[Y_0^-]$ products at *m/z* 316 and 317, indicating myricetin aglycone. Neutral loss of 308, 162 and 146 was suggesting the tentative structure for compound **31** myricetin-3-*O*-hexosyl-desoxyhexoside, for compound **32** and **36** myricetin-3-*O*-hexoside isomers and for compound **52** myricetin-3-*O*-desoxyhexoside, respectively. Fragmentation pattern of compound **52** was compared with that of authentic standard, and was identified as myricitrin.

Flavonoid compounds of the Hungarian native *Lysimachia* sepcies are summarised in Figure 27. In our study one methoxy flavonoid, isorhamnetin-3-*O*-desoxyhexosyl-hexoside (**62**) was characterised only in *L. vulgaris* in accordance with Yasukawa and Takido's (1988) findings. Presence of myricetin-3-*O*-hexoside (**36**), kaempferol-3-*O*-hexosyl-desoxyhexoside-7-*O*-desoxyhexoside (**40**), quercetin-3-*O*-hexoside (**57**), quercetin-3-*O*-pentoside (**64**), quercetin-3-*O*-desoxihexoside (**65**) in Hungarian native species is in good accordance with other studies (Hänsel et al., 1975, Yasukawa et al., 1988, Yasukawa et al., 1990, Méndez 1970). Chemical structures of the main identified compounds of Hungarian native *Lysimachia* species are depicted in Fig. 27.



	R_3	R_7	$R_{3'}$	$R_{5'}$	
quercetin-3-*O*-hexosyl-desoxyhexoside-7-*O*-desoxyhexoside	hexose-desoxyhexose	desoxyhexose	OH	H	(**28**)
myricetin-3-*O*-hexosyl-desoxyhexoside	hexose-desoxyhexose	OH	OH	OH	(**31**)
myricetin-3-*O*-hexoside	hexose	OH	OH	OH	(**36**)
kaempferol-3-*O*-hexosyl-desoxyhexoside-7-*O*-desoxyhexoside	hexose-desoxyhexose	desoxyhexose	H	H	(**40**)
quercetin-3-*O*-hexosyl-desoxyhexoside	hexose-desoxyhexose	OH	OH	H	(**47**)
myricetin-3-*O*-desoxyhexoside	desoxyhexose	OH	OH	OH	(**52**)
quercetin-3-*O*-hexoside	hexose	OH	OH	H	(**57**)
kaempferol-3-*O*-hexosyl-desoxyhexoside	hexose-desoxyhexose	OH	H	H	(**60**)
isorhamnetin-3-*O*-hexosyl-desoxyhexoside	hexose-desoxyhexose	OH	OCH_3	H	(**62**)
kaempferol-3-*O*-hexoside	hexose	OH	H	H	(**63**)
quercetin-3-*O*-pentoside	pentose	OH	OH	H	(**64**)
quercetin-3-*O*-desoxyhexoside	desoxyhexose	OH	OH	H	(**65**)
quercetin	OH	OH	OH	H	(**79**)
kaempferol	OH	OH	H	H	(**82**)

Figure 27. Flavonol compounds in the Hungarian native *Lysimachia* sepcies

The main compounds were quercetin-3-*O*-hexosyl-desoxhexoside-7-*O*-desoxihexoside (**28**) and rutin (**47**) in yellow loosestrife samples, and myricitrin (**52**) in moneywort and spotted loosestrife samples. Based on chromatographic data, the main compound of gooseneck loosestrife was an isomer of quercetin-3-*O*-hexoside (**57**), and main compound of fringed loosestrife was another isomer of quercetin-3-*O*-hexoside (**53**).

Amongst flavonol-*O*-glycosides, hyperoside, quercitrin, isoquercitrin, rutin, were identified in *L. vulgaris*, and the latter three in *L. nummularia* published by Hanganu and co-workers (2016). Interestingly, the presence of flavonol di- and tri-glycosides, as well as kaempferol – and myricetin derivatives is not mentioned. Contrary to our results, in addition to kaempferol and quercetin free flavonol aglycones, accumulation of apigenin and luteolin in *L. vulgaris* and the latter one in *L. nummularia* was confirmed by Hanganus' research group (2016). Our results are in good accordance with the paper of Csepregi and co-workers (2020). *L. nummularia* contained flavonol gylcosides, dominated by the presence of myricetin glycosides, such a myricetin-desoxyhexosyl-hexoside, myricetin-hexoside, myricetin-desoxyhexoside and other derivatives. Equally with our data, myricetin-3-*O*-desoxyhexoside presumably myricitrin was found as main compound. However, in contrast to our results, occurrence of coumaroyl and feruloyl glucarate isomers was reported (Csepregi et al., 2020).

Presence of quercetin- and kaempferol and their mono- and di- *O*-glycosides in gold coin grass and gooseneck loosestrife are described here in detail and our results are in good accordance with other papers (Liu et al., 2019, Shin-Kim et al., 1993, Ren et al., 2001, Wan et al., 2011, Sun et al., 2013, Wei et al., 2017, Kim et al., 2018, Jiang et al., 2007, Liu et al., 2010). Compound **28** is a quercetin-tri-*O*-glycoside, while compound **40** is a kaempferol-tri-*O*-glycoside. Although our (-)ESI-MS/MS investigation did not give information about the exact structure, according to Liu and co-workers (2010), formation of kaempferol-2'-α-L-rhamnopiranosyl-3-*O*-β-rutinoside and quercetin-2'-α-L-rhamnopiranosyil-3-*O*-β-rutinoside is presumed. Our results suggest only the presence of myricetin-3-*O*-hexoside in *L. christinae*, yet literature data also describe additional derivatives of myricetin in this species (Liu et al., 2019).

Quercetin-3-*O*-hexosyl-desoxyhexoside-7-*O*-desoxyhexoside (**28**), myricetin-3-*O*-hexoside (**32**, **36**), quercetin-3-*O*-hexosyl-desoxyhexoside (**39**), kaempferol-3-*O*-hexosyl-desoxyhexoside-7-*O*-desoxyhexoside (**40**), kaempferol-3-*O*-hexosyl-

desoxyhexoside (**51**) and quercetin-3-*O*-hexoside (**53**) have been described in *L. ciliata* for the first time.

Flavone-*C*-glycosides

The application of mass spectrometry in the structural analysis of flavonoid-*C*-glycosides has been reviewed in detail (Ferreres et al., 2003, Marin et al., 2004, Ferreres et al., 2007, Guo et al., 2013, Cao et al., 2014, Singh et al., 2015). The ESI-MS/MS fragmentation patterns of *C*-glycosyl flavonoids are different from *O*-glycosyl flavonoids. Neutral losses of 90, 120 and 150 amu were observed for 6-*C*- and 8-*C*-glycosyl flavonoids by Ferreres and co-workers (2003). Furthermore, based on fragmentation pattern, 6-*C*- and 8-*C*-glycosides can be distinguished. Guo and co-workers (2013) confirmed that the presence of $[M-H-H_2O]^-$ fragment ions in the CID spectra by loss of H_2O, and the high relative intensity of $[^{0,3}X_0-H]^-$ and presence of $[^{0,3}X_0-H-H_2O]^-$ product ions are characteristic of flavon-6-*C*-glycosides, in contrast to flavon-8-*C*-glycosides, where CID spectra showed more abundant $[^{0,2}X_0-H]^-$ ions compared to $[^{0,3}X_0-H]^-$ ions. Ferreres and co-workers (2003, 2007) noted that for di-*C*-glycosides, characteristic fragment ions are at *m/z* Agly+113 and Agly+83, while, *O*-substituted *C*-glycosidic chain gave fragment ions at *m/z* Agly+41, Agly+(41-18), Agly+71 and Agly+(71-18).

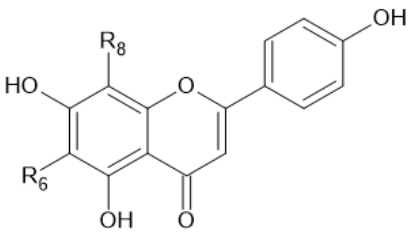
Compound **29** provided molecular ion $[M-H]^-$ at *m/z* 447 with neutral loss of 90, 120 and 150 amu indicating *C*-glycoside. The deprotonated aglycone $[Y_0-H]^-$ at *m/z* 285 and the presence of $[M-H-H_2O]^-$ at *m/z* 429 and relative high abundance of $[^{0,3}X_0-H]^-$ fragment ion at *m/z* 357 indicates luteolin-6-*C*-hexoside, presumably isoorientin. At the same time, compound **56** at *m/z* 447 yielded fragment ions at m/z 357, 327, and 285. The high relative intensity of $[^{0,2}X_0-H]^-$ at *m/z* 327 assumed to be identified as luteolin-8-*C*-hexoside, probably orientin. Comparison of data with mass spectra of standard substances confirmed these results. Compound **35** with molecular and fragments ions at *m/z* 429, 357, 327, 297, 285 was supposed to be a luteolin-6-*C*-hexoside isomer.

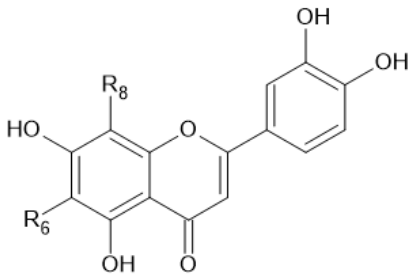
Compound **11** exhibited ions at *m/z* 609 $[M-H]^-$, 519 $[M-H-90]^-$, 489 $[M-H-120]^-$, 429 $[M-H-(120+60)]^-$, 399 $[M-H-(120+90)]^-$, 369 $[M-H-(120+120)]^-$. Based on fragmentation pattern and literature data (Singh et al., 2015), compound **11** was tentatively identified as luteolin-6,8-di-*C*-glycoside, presumably orientin-6-*C*-hexoside.

Compounds **12**, **16**, **20**, **23** and **24** provided $[M-H]^-$ at *m/z* 579, with loss of 60, 90, 120, 150 amu, thus supposed to be a *C*-glycoside derivative. The characteristic product ions were $[M-H-60]^-$ and $[M-H-150]^-$ at *m/z* 519 and 429, respectively, pointing to a pentose unit. Furthermore, the fragmentation pattern was typical for di-*C*-glycosyl flavones, due to the presence of the ions Agly+113 and Agly+83 at *m/z* 399 and 369 respectively. A 6-*C*-pentosyl-8-*C*-hexosyl-substitution is indicated for these compounds, because of the higher abundance of the ion $[M-H-90]^-$ relatively to $[M-H-120]^-$. Thus, according to the considerations previously described and literature data (Ferreres et al., 2003), these compounds were tentatively identified as isomers of luteolin-6-*C*-pentosyl-8-*C*-hexoside. Compound **48** yielded molecular ion $[M-H]^-$ at *m/z* 431. Further product ion $[M-H-H_2O]^-$ at *m/z* 413, and product ion with high relative abundance $[^{0,3}X_0-H]^-$ at *m/z* 341, and deprotonated aglycone $[Y_0-H]^-$ at *m/z* 285 supposed to be apigenin-6-*C*-hexoside, presumably isovitexin. However, compound **67** at *m/z* 431 exhibited product ions at m/z 341, 311, 269. The high relative intensity of $[^{0,2}X_0-H]^-$ (*m/z* 311) assumed to be identified as apigenin-8-*C*-hexoside or vitexin. Comparison of data with mass spectra of standard substances confirmed these results.

Compound **17**, **30**, and **41** provided molecule ion $[M-H]^-$ at *m/z* 593. The further produced ions at *m/z* 575 $[M-H-18]^-$, 503 $[M-H-90]^-$ and 473 $[M-H-120]^-$, exhibiting a fragmentation pattern of flavone di-*C*-glycosides, indicate apigenin as an aglycone substituted with two hexose moieties. Therefore, these compounds were tentatively assigned as isomers of apigenin-6,8-di-*C*-hexoside, presumably isovitexin-8-*C*-hexoside. Compounds **22**, **25** and **27** showed the same $[M-H]^-$ ion at *m/z* 563 in the MS scan and produced the same fragments in ESI-MS/MS, indicating isomeric structures. In the MS/MS spectrum, the presence of the $[M-H-18]^-$ ion at *m/z* 545, and the relative abundance of the $[M-H-120]^-$ ion was higher at *m/z* 443 compared to the $[M-H-90]^-$ ion at *m/z* 473 indicating the presence of a 6-*C*-hexosyl-8-*C*-pentosyl substitution. The ions at *m/*z 383 $[M-H-(120+60)]^-$ and 353 $[M-H-(120+90)]^-$ indicated the presence of apigenin substituted with 6-*C*-glucoside-8-*C*-arabinoside (Cao et al., 2014). Therefore, compound **22**, **25** and **27** are tentatively identified as isomers of isovitexin-8-*C*-pentoside. Compound **42**, **45**, **46** and **50** gave molecular ion at *m/z* 533. Major fragment ions at *m/z* 473 $[M-H-60]^-$ and 433 $[M-H-90]^-$ indicating the presence of a 6-*C*-pentosyl residue.

Further fragment ions at *m/z* 383 $[M-H-(60+90)]^-$ and 353 $[M-H-(90+90)]^-$ pointed to a 8-*C* pentose unit. These data suggested apigenin-6,8-di-*C*-pentosides (Cao et al., 2014).
Compound **34** provided molecular ion at *m/z* 475. Product ions at *m/z* 431, 341 and 311 are characteristic for vitexin. As no further structural information could be interpreted, this compound supposed to be a vitexin-derivative.
For compounds **43** and **49** with molecular ion at *m/z* 433, and for compound **55** and **58** with molecular ion at *m/z* 461 and for compound **44** with molecular ion at *m/z* 577, the neutral loss of 90 and 120 amu indicates flavone-*C*-glycosides, however no further detailed structure was characterised.
Liu's researcher group (2019) described the detailed investigation of eighty flavonoids of *L. christinae* herb. Our results are in good accordance with this paper. Flavone-mono-*C*-glycosides, especially vitexin, isovitexin, orientin, and isoorientin, furthermore flavone-*C*-diglycosides are tentatively identified. However no evidence of naringenin- and diosmetin-*C*-glycosides was found. Structures of the most abundant flavon-*C*-derivatives are summarised in Figure 28.
Presence of isoorientin (**29**) luteolin-6-*C*-hexoside (**35**) and isovitexin (**48**) have been described in *L. ciliata* for the first time.





	R6	**R8**			**R6**	**R8**	
m/z 431	hexose	H	(**48**)	*m/z* 447	hexose	H	(**29, 35**)
m/z 431	H	hexose	(**67**)	*m/z* 447	H	hexose	(**56**)
m/z 533	pentose	pentose	(**42**,**45**,**46**,**50**)	*m/z* 579	pentose	hexose	(**12**,**16**,**20**, **23**,**24**)
m/z 563	hexose	pentose	(**22**,**25**,**27**)	*m/z* 609	hexose	hexose	(**11**)
m/z 593	hexose	hexose	(**17**,**30**,**41**)				

Figure 28. The most abundant flavon-*C*-compounds in the *Lysimachia* extracts

Further flavone and flavanone derivatives

Compound **83** exhibited fragment ions at *m/z* 269 $[M-H]^-$, 225 $[M-H-44]^-$ 197 $[M-H-44-28]^-$ and 151 characteristic for flavones, thus compound **83** tentatively identified as trihydroxyflavone, presumably apigenin (Fabre et al., 2001). The same conclusion could be drawn about compound **77**. Molecule ion at *m/z* 253 and the fragment ion mentioned above (*m/z* 269) showed 16 Da difference from those of trihydroxyflavone, thus the presence of two hydroxyl groups in the molecule was also presumed, so compound **77** was tentatively identified as dihydroxyflavone. Compounds **33** and **37** yieled molecule ion and deprotonated aglycone ion at *m/z* 563 and 269 respectively. The latter one is probably the trihydroxyflavone aglycone based on the above, while the neutral loss of 294 Da between *m/z* 653 and 269 refers to a hexose (162) and pentose (132) moiety, thus compound **33** and **37** are tentatively characterised as isomers of trihydroxyflavone-pentosyl-hexoside. Compound **80** exhibits $[M-H]^-$ at *m/z* 283, 30 Da difference from those of dihydroxyflavone referred to further methoxy (CH_3O-) group on the molecule, and assumed a dihydroxy-methoxy-flavone. Compound **81** showed molecular ion at *m/z* 491 and further fragment ions at 283 and 268, thus some glycosides derivative of dihydroxy-methoxy-flavone is feasible. Compound **70** is tentatively identified as dihydroxy-methoxy-flavone-hexoside due to the molecular ion $[M-H]^-$ detected at *m/z* 445, neutral loss of 162 amu, and deprotonated aglycone ion at $[Y_0-H]^-$ at *m/z* 283. Compound **78** exhibited molecular ion and fragment ion at *m/z* 285 and 270, 2 Da difference from those of compound **80**. The 2 Da difference referred to a saturated double bond, therefore, compound **78** was probable as dihydroxy-methoxy-flavanone.

Compound **85** and **86** were tentatively identified as trihydroxy-dimethoxy-flavone. The neutral loss of 15 Da indicated the cleavage of a methyl radical, thus the presence of a methoxy group in the molecules are presumed. However without authentic standard, exact location of methyl groups can not be determined by MS/MS. Its CID spectra showed $[M-H]^-$ at *m/z* 329, and fragment ions at *m/*z 314 $[M-H-CH_3]^-$ and 299 $[M-H-2CH_3]^-$ were also detected. Another fragment ion at *m/z* 271 was due to $[M-H-2CH_3-CO]^-$.

Compounds **5**, **21** and **59** were tentatively identified as isomers of dihydrokaempferol–hexoside. Neutral loss of 162 amu refers to a hexose moiety, while fragment ions at *m/z* 287, 269, 259, 179, 125 are characteristic for dihydrokaempferol based on literature data (Lech 2020).

In our study one methoxy flavonoid, isorhamnetin-3-*O*-desoxyhexosyl-hexoside (**62**) was characterized only in *L. vulgaris*. Methylated flavonoid, as tricin, isorhamnetin, diosmetin and laricitrin and flavone aglycones, as apigenin, luteolin derivatives have been described in *L. christinae* (Sun et al., 2013, Liu et al., 2019). Presence of di- and trihydroxy-flavone were confirmed, and accumulation of methylated flavonoids in *L. christinae* herb are presumed also, however without authentic standard the exact structure cannot be determined by our method.

5.2.1. Comparison of the phenolic profile of the *Lysimachia* extracts

The HPLC-DAD-ESI-MS analyses revealed that the main compounds of all the *Lysimachia* extracts were flavonoid derivatives, however accumulation of structurally different flavonoids showed great variability in the species. Phenolic metabolite pattern of six *Lysimachia* species summarised in Table 17 and the following findings can be made.

Table 17. Phenolic profile of the *Lysimachia* extracts studied

Phenolic compounds		Subgenus					
		Seleucea	Lysimachia gr. B		Lysimastrum	Nummularia	Palladia
		L. ciliata	*L. num.*	*L. punct.*	*L. vulg.*	*L. christ.*	*L. clethr.*
Phenolic acids		X	X	X	X	X	X
Catechin derivatives		-	-	-	-	-	X
Kaempferol derivatives	Aglycone	-	-	X	X		-
	monoglycosides	-	X	X	X	X	X
	diglycosides	X	-	-	X	X	X
	triglycosides	X	-	-	X	-	X
Quercetin derivatives	Aglycone	-	-	X	X	X	-
	monoglycosides	X	X	X	X	X	X
	diglycosides	X	-		X	X	X
	triglycosides	X	-	-	X		X
Myricetin derivatives	Aglycone	-	-	-	-	-	-
	monoglycosides	X	X	X	-	X	X
	diglycosides	-	-	-	X	-	-
	triglycosides	-	-	-	-	-	-
Flavone-C-glycosides		X	-	-	-	X	-
Methoxy derivatives		-	-	-	X	X	-

Among the phenolic acids identified in methanolic extracts, one caffeic acid derivative was present in all six *Lysimachia* species. (Epi)catechins and procyanidins are accumulated only in *L. clethroides* species belonging to the Palladia subgenus. Regarding the occurrence of unmethylated flavonoid-*O*-glycosides, great variability is observed in the studied species. The results of the qualitative analyses by HPLC-DAD-ESI-MS/MS clearly indicated that the main flavonoids are kaempferol- quercetin and myricetin mono- and di-*O*-glycosides. Among flavonols, kaempferol appeared in aglycone form only in *Lysimachia vulgaris* and *L. punctata*, while quercetin accumulated in *L. christinae* also in addition to the aformentioned two species. The quantitative amount of quercetin in the latter one has diagnostic significance, given that *L. christinae* is official in the Pharmacopoeia of People's Republic of China. However, myricetin-monoglycoside was identified in almost every species, exept in *L. vulgaris*. In contrast, this flavonoid does not appear in aglycone form in any species, even in diglycosidic form it occurs only in the aerial part of *L. vulgaris*. Accumulation of quercetin- and kaempferol-tri-*O*-glycosides are characteristic of three species: *L. vulgaris*, *L. ciliata* and *L. clethroides*, which species show macromorphological similarity. A large number of structurally different flavone-*C*-glycosides, especially orientin, isoorientin, vitexin and isovitexin and their derivatives were identified in the extracts, which are characteristic only in two species, *L. ciliata* and *L. christinae*. Nevertheless, these two species are macromorphologically and phylogenetically relatively far apart.

The distinguishing features of *L. clethroides* among the studied species are the presence of catechins and dimer and trimer forms of procyanidins, as well as the simultaneous absence of methylated flavonoids and *C*-glycosides.

L. christinae had the richest phenolic metabolite spectrum. Its distinguishing features are the simultaneous presence of flavonol-*O*-glycosides, flavone-*C*-glycosides and methylated derivatives. Among the methylated derivatives, mono- and dimethoxy compounds were probable.

In addition to flavonol derivatives *L. ciliata* contains *C*-glycosides similar to *L. christinae*. Based on the fragmentation pattern, flavone-6-*C*-derivatives, isoorientin and isovitexin are synthetized in the herb. Unlike the aforementioned species, methylated flavonoids are absent secondary metabolites. This work is the first to describe the presence of 2 caffeic acid derivatives and 10 flavonoid compounds in *L. ciliata* herb (not published yet).

Regarding Hungarian species, the presence of flavonol-*O*-mono-di- and triglycosides are characteristic. However, there are significant differences in the phenolic metabolites of the three closely related *Lysimachia* species from the same family. The morphological properties of spotted loosestrife are similar to yellow loosestrife. According to chemotaxonomic data, it belongs to *Lysimachia* subgenus group B, as well as creeping jenny, compared to yellow loosestrife which belongs to Lysimastrum subgenus. Based on our examination the similarity in the phenolic secondary metabolites of the other two species is considerable. In view of the accumulation of myricitrin and some further major components, *L. punctata* is close to *L. nummularia*, while accumulation of free flavonoid aglycones, which are completely absent from creeping jenny, but characteristic in yellow loosestrife makes it similar to the latter one.

5.3. Quantitative analysis of Hungarian native species by HPLC-DAD

Due to the incomplete scientific investigations and the more deteailed phytochemical characterisation of Hungarian native species, quantitative analysis of the main phenolic compounds in *L. vulgaris*, *L. nummularia* and *L. punctata* was performed. A liquid chromatographic method was developed for the quantitative analyses of phenolic compounds in methanolic extracts of three Hungarian native *Lysimachia* species. The quantity of compounds was determined by LC-DAD. Detection wavelength of 350 nm was chosen for the quantitation of flavonoid derivatives and chlorogenic acid. The performed gradient elution ensured a good resolution for the main compounds, and linear regression analyses were applied by the use of external calibration.

Linearity was determined by analysing kaempferol, quercetin, rutin, myricetin-3-*O*-rhamnoside and chlorogenic acid authentic standards. However, it was not possible to obtain commercial standards for each compound or isolate them. Only the listed compounds were available as authentic standard, therefore only these amounts were exactly determined. Nevertheless, an estimate value of other components can also be given. The amounts of different flavonol glycosides were inferred from the calibration data of the standard compound with the corresponding aglycone. This quantitation method provides good linearity, precision and accuracy (Tables 8-9).

Based on the result of qualitative analyses, the two main compounds of yellow loosestrife are rutin and quercetin-3-*O*-neohesperoside-7-*O*-rhamnoside (quercetin-3-*O*-hexosyl-

desoxyhexoside-7-*O*-desoxyhexoside), which are accumulated in the herb nearly the same amount (14.2±0.3 and 12.7±0.3 mg/g dried extract, respectively). The two flavonoid aglycones, kaempferol and quercetin were measured in very low amount (less than 0.4mg/g dried extract). In contrast, spotted loosestrife is containing quercetin nearly the same amount but kaempferol aglycones represents twice higher (0.8±0.0 mg/g dried extract) in the sample. The ration of main compound myricitrin is 8.26±0.2 mg/g dried extract, which is much lower than in creeping jenny (26.22±0.6 mg/g dried extracts). Results of the quantitative analysis by HPLC-DAD are depicted in Figure 29.

Hanganu and co-workers (2016) quantified flavonoids in moneywort and yellow loosestrife collected in Transylvania, however, the extraction method and expression of results were different, therefore their data are not fully comparable with our results. For that reason, comparison of quantitative results are omitted.

According to the literature, quantitative data of flavonoids in *L. punctata* herb have not been reported at all.

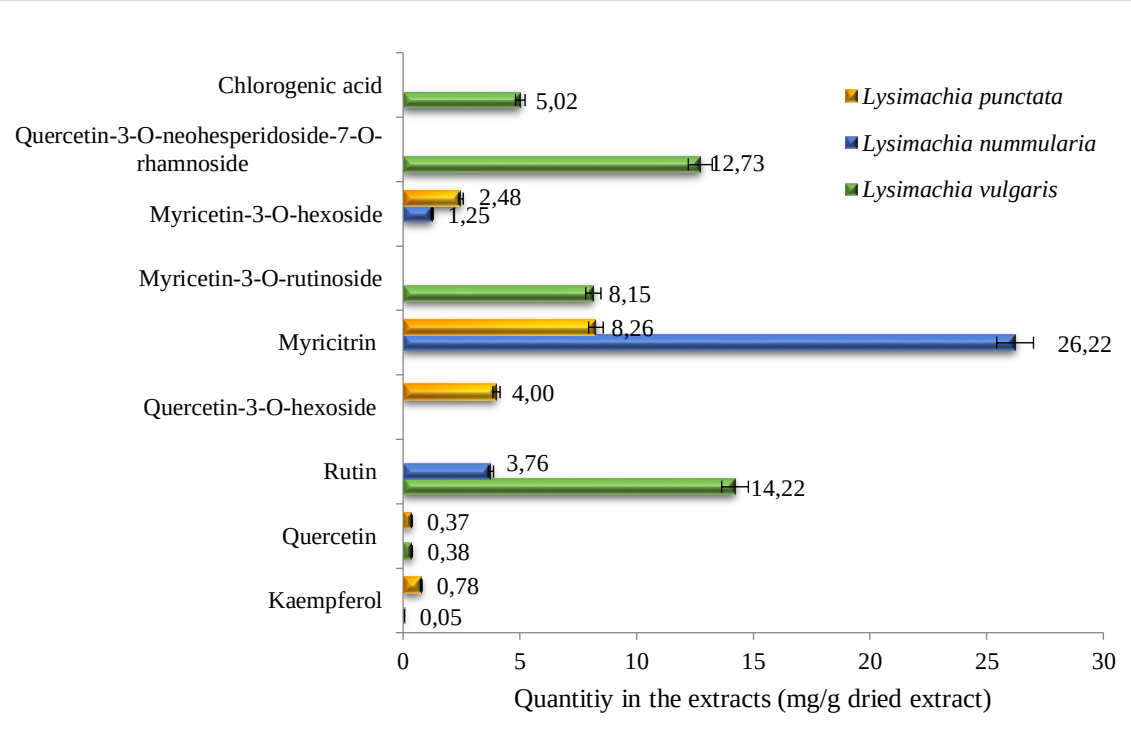


Figure 29. Results of the quantitative analyses of methanolic extracts of Hungarian native *Lysimachia* species by HPLC-DAD

5.4. 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 presence of kampferol- quercetin- and myricetin-O-glycosides are characteristic of all six species studied. 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 (section **3.3**.). Under the used conditions, *C*-glycosidic bonds are unimpaired. For the quantitation of flavonoid aglycones, kaempferol, quercetin and myricetin 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 optimised UPLC method was validated in terms of linearity, limits of quantification (LOQ) and detection (LOD), precision and accuracy. The validation parameters are summarised in Table 11-12 and in section **3.8.** In our studies flavonol aglycones, kaempferol quercetin and myricetin were present in all the extracts in amounts above the LOQ of the applied UPLC-DAD method. The quantitative results are summarised in Figure 30.

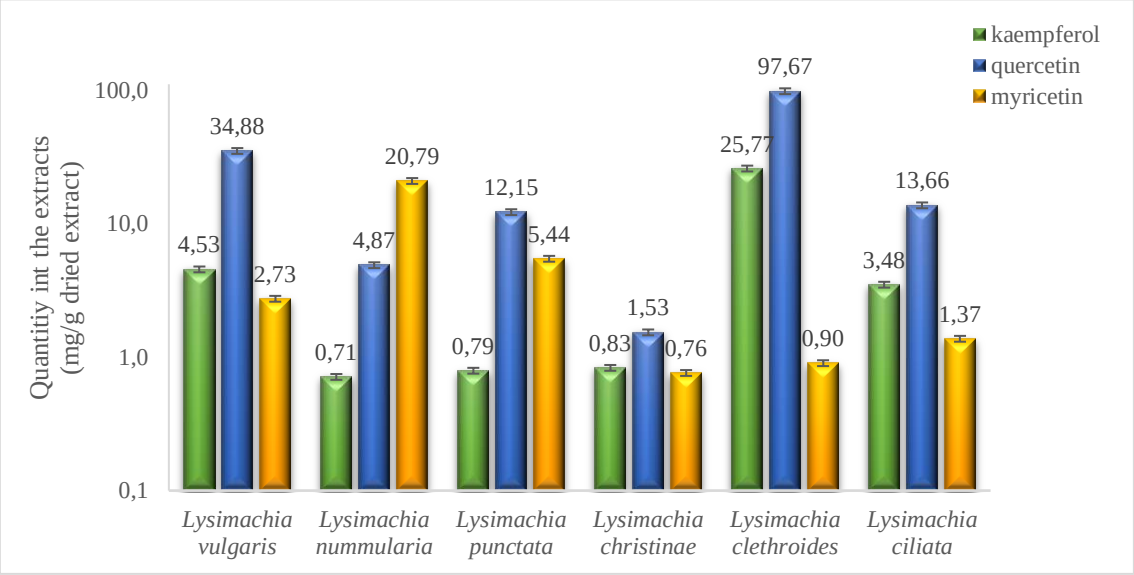


Figure 30. Results of the quantitative analyses of hydrolysed methanolic extracts by UPLC-DAD

Interesting to note, that the main compound of hydrolysed extract of spotted loosestrife was quercetin (Fig. 22) in contrast of the methanolic extract, where myricetin-3-*O*-rhamnoside (myricitrin) (Fig. 18). The apparent discrepancy may be explained by the different measurement conditions (section **3.7.-3.8.**). In addition, several quercetin-glycosides, as minor compounds contributed to the quercetin content of the hydrolysed extracts. Furthermore, it is possible that the hydrolysis has not been completed yet, or the degradation of aglycones has started, but no studies have been performed due to the use of the Pharmacopoeia protocol.

The calculated total flavonol aglycone content (sum quantity of kaempferol, quercetin and myricetin) was the highest in *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 *Lysimachia vulgaris* sample revealed relatively high amount of quercetin, also confirmed by our previous work (Toth et al., 2014).

The lower quantity of flavonol aglycones, however, according to literature data, other type of flavonoids: flavone-, flavanone- and methylated flavonoid derivatives are cumulated in the herb. Furthermore, *C*-glycosides are also reported in *Lysimachia* species (Sun et al., 2013).

To the best of our knowledge, no literature data are available regarding the flavonoid composition of *L. ciliata*. Presence of kaempferol-, quercetin- and myricetin aglycones in the hydrolysed extract and their quantity were described for the first time. The cultivated varieties 'Firecracker' contains similarly to *L. vulgaris,* mainly quercetin and kaempferol, while myricetin is present in lower amounts. In *L. clethroides*, significant amount of quercetin and kaempferol was measured, which is in good agreement with literature data that show presence of numerous glycosides of kaempferol and quercetin (Liu et al., 2010). The amount of myricetin was the lowest in both *L. clethroides* and *L. christinae*.

5.5. Antioxidant activity assays

Antioxidant properties of plant derived flavonoids are widely known. Several mechanisms have been proved to play role in the antioxidant effect of polyphenols. They exerts their effect by direct free radical neutralization, which can occur by HAT, SET-PT or SPLET mechanism (section **1.2.3.**), while they are able to delocalize the unpaired

electron leading to the formation of a stable phenoxyl radical. They also contribute to reducing lipid peroxidation and maintaining redox homeostasis in the body by chelating heavy metal ions and neutralizing singlet oxygen (Wright el al., 2001, Heim et al., 2002, Procházková, 2011, Gülcin 2012, Zheng et al. 2019a).

Several *in vitro* methods have been developed to determine antioxidant activity (section **1.3.**), the most commonly used for the analysis of plant extracts being the neutralization reaction of DPPH (Blois 1958) and ABTS (Miller et al., 1993, Re et al., 1999).

Both scavenging methods have been used widespread to evaluate the antioxidant activity of compounds due to the simple, rapid and sensitive procedures. ABTS radicals are more reactive than DPPH radicals, and unlike the reactions with DPPH radical, which involve mainly HAT, and much less frequently SET-PT mechanism, the reactions with ABTS radicals involve both HAT or SET-PT and even SPLET mechanism depending on the structure of the compounds (Gülcin 2012, Spiegel et al., 2018, Gülcin 2020, Ilyasov et al., 2020).

In order to explain our result more accurately, the structural features of antioxidant flavonoids are summarised in section **1.2.2**. Repeating briefly, the phenolic hydroxyl group can also function as a H-donor and acceptor, so the ortho-dihydroxyl group of ring B (catechol or galloyl function) is involved in electron delocalization. The double bond between carbon atoms 2 and 3 in conjugation with the oxo group at position 4 of ring C contributes to the extension of delocalization. Additional hydroxyl groups in the C3- and C5-positions are able to form a H-bond with the oxo group in the 4-position, thereby increasing the free radical scavenging potential of the compound. These properties stabilize the phenoxyl radical formed after the removal of the H atom. The glycosylation and methylation patterns of the hydroxyl group, especially at the C3 position, may reduce the free radical scavenging ability. Based on these facts, the *in vitro* antioxidant activity of flavonoids depends on the number and location of free hydroxyl groups (Blois 1958, Bors et al., 1995, Burda & Oleszek 2001, Choe & Min 2009, Gülcin 2012, Spiegel et al., 2020).

DPPH and ABTS radical systems were used for the primary screening of the antiradical activities of *Lysimachia* aerial parts and reference compounds. The results demonstrate that the various samples investigated have a significant hydrogen – and electron-donating

ability, which are concentration dependent in the presence of DPPH and ABTS radicals (Tables 14-15, Fig. 31).

Based on the results obtained, the following conclusions could be drawn. The flavonoid standards confirmed that the free-radical scavenging activity is in strong relation to the number and glycosylation status of the phenolic OH groups. The higher number of free phenolic hydroxyl groups comes with higher scavenging activity, (Tables 14-15, Fig. 31), e.g. quercetin with 2 free OH groups vs. kaempferol with 1 free OH group on ring B. The latter is weaker antioxidant. Similarly, the difference in radical scavenging activity can be explained between glycosidic bond containing rutin and their free OH group containing quercetin.

The mentioned *in vitro* assays were employed to determine the free radical scavenging potency of the extracts. Table 14-15. and Figure 31. summarise the results of the antioxidant capacity of the standards and methanolic and hydrolysed *Lysimachia* extracts. All the plant extracts were capable of scavenging the mentioned radicals in a concentration-dependent manner, however the radical scavenging activity of herbal extracts are lagged behind standards, because the identified main flavonoids of *Lysimachia* species are present as glycosides, which have obviously lower scavenging activity than flavonoid aglycones as minor components.

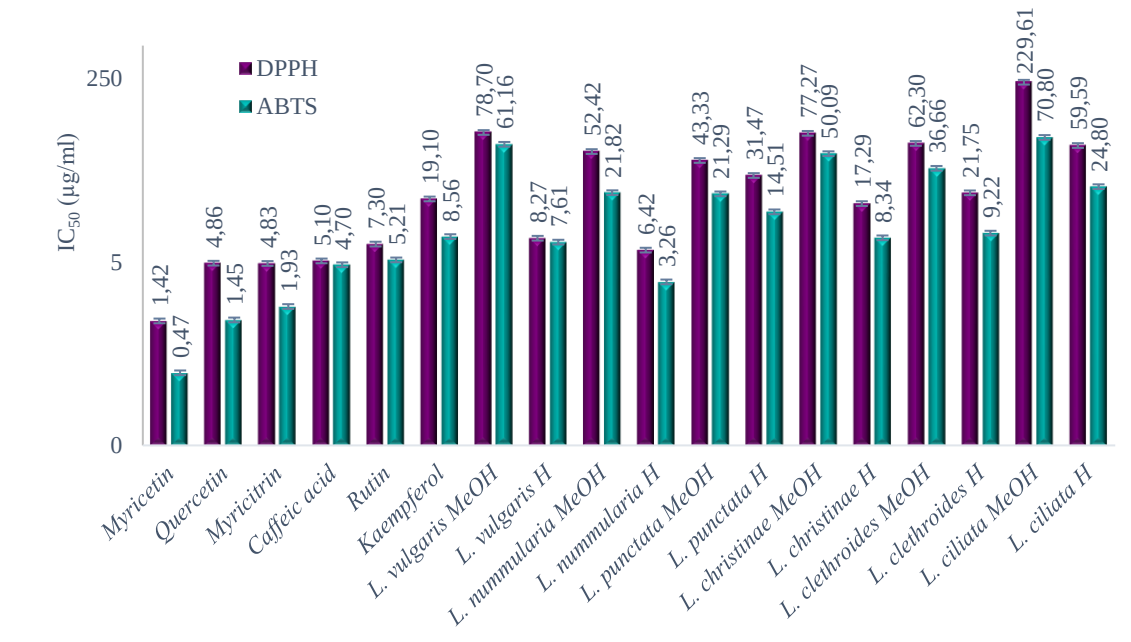


Figure 31. Half maximal inhibiton concentration (IC_{50}) of the *Lysimachia* extracts in the DPPH and ABTS *in vitro* tests. Abbreviations: MeOH: methanolic extract, H: hydrolysed methanolic extract

Among the herbal extracts *L. punctata* proved to have the strongest hydrogen donor capacity with a mean IC_{50} of 43.3±2.2 μg/ml, followed by *L. nummularia* 52.4±3.5 μg/ml. Flavonoid content of these species (see section **4.1.**) is the highest, however, the order is reversed between them. The presence and high number of flavonoids with myricetin aglycone (Table 7) 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 (Table 5-6, Figure 24-25).

Hanganu et al., (2016), Yildirim et al., (2017), Wei et al., (2017) and Csepregi and co-workers (2020) reported DPPH or ABTS radical scavenging activity results of *Lysimachia* species, however, comparison of the data is difficult due to the differences in the expression of the results and in the methods used.

In the ABTS system nearly the same trend was observed for 50% inhibition values, with the highest activity in *L. punctata* and *L. nummularia*, (21.3±0.6 μg/ml, 24.8±0.7 μg/ml, respectively) and lowest in *L. ciliata* 70.80±5.94 μg/ml.

Hydrolysed extracts of *Lysimachia* species showed stronger radical scavenging activity than herbal methanol extracts. 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 system *L. nummularia* hydrolysed extract showed the highest antioxidant activities (IC_{50}=6.42±0.30 μg/ml) which can be explained by the high amount of myricetin in the sample. Besides *L. nummularia*, *L. vulgaris* (IC_{50}=8.27±0.39 μg/ml) and *L. christinae* (IC_{50}=17.29±0.81 μg/ml) hydrolysed extracts have higher scavenging activity than kaempferol (IC_{50}=19.10±0.90 μg/ml). The hydrolysed samples of endemic species contain quercetin and myricetin in relatively high amounts (see section **4.4.2.**). Moreover, besides flavonol-O-glycosides, C-glycosides and methylated flavonoids mas also be present in the *L. christinae* hydrolysed sample (Table 6), which contribute to the stronger radical scavenging effect.

It is important to note that *L. clethroides* hydrolysed extract showed lower antioxidant capacity (IC_{50}=21.75±1.02 μg/ml) despite the high amount of flavonol aglycones. This may be explained by antagonism or proodixant effect between flavonoids, due to the occurring intermolecular H-bond, which decreases the DPPH radical scavenging

capability (Hidalgo et al., 2010) Fruthermore, other, currently not identified and quantified components may impair the free radical scavenging properties, too.

5.6. HPLC-based DPPH scavenging assay

The antioxidant flavonoid pattern of six *Lysimachia* samples was investigated in aglycone form after acidic hydrolysis. The extracts possessed notable DPPH free radical quenching ability (with IC_{50} values less than 60 µg/ml, Table 14). 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 main flavonoid compounds was examined. The magnified detail of the chromatograms of *L. nummularia* hydrolysed methanolic extract are presented in Figure 23 section **4.6.** as a representative example. The following trend was observed regarding the decrease in the peak area: myricetin (88.7±7.1%) > quercetin (76.4±8.7%) > kaempferol (65.3+8.0%). The *in vitro* antioxidant activity of flavonoids depends on the arrangement and number of free OH functional groups. Thus, according to these criteria, an increase in the number of hydroxyl groups increases the effectiveness against free radicals (section **1.2.2.**). Our results show good accordance with this statement, myricetin and quercetin that bear three and two free hydroxyl groups in the B ring, respectively, presented enhanced radical quenching activity compared with kaempferol. The contribution of certain compounds to the total antioxidant capacity of the *Lysimachia* extracts has also been investigated. It could be estimated by calculating the ratio of the decrease in the compounds' peak area and the sum of total peak area decrement. It has to be noted that the percentages reported here cannot be considered as exact results, rather estimation (Table 16, Figure 32-34).

In *L. punctata* extract relatively high amounts of quercetin and myricetin were determined (12.15±1.94 and 5.44±0.26 µg/mg dried extract, respectively). The contribution of the two compounds to the DPPH scavenger activity was nearly 75% (Fig. 32) that resulted in moderately low DPPH activity of the extract, with 31.47±1.94 µg/ml inhibition value.

Similar results were obtained regarding the cultivated *L. ciliate* herb extracts; medium high level of kaempferol (3.48±0.17 µg/mg) and quercetin (13.66±0.65 µg/mg), with approximately 60% contribution to the scavenger capacity determined the lowest DPPH scavenging activity (IC_{50}=59.59±2.79 µg/ml) for the whole extract (Fig. 32). This let us suppose impaired free radical neutralization reaction by the presence of currently not identified phenolic compounds.

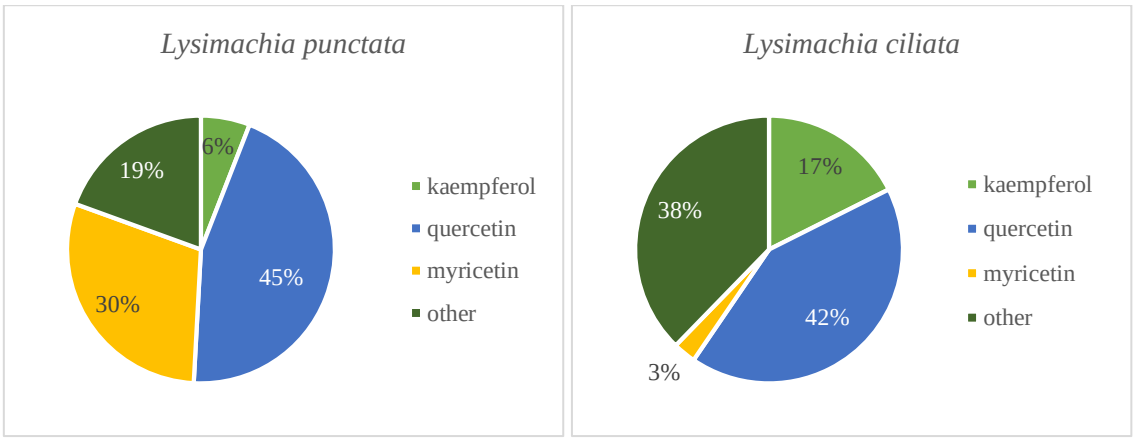


Figure 32. Contribution of the main compounds detected in the *L. punctata* and *L. ciliata* hydrolysed extracts to the DPPH scavenger activity

The strongest scavenging activity (IC_{50}=6.42±0.30 µg/ml) was detected in *L. nummularia* extract. 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%) see Figure 33.

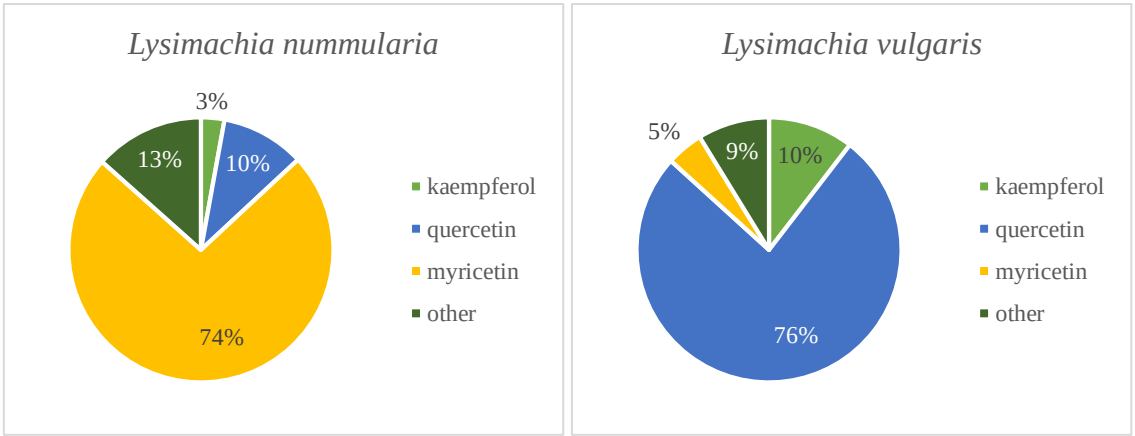


Figure 33. Contribution of the main compounds detected in the *L. nummularia* and *L. vulgaris* hydrolysed extracts to the DPPH scavenger activity

The *L. vulgaris* sample revealed higher amounts of quercetin (34.88±1.67 µg/mg) and kaempferol (4.53±0.23 µg/mg) than the former discussed species. Although the myricetin content was lower, the extract showed the second highest DPPH scavenging activity (IC_{50}=8.27±0.39 µg/ml). 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* (Fig. 33).

The lowest quantities of the three measured flavonoid aglycones, were cumulated in the *L. christinae* herb (Table 13, Figure 30). Their contribution to the DPPH scavenger capacity was also very low, less than 30% (Fig. 34). In contrast to these results, the hydrolysed extract possessed moderate scavenging activity ($IC_{50=}$17.29±0.81 µg/ml). Therefore, synergistic interaction between the antioxidant compounds was supposed. Furthermore, according to literature data besides flavonol-*O*-glycosides, *C*-glycosides and methoxylated flavonoids may also be accumulated in the plant (Sun et al., 2013, Liu et al., 2019). The latter ones are unmodified during the hydrolysis and are present in the investigated samples. They may also influence the DPPH scavenger activity. This is in good agreement with our results, since contribution of other compounds to the antioxidant activity is 70%

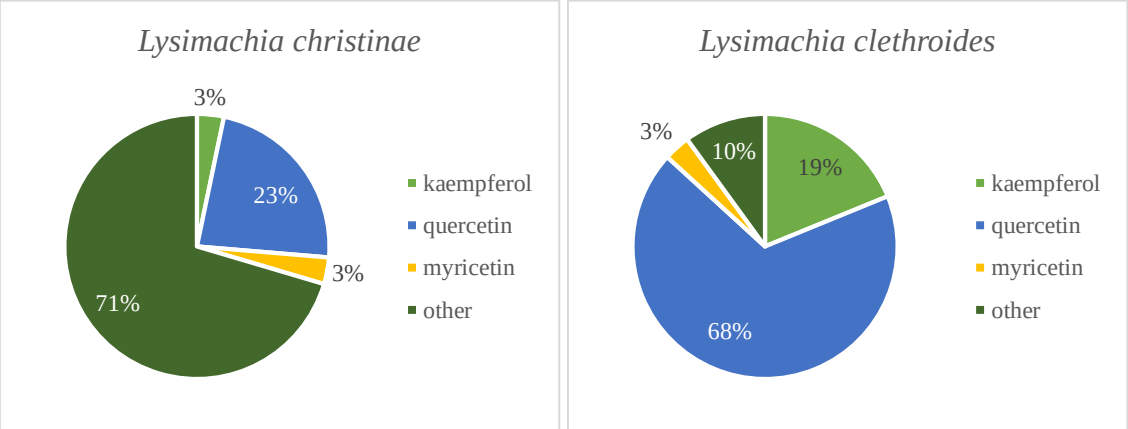


Figure 34. Contribution of the main compounds detected in the *L. christinae* and *L. clethroides* hydrolysed extracts to the DPPH scavenger activity

In *L. clethroides*, the highest kaempferol (25.77±1.29 µg/mg) and quercetin (97.67±4.61 µg/mg) content was measured. Although, the contribution of the three flavonoid

compounds to the antioxidant activity was high (90%) (Fig. 34), only moderate scavenger capacity was measured. These results indicate that other compounds may act as antagonists or prooxidant effect may also play a role. However, the total amount of three flavonoid aglycones (sum of the kaempferol, quercetin and myricetin amount) was the highest (124.34 μg/mg dried extract) among the investigated species, and the contribution of these flavonoids to the total radical scavenging activity was almost the highest (90%). Although, the contribution of the flavonoids to the scavenger capacity showed good relationship in the Hungarian endemic species, in other extracts the presence of different interactions between the compounds may influence the antioxidant activity of the whole extracts. Therefore, further studies aiming for the clarification of the interactions (additive effect, synergism, antagonism) between the antioxidant compounds are needed.

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6. CONCLUSION

1. Total phenolic, flavonoid, hydroxycinnamic and tannin contents of the *Lysimachia* herbal drugs were determined according to the spectroscopic methods described in Ph. Hg. VIII. It was concluded that all the studied herbal 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. For the characterisation of the compounds 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: *L. vulgaris* L., *L. nummularia* L., *L. punctata* L., *L. christinae* Hance, *L. clethroides* Duby and *L. ciliata* L. *var.* "Firecracker" altogether eighty six compounds were tentatively identified, amongst them seventeen phenolic acids, six catechin derivatives, nineteen flavonol aglycones and *O*-glycosides, twenty seven flavone-*C* derivatives and seventeen other flavonoids were presumable.

   The applied HPLC-DAD-ESI-MS methods were successfully utilised for the identification and differentiation of the six *Lysimachia* extracts. The phytochemical investigation of *L. ciliata* and the characterisation of its phenolic compound have been reported in this study for the 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. This work is the first to describe a validated quantitative HPLC-DAD method for the analysis of the main phenolic compounds, namely, chlorogenic acid, myricitrin, rutin, quercetin and kaempferol in the Hungarian native species. The results contribute to the description of members of the domestic flora. In addition, the

method is applicable for the investigation of the influence of geographical 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. For the quantitative determination of flavonoid aglycones, hydrolysis step and a fast, selective and validated UPLC-DAD quantitation method was applied to measure the most abundant flavonoid aglycones (kaempferol, quercetin and myricetin) in six *Lysimachia* species for the first time.
5. As *Lysimachia* herbal drugs were proved to be rich in antioxidant flavonoids, two *in vitro* tests using DPPH and ABTS as free radicals were utilised. The 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 herbal 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 their antiradical power and their 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 each *Lysimachia* extracts studied.

   A high-performance liquid chromatographic-mass spectrometric method coupled with DPPH free radical scavenging assay was applied to reveal the contribution of kaempferol, quercetin, myricetin and other phenolics in *Lysimachia* extracts to the total scavenger for the first time. We could conclude that in extracts of some *Lysimachia* species the quality of the main flavonoid aglycones strongly affected the scavenging activity, in other extracts further compounds (flavones, flavone-*C*-derivatives, phenolic acids) played role in the antioxidant activity. Thus, additional studies aiming the clarification of the interactions (additive effect, synergism, antagonism) between the antioxidant compounds are needed.

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7. SUMMARY

The members of the *Lysimachia* genus (Primulaceae family) are distributed worldwide in the temperate climates of the northern hemisphere, and are widely known both as traditional herbal remedies and ornamental garden plants. However, the phytochemical exploration of these species is incomplete, therefore the primary aim of our study was the detailed phytochemical evaluation of phenolics in six *Lysimachia* species: *L. vulgaris*, *L. nummularia*, *L. punctata*, *L. christinae*, *L. clethroides* and *L. ciliata*.

The total phenolic, flavonoid, hydroxycinnamic and tannin contents of the herbal drugs of the six *Lysimachia* species were determined by spectroscopic methods. All the plant samples were found to be rich in phenolic compounds.

For structural characterisation of the detected phenolic composition of the *Lysimachia* methanolic extracts, HPLC-DAD and HPLC-ESI-MS/MS methods were developed. Altogether eighty six compounds were tentatively identified, amongst them seventeen phenolic acids, six catechin derivatives, nineteen flavonol - aglycones and *O*-glycosides, twenty seven flavone-*C* derivatives and seventeen other flavonoids were presumed.

A HPLC-DAD method for the quantitative determination of the main phenolic compounds in the Hungarian native *Lysimachia* methanolic extracts was applied and validated.

Given the well-known antioxidant effects of flavonoids, antiradical activity of *Lysimachia* herbal and hydrolysed extracts were investigated in DPPH and ABTS system. Our results indicated that all the extracts possessed notable activity in both *in vitro* tests, and the acidic treatment increased the free radical scavenging potential of the herbal extracts. For the reduction of the complexity of genuine flavonoid compounds mild hydrolysis method was applied, followed by validated UPLC-DAD method for quantification of the main flavonol aglycones, kaempferol, quercetin and myricetin.

A HPLC-DAD-ESI-MS method coupled with DPPH free radical scavenging assay was developed and successfully utilised for the investigation of the contribution of certain compounds to the total radical scavenging activity.

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8. ÖSSZEFOGLALÁS

A *Lysimachia* nemzetség képviselői (Primulaceae) az északi félteke mérsékelt övi területein előforduló évelő, lágyszárú növények, és a hagyományos orvoslásban betöltött szerepük mellett kerti dísznövényként is elterjedtek. Fitokémiai jellemzésük széleskörű alkalmazásuk ellenére kevéssé feltárt, ezért kutatásunk elsődleges célja három Magyaroroszágon fellelhető lizinka faj: a *L. vulgaris* – közönséges lizinka, *L. nummularia* – pénzlevelű lizinka és a *L. punctata* – pettyegetett lizinka, illetve további három termesztett faj, a *L. christinae* – kínai lizinka, *L. clethroides* – hattyúnyakú lizinka és a *L. ciliata* – vöröslevelű lizinka fenoloidjainak részletes fitokémiai feltárása volt.

Vizsgálataink kiindulópontját a *Lysimachia* herba drogok összes fenoloid, flavonoid, hidroxifahéjsav és tannin tartalmának a Ph. Hg. VIII. spektroszkópiás módszereivel történő meghatározása képezte. Minden minta esetén magas fenoloid tartalom volt kimutatható.

A mintákból metanollal készült kivonatok fenoloid-profilját HPLC-DAD-ESI-MS/MS módszerrell vizsgáltuk. A Magyaroroszágon honos fajokban összesen 16 fenolos komponenst, két növényi sav származékot és 14 flavonolt azonosítottunk. A termesztett fajokban a detektált 81 vegyület között 16 növényi savszármazékot, 6 katechin típusú vegyületet, és összesen 59 flavonoidot, köztük flavonol-*O*-glikozidokat, flavon-*C*-glikozidokat és metilált származékokat írtunk le.

A Magyarországi fajokra jellemző fenoloidok mennyiségi meghatározása céljából HPLC-DAD módszert fejlesztettünk és validáltunk.

Tekintettel a flavonoidok ismert antioxidáns aktivitására, a kivonatok szabadgyökfogó képességét DPPH és ABTS *in vitro* tesztrendszerekben vizsgáltuk. Eredményeink alapján a *Lysimachia* kivonatok jelentős antioxidáns hatással rendelkeznek. Megállapítottuk, hogy a savas hidrolízis növeli a minták szabadgyökfogú aktivitását.

Az egyes fajok összehasonlításának egyszerűsítése céljából hidrolízist követően validált UPLC-DAD módszerrel határoztuk meg a három legjelentősebb flavonoid aglikon, a miricetin, kvercetin és kempferol mennyiségét.

Sikeresen alkalmaztuk az off-line módon kapcsolt DPPH-HPLC módszert az egyes komponensek összes antioxidáns hatáshoz való hozzájárulásának vizsgálatára.

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10. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATION

10.1. 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.

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10.2. Further scientific publications

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