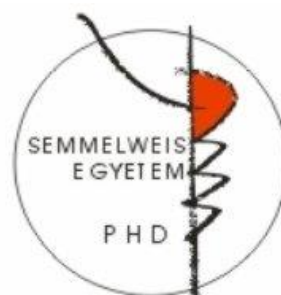


LC-ESI-MS/MS methods in profiling of flavonoid
glycosides and phenolic acids in traditional
medicinal plants: *Sempervivum tectorum* L. and
Corylus avellana L.

Ph.D. Dissertation

Ágnes Alberti-Dér

Semmelweis University
Doctoral School of Pharmaceutical Sciences



Supervisor:

Dr. Ágnes Kéry, Ph.D.

Reviewers:

Chair of final examination committee:

Dr. Sylvia Marton, Ph.D.

Members of final examination committee:

Dr. Imre Máthé, D.Sc.

Dr. Éva Sátyor, D.Sc.

Budapest
2013

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ALP	alkaline phosphatase
amu	atomic mass unit
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
APPI	atmospheric pressure photo ionization
BPC	base peak chromatogram
CAM	crassulacean acid metabolism
CE	capillary electrophoresis
CEC	capillary electrokinetic chromatography
CID	collision-induced dissociation
DAD	diode array detector
DART	direct analysis in real time ionization
DESI	desorption electrospray ionization
DPPH	2,2-diphenyl-1-picrylhydrazyl
ESI	electrospray ionization
FAB	fast atom bombardment
FT-ICR	Fourier-transform ion cyclotron resonance
GC	gas chromatography
GGT	gamma-glutamyl transpeptidase
GPT	glutamate-pyruvate transaminase
HDL	high-density lipoprotein
HPLC	high-performance liquid chromatography
IC ₅₀	half maximal inhibitory concentration
i.p.	intraperitoneal
IT	ion trap mass analyzer
LC	liquid chromatography
LC-MS	liquid chromatography coupled to mass spectrometry
MALDI	matrix-assisted laser desorption ionization
MS	mass spectrometry

MS/MS	tandem mass spectrometry
MS ⁿ	multiple stage mass spectrometry
MSPD	matrix solid-phase dispersion
<i>m/z</i>	mass-to-charge ratio
NI	negative ionization
NMR	nuclear magnetic resonance spectroscopy
NO [•]	nitric oxide radical
O ₂ ^{•-}	superoxide radical
•OH	hydroxyl radical
ONOO ⁻	peroxynitrite anion
PEP-C	phosphoenolpyruvate-carboxylase
PI	positive ionization
Q	quadrupole mass analyzer
QQQ	triple-quadrupole mass analyzer
Q-TOF	quadrupole – time-of-flight mass analyzer
RDA	retro-Diels-Alder
RP-HPLC	reversed-phase high-performance liquid chromatography
s.c.	sub cutan
S.D.	standard deviation
S.E.M.	standard error of the mean
SFE	supercritical fluid extraction
SPE	solid-phase extraction
SPME	solid-phase micro-extraction
SRM	single reaction monitoring mode
TEAC	trolox equivalent antioxidant capacity
TIC	total ion current chromatogram
TOF	time-of-flight mass analyzer
TLC	thin layer chromatography
UHPLC	ultrahigh-pressure liquid chromatography
UV	ultraviolet light
vis	visible light
VLDL	very-low density lipoprotein

1. INTRODUCTION

In recent years a number of medicinal plants have been proved to offer an alternative to synthetic drugs in preventing and treating diseases. Quality control of standardized herb extracts is an essential part of investigations regarding safety, efficacy and therapeutical reproducibility. However, it is not an easy task, because medicinal plant extracts or preparations comprising several herbs are complex mixtures of numerous different compounds among which a substantial proportion is unknown. This applies particularly to extracts containing plant phenolics, since these are present in plant material in great structural variability. Characterization of quality includes phytochemical screening of medicinal plants with the objectives of authentication and revelation of diagnostic compounds to avoid adulteration.

In order to develop modern evidence-based phytomedicines or registered herbal extracts, standardization is a crucial step. It comprises identification and determination of the active substances in a herbal remedy, as well as development of validated methods for comprehensive chemical characterization and quantification of the main compounds. However, pharmacological effects often can not be attributed to a certain active substance, but a sort of constituents contribute to them or a synergistic effect between compounds exists.

For all these phytoanalytical challenges provides mass spectrometry (MS) coupled to high-performance liquid chromatography (HPLC) an adequate tool. Mass spectrometry offers great selectivity and sensitivity and with the separative power of high-performance liquid chromatography enables simultaneous structural analysis of compounds present in complex matrices.

Evaluation of two traditional medicinal plants, *Sempervivum tectorum* L. and *Corylus avellana* L. has been chosen as scope of our work. Mainly kaempferol glycosides have been reported for *Sempervivum tectorum*, however, its phytochemical characterization is incomplete. Its flavonoid profile has been studied only at the aglycone level, although glycosyl substitution can have a great impact on bioavailability of compounds. Phenolics in kernels of *Corylus avellana* have been studied extensively, nevertheless data regarding leaves are less detailed.

Phenolics are present in plant material in great structural variability and have been reported by some recent studies to exhibit various pharmacological activities, such as anti-inflammatory, anti-cancer, hepatoprotective, anti-atherogenic, antimicrobial and estrogenic effects. Flavonoids and phenolic acids have drawn considerable attention because of their biological activities mentioned above. In addition, phenolics are considered as chemotaxonomic markers in plants and are regarded as chemical markers for authentication of herbal extracts and pharmaceutical preparations. Due to the great structural variety of flavonoids and phenolic acids and their presence as complex mixtures in medicinal plants, their qualitative and quantitative phytochemical characterization requires sensitive and reliable analytical methods. HPLC-MS is the method of choice for phenolic analysis, since it provides a powerful, robust, versatile and available technique.

2. LITERATURE OVERVIEW

2.1. Plant phenolics

Phenolics form a vast group of secondary metabolites in plants and are characterized by the presence of at least one aromatic ring substituted by at least one hydroxyl group, free or engaged in another function: ether, ester, or glycoside. However, structural requirements are insufficient to characterize plant phenolics, since these would include many other substances, which belong to different phytochemical groups, e.g. alkaloids, terpenes, etc. Therefore a definition based on biosynthetic origin is necessary. Plant phenolics originate from two main aromatization pathways.

- Shikimate pathway:

The most common pathway leads from D-erythrose-4-phosphate and phosphoenol-pyruvate via shikimate to aromatic amino acids, then by deamination of the latter, to cinnamic acids and their derivatives: benzoic acids, acetophenones, lignans, lignins and coumarines.

- Acetate pathway:

The pathway leads from acetate to poly- β -ketoesters (polyketides) of variable length, then by cyclization (Claisen or aldol condensation) of the latter to chromones, isocoumarins, depsides, xanthenes and quinones.

Due to the combination of both pathways compounds of mixed origin, e.g. flavonoids, stilbenes, pyrones and xanthenes are generated. In less frequent cases a third pathway, the mevalonate pathway is involved. These mixed derivatives of shikimate and mevalonate pathways are certain quinones, furano- and pyrano-coumarins, while those of acetate and mevalonate pathways are cannabinoids [1].

Phenolic compounds can be categorized into several classes including simple phenols, hydroxybenzoic acid derivatives, hydroxycinnamic acid derivatives, xanthenes, stilbenes, flavonoids, lignans, lignins and condensed tannins (Table 1.) [2].

Table 1. Classes of phenolics in plants [2].

Classes of phenolic compounds in plants	Structure
Simple phenolics, benzoquinones	C_6
Hydroxybenzoic acids	C_6-C_1
Acetophenones, phenylacetic acids	C_6-C_2
Hydroxycinnamic acids, phenylpropanoids (coumarins, isocoumarins, chromones, chromenes)	C_6-C_3
Naphtoquinones	C_6-C_4
Xanthones	$C_6-C_1-C_6$
Stilbenes, anthraquinones	$C_6-C_2-C_6$
Flavonoids, isoflavonoids	$C_6-C_3-C_6$
Biflavonoids	$(C_6-C_3-C_6)_2$
Lignans, neolignans	$(C_6-C_3)_2$
Lignins	$(C_6-C_3)_n$
Condensed tannins (proanthocyanidins)	$(C_6-C_3-C_6)_n$

2.1.1. Phenolic acids

Phenolic acids are widely distributed in the plant kingdom. The term phenolic acid applies theoretically to all organic compounds with at least one carboxyl group and one phenolic hydroxyl group, however according to the current practice, this term is reserved to benzoic and cinnamic acid derivatives only.

Hydroxycinnamic acids, also called phenylpropanoids are compounds with a C_6-C_3 skeleton. They are the most common metabolites of shikimic acid. Though their side chains may be in different degrees of oxidation (alcohol, aldehyde, propene or other), they all derive from cinnamic acid. They may occur in the free acid form only exceptionally, combined states are more prevalent, e.g. esters of aliphatic alcohols (mono- and dicaffeoyltartaric acids, caffeoylmalic acid), esters of quinic acid (chlorogenic acid), depsides (rosmarinic acid). However most frequently they are linked through ester, ether or acetal bonds to other metabolites, such as flavonoids,

anthocyanins, saponins, proteins, cellulose, glucose, etc. Furthermore, the phenylpropanoid moiety may cyclize (coumarins), dimerize (lignans), polymerize (lignins), or undergo side chain elongation (stilbenes, flavonoids) [1, 3-4].

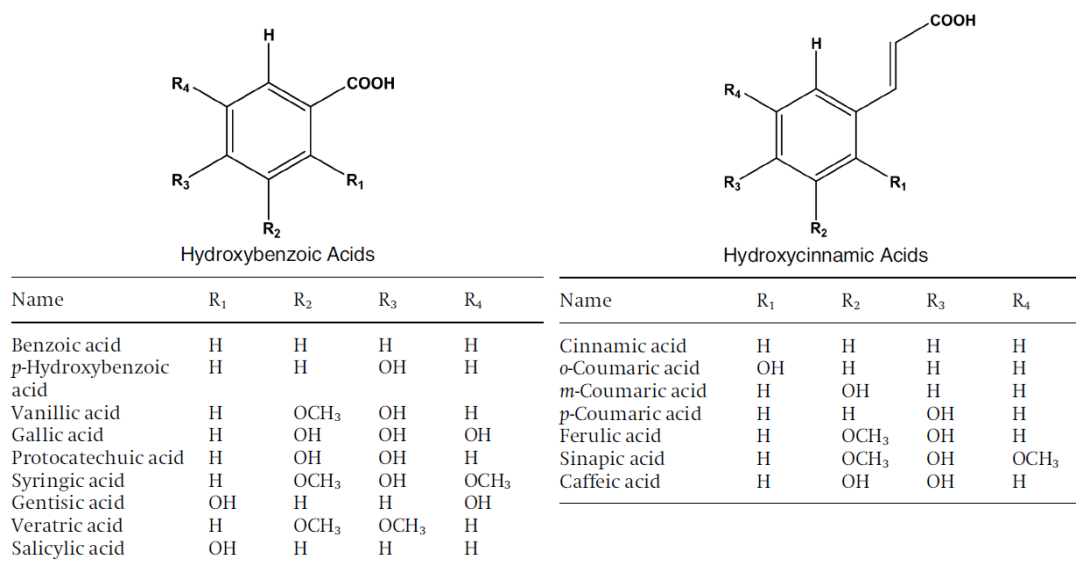


Fig.1. Structures of the most common hydroxybenzoic and hydroxycinnamic acids [3].

Hydroxybenzoic acids are C₆-C₁-type compounds and they may arise whether from shikimic acid (like gallic acid) or universally from side chain degradation of the corresponding cinnamic acids. They are quite common in the free state, as well as combined into esters or glycosides [1, 4]. Structures of the most common hydroxybenzoic and hydroxycinnamic acids are shown in Fig. 1.

Although the role of phenolic acids in plants is not fully explained, they have been associated with nutrient uptake, protein synthesis, enzyme activity, photosynthesis and allelopathy [3].

2.1.2. Flavonoids

Flavonoids are a widely distributed group of structurally related compounds with a 2-phenylchromane skeleton with a phenyl substituent in the C₂ or C₃ position. They are divided into subclasses depending on the degree of oxidation of the central pyran ring, the main subclasses are shown in Fig. 2.

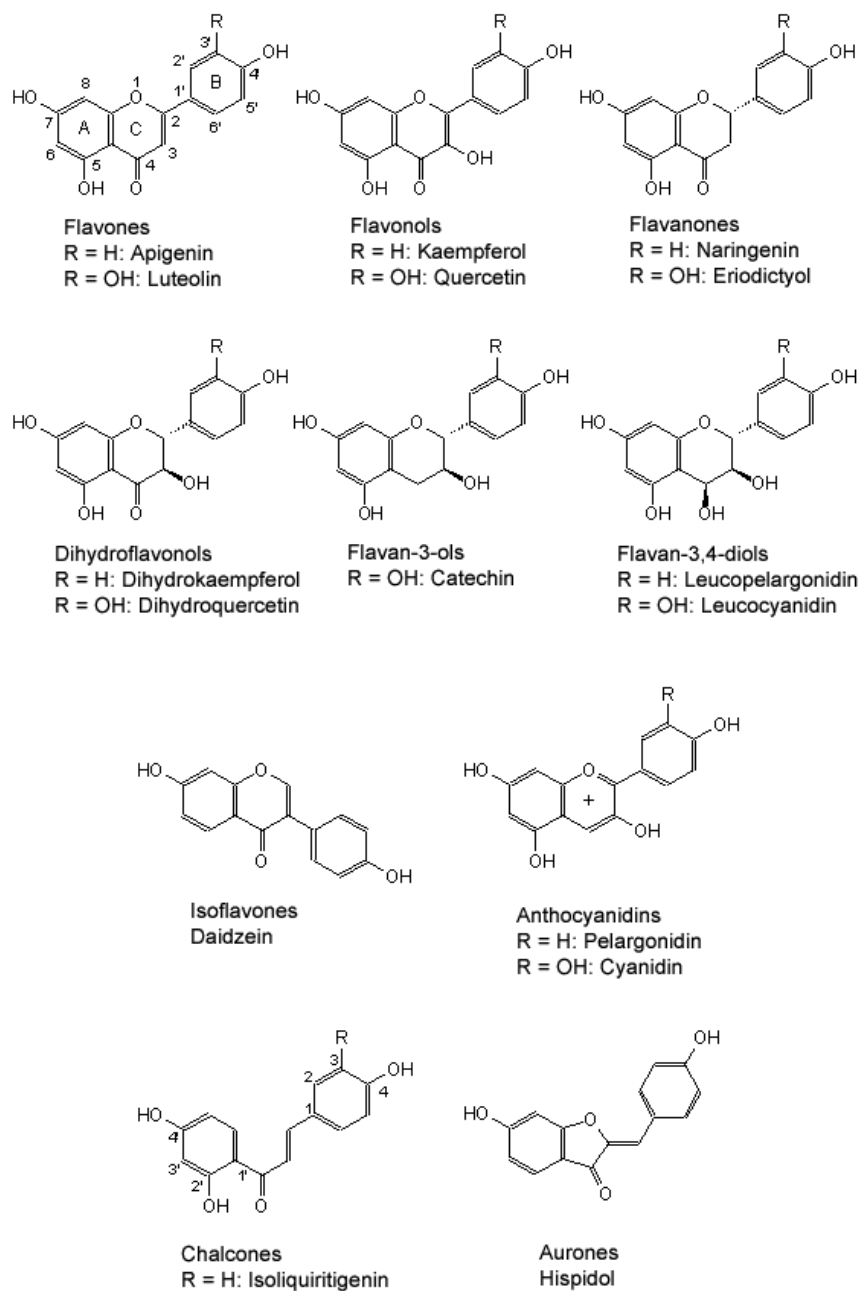


Fig. 2. Structure of the main flavonoid subclasses [1].

Flavonoids are often hydroxylated in positions 3, 5, 7, 3', 4' and 5'. Frequently, one or more of the hydroxyl groups are methylated, acetylated, prenylated or sulphated. Although sometimes they are found as their aglycones, in plants flavonoids most commonly occur as *O*-glycosides and less frequent as *C*-glycosides. Flavones,

flavanones and isoflavones are usually glycosylated at the 7-hydroxyl group, flavonols and flavanols at the 3- and 7-hydroxyl groups, while common glycosylation sites in anthocyanidins are 3- and 5-hydroxyls. C-glycosides have their sugar groups bound to a carbon of the aglycone, usually at C-6 or C-8. The most common sugar moieties are glucose, galactose, rhamnose, xylose and arabinose, while they include allose, apiose, glucuronic acid and galacturonic acid, too. Flavonoids occur frequently as diglycosides, very common disaccharides are neohesperidose and rutinose containing glucose and rhamnose, (1 → 6) linked and (1 → 2) linked, respectively. Disaccharides containing two glucose units are also familiar: (1 → 6) linked in sophorose and (1 → 2) linked in gentiobiose. There are also flavonoid tri- or tetraglycosides, where sugar units can be linear or branched. The sugars are often further substituted by *p*-coumarate, malonate or acetate [1, 5-8].

Flavonoids are of biological and physiological importance, they play an important role in plants as defense and signalling compounds in reproduction, pathogenesis and symbiosis. Flavonoids are sources of flower, fruit and leaf colouring pigments, e.g. chalcones, aurones and yellow flavonols are yellow, anthocyanins may be red, blue or purple. There are not directly visible flavonoids which can contribute to the colour as co-pigments by protecting anthocyanins, while near-UV absorbing flavonoids attract insects and in this way ensure pollen transport. Flavonoids are involved also in tissue protection against UV radiation [1, 5-6].

2.2. Biological activities

Phenolic acids and especially flavonoids have been reported by some recent studies to exhibit various effects including antioxidant [9-14], anti-cancer [15-21], anti-allergic [22-25], anti-inflammatory [26-30], antimicrobial [31-34], anti-atherogenic [35-36], anti-thrombotic and vasodilatory [36-40], estrogenic [41-43] and capillary fragility and permeability decreasing [44] actions.

2.3. Extraction and analysis of phenolic acids and flavonoids

Flavonoids and phenolic acids have drawn considerable attention because of their pharmacological activities. In addition, phenolics are considered as chemotaxonomic markers in plants and are regarded as chemical markers for authentication of herbal extracts and pharmaceutical preparations. The number of identified phenolic compounds mounts up to several thousands and is still increasing. Due to the great structural variety of flavonoids and phenolic acids and their presence as complex mixtures in medicinal plants, for their qualitative and quantitative phytochemical characterization sensitive and reliable analytical methods are required.

2.3.1. Sample preparation

Sample preparation is the initial and crucial step of both qualitative and quantitative analyses. According to the classification of de Rijke et al. [6] and Stalikas [3], there are three main types of phenolic-containing matrices: plants, food and liquid samples (including biological samples and beverages). Sample pretreatment steps may vary to a great extent, depending on the matrix of the particular sample. Several liquid samples such as urine, serum, plasma, and some beverages can be injected directly into the separation system after filtration and / or centrifugation [3, 6-7], while solid samples are usually first air-dried or freeze-dried then subjected to milling or grinding and homogenization before further extraction and purification steps [3, 6].

2.3.2. Extraction

Solvent, liquid-liquid and Soxhlet extraction are frequently used procedures for isolation or enrichment of phenolic acids and flavonoids, while supercritical fluid extraction (SFE) offers an alternative technique for extraction of lipophylic flavonoids [3, 6, 47]. Biesaga studied the effect of extraction methods (reflux heating, sonication, maceration and microwave) on the stability of maize flavonoids belonging to four subgroups (flavonols, flavones, flavanones and isoflavones) [45]. Degradation of flavonoids was influenced by the extraction mode, as well as by their chemical

structure. The highest stability was achieved by heated reflux extraction within 30 minutes and by microwave assisted extraction under 160 W for 1 minute. Degradation of flavonoids was inhibited by smaller number of substituents, furthermore a sugar moiety also stabilized flavonoids.

Commonly used extraction solvents are alcohols (methanol, ethanol), acetone, diethyl ether, ethyl acetate and acetonitrile [3, 6-7, 46-47]. For more polar compounds, e.g. hydroxycinnamic acid derivatives, flavonoid tri- or tetraglycosides, the use of aqueous methanol or aqueous acetone is recommended [3]. Depending on the purpose of the analytical evaluation a hydrolysis step can be included in sample preparation. Chemical hydrolysis is employed to release insoluble phenolic acids and to remove the sugar moieties from flavonoid aglycones. Usually it is performed with hydrochloric acid (or formic acid) at reflux or above reflux temperatures in aqueous or alcoholic solvents [3, 6, 47]. Alternatively, enzymatic hydrolysis with pectinases, cellulases or amylases for phenolic acids and with β -glucuronidase or β -glucosidase for flavonoids can be used [3, 6-7, 47]. If soluble phenolic acids or flavonoids in their conjugated forms are the target analytes, hydrolysis should be prevented, thus harsh extraction conditions and heating should be avoided [6].

2.3.3. Purification and fractionation

There are many phenolics present in low concentrations in plant extracts, which remain unidentified but whose significance may far outweigh their concentration level. Isolation and structure elucidation of these compounds are the initial steps to understand their significance and action [48].

Solid-phase extraction (SPE) is widely used for purification, analyte isolation and concentration from crude plant extracts or biological samples. Most frequently, the sorbent is C18-bonded silica and the sample solution and solvents are usually slightly acidified to prevent ionization of the phenolics, which could reduce their retention [3, 6]. Column chromatography has been frequently employed for fractionation of phenolics, with polyamide, Silicagel and Sephadex LH-20 as the most common stationary phases [46, 49-50].

Application of other sample preparation techniques is also discussed in the literature. By the use of solid-phase micro-extraction (SPME) considerable analyte enrichment can be achieved besides reduced organic solvent consumption. It is usually combined with gas chromatography (GC) for the extraction of (semi-) volatile organic compounds, and is therefore less suitable for extraction of highly polar and non-volatile flavonoids and phenolic acids. Matrix solid-phase dispersion (MSPD) enables simultaneous sample extraction and clean-up [3, 6].

2.3.4. Separation

Since the early 1960's thin layer chromatography (TLC) has been widely used in the analysis of plant phenolics. Though it has lost on significance, TLC still plays a distinct role in phenolic analysis, since it provides rapid preliminary screening of many samples simultaneously prior to detailed analysis by instrumental techniques [3, 50].

Gas chromatography affords high resolution and low detection limit, though it has some disadvantages in phenolic analysis, i.e. the need for derivatization of phenolics to improve their thermal stability and increase their volatility. It has to be mentioned that analysis of highly polar, non-volatile and thermally unstable flavonoid glycosides by conventional GC is very difficult even after derivatization. Other separation technique in phenolic analysis is capillary electrophoresis (CE), while promising new fields are capillary electrokinetic chromatography (CEC) and chip technology [3, 6, 50-51].

High-performance liquid chromatography (HPLC) is by far the most relevant and important separation technique in phenolic analysis. LC is usually performed in the reversed-phase mode, on C8- or C18-bonded silica columns. Both isocratic and gradient elution systems are applied. Gradient elution is generally performed with binary solvent systems, i.e. with water containing acetate or formate buffer, and methanol or acetonitrile as organic modifier [3, 6]. Though for separation of complex mixtures of phenolics such as flavonoid conjugates – glycosides, malonates and acetates – is usually gradient elution and run times of up to 2 hours necessary, Zgóřka and Kawka [52] could separate free phenolic acids using a simple isocratic mobile phase. LC is usually performed at room temperature, but in some cases the use of thermostated columns (up

to 40 °C) is recommended to reduce analysis time and improve repeatability of elution times [3, 6].

Abad-García and co-workers [8] studied retention times of different phenolics using RP-HPLC. The polar the compounds were the early they eluted, in the following order: hydroxybenzoic acids, flavan-3-ols, hydroxycinnamic acids, coumarins, flavanones, dihydrochalcones, flavonols and flavones. However, because of the structural diversity of the compounds, overlap of the individual members of different classes is usually observed. Within the same polyphenol class: a) the retention time decreased with the increase of hydroxyl groups, b) the retention time increased when apolar substituents, e.g. methoxy groups were present, c) phenolic glycosides eluted before their corresponding aglycones, d) the retention time increased when sugar moieties were acylated, e) the retention time decreased when quinic acid and tartaric acid substituents were present. The glycosylation affected retention times differently, based on the nature and position of the glycan substitution. For glycosylated phenolics, with the sugar moiety in the same bond position, elution order was: di-*O*-glycoside, *O*-galactoside, *O*-glucoside, *O*-rutinoside, *O*-neohesperidoside, *O*-rhamnoside, aglycones. However, the position of the sugar substitution was also important for the retention times, showing the following elution order for glucosides: 8-*C*, 6-*C*, 3',7-di-*O*, 7-*O*, 4'-*O* and 3-*O*.

2.3.5. HPLC detection

All phenolics contain conjugated double and aromatic bonds and exhibit consequently absorption in the UV or UV / vis region. Hydroxybenzoic acids have their maxima in the 200-290 (255-280) nm range which is attributed to the benzoyl group as chromophore. Hydroxycinnamic acid derivatives show an absorption band from 270 to 360 (310-325) nm due to the additional conjugation of the cinnamoyl system. Flavonoids exhibit two major absorption bands. Band I is associated with absorption due to the cinnamoyl system of B-ring, while band II is ascribed to the absorption involving the benzoyl system in A-ring. Evaluation of UV spectra of flavonoid glycosides offers additional information for structural characterization. Characteristic absorption maxima of different flavonoid classes are as follows [6, 8, 49]:

- Flavones and flavonols exhibit band I usually in the 300-380 nm region and band II from 240 to 280 nm. Depending on the B-ring oxidation pattern, band II can be observed as either one or two peaks, for 4'-oxygenated and for 3',4',(5')-oxygenated flavones and flavonols, respectively.
- Isoflavones, flavanones, and dihydroflavonols exhibit an intense band II absorption with a shoulder or low intensity peak representing band I, as a result of the absence of conjugation between the A- and B-rings. Isoflavones exhibit band II in the 245-270 nm region, while that of dihydroflavonols and flavanones occurs in the 270-295 nm range.
- Chalcones exhibit an intense band I absorption from 340 to 390 nm, while band II appears as a minor peak in the 220-270 nm range. Aurones show the major band I absorption in the 370-430 nm region.
- Anthocyanins exhibit two major absorption bands due to the aromaticity of C-ring: band II in the 275-285 nm UV region and band I in the 520-545 nm visible region.

Furthermore, information on substitution pattern of flavonoids can be gained by evaluation of their UV / vis spectra. Additional hydroxyl groups in A- or B-ring are responsible for bathochromic shifts (i.e. to longer wavelengths) of band II or I, respectively. Glycosylation or methylation of 3-, 5- or 4'-hydroxyl groups on the flavone or flavonol nucleus causes hypsochromic shifts (i.e. to shorter wavelengths), especially in band I [8, 49]. Additionally, intensity of band I (347-370 nm) in UV spectra of 3-*O*-glycosylated flavonols is lower than that of band II (250-267 nm) [8].

Fluorescence detection is rarely used in phenolic analysis because of the limited number of phenolic compounds exhibiting native fluorescence. This group includes, e.g. isoflavones without 5-hydroxyl group, methoxylated flavones, 3-hydroxylated flavonoids and it can be extended by derivatization of other phenolics. Due to the presence of phenolic groups, most phenolics are electroactive, thus electrochemical detection can also be used [3, 6, 53].

Mass spectrometric (MS) detection coupled to HPLC has become the dominating technique in phenolic analysis. Different mass analyzers: quadrupole (Q), triple-quadrupole (QQQ), time-of-flight (TOF), ion trap (IT), Orbitrap and Fourier-transform ion cyclotron resonance (FT-ICR), as well as hybrid, e.g. quadrupole – time-of-flight (Q-TOF) instruments are applied. Single-stage MS is usually used in combination with UV detection to confirm identity of known components in plant extracts with the help of literature data and reference compounds. Because of its better sensitivity and the access to more detailed structural information, leading role of single-stage MS in phenolic analysis has recently been taken over by tandem mass spectrometry (MS/MS or MSⁿ) which can be used for the identification and quantification of unknown constituents. In tandem mass spectrometry only the ion of interest is transmitted by the first analyzer which is then analyzed by the second one. MS/MS experiments can be achieved on, e.g. quadrupole – time-of-flight (Q-TOF), time-of-flight – time-of-flight (TOF-TOF) or QQQ instruments, application of an IT analyzer, however, allows also MSⁿ analyses. Combination of multi-stage MS techniques with the improved performance of ultrahigh-pressure liquid chromatography (UHPLC) systems has led to extremely high resolution, sensitivity and throughput [6-7, 51, 54-57].

In the LC-MS of phenolics atmospheric pressure ionization (API) interfaces, such as ESI (electrospray ionization) and APCI (atmospheric pressure chemical ionization) are almost exclusively applied. They complement one other regarding polarity and molecular mass of analytes, as ESI is suitable for polar/very polar compounds with high molecular weights, while APCI is suitable for non-polar compounds with molecular weights up to several thousands Da [57-58]. Other ionization techniques employed include fast atom bombardment (FAB), atmospheric pressure photo ionization (APPI) and matrix-assisted laser desorption ionization (MALDI). Ambient ionization techniques, e.g. direct analysis in real time (DART) and desorption electrospray ionization (DESI) have been proposed for the direct evaluation of molecules present in food without using HPLC and with a minimal or no sample preparation [56].

Despite of the wide range of information provided by mass spectrometry, unambiguous structural identification can only be carried out by combining nuclear magnetic resonance spectroscopy (NMR) with LC-DAD and LC-MS/MS. On-line coupling of

HPLC and NMR is also gaining in importance in the analysis of plant-derived compounds [6-7, 51, 54-55].

2.3.6. LC-MS/MS in structural characterization of phenolics

Regarding the structural characterization of phenolics, tandem mass spectrometry with combination of collision-induced dissociation can present more detailed information beyond molecular mass of the analytes. Information can be obtained on [8, 59]:

- the aglycone moiety
- the types of sugar moieties (mono-, di-, tri- or tetrasaccharides and hexoses, desoxyhexoses or pentoses) and other substituents
- the stereochemical assignment of terminal monosaccharide units
- the sequence of the glycan part
- the interglycosidic linkages
- the attachment points of the substituents to the aglycone.

As mentioned above, application of ESI and APCI ionization is dominating in the analysis of phenolics. For both techniques negative ionization (NI) mode provides better sensitivity, however – despite the stronger background noise – positive ionization (PI) mode can also be employed. The method of choice for the analysis of low-molecular-mass phenolics is yet HPLC-(–)-ESI-MS/MS [6, 58, 60-61].

Mass spectrometry, particularly MS/MS provides high sensitivity and selectivity, even for complex biological matrices, e.g. phenolic-containing extracts comprising a number of target analytes of analogous structures. Mauri and Pietta analyzed different plant extracts without prepurification and chromatographic separation, by direct infusion into an ESI-MS apparatus with the aim to obtain their finger-prints [62]. Gioacchini et al. concluded also that use of the ESI-MS system with selected ion monitoring mode (i.e. when only selected ions pass through the first analyzer and are then analyzed by the second one) did not required complete HPLC separation of the phenolic acids and aldehydes studied, unless isomers were to be identified [63]. Nevertheless, sample purification and chromatographic separation should not be neglected, since co-eluting components may cause problems with the MS response due to ion suppression and other

matrix effects. HPLC-ESI-MS/MS methods are adversely affected by matrix effects in terms of sensitivity, accuracy and precision. Consequently, almost all mass spectrometric methods in phenolic analysis include a high-performance separation method, as adequate chromatographic resolution improves LC-MS sensitivity [58, 64-65].

It should be kept in mind that in all four operation modes: (-)-ESI, (+)-ESI, (-)-APCI and (+)-APCI, composition and pH of the LC eluent and the nature of the buffer components added can have a distinct influence on analyte responses and ionization efficiency. The most common additives in LC-MS are acetic acid, formic acid, ammonium-acetate and ammonium-formate [6-7]. Trifluoroacetic acid is also used, despite its ion suppressing effects due to ion-pairing with basic analytes and its adverse effect on efficient spray formation due to its high surface tension [6-7]. Phosphate buffers are not employed, because of contamination of the ion source [6, 54]. However, sensitivity of ESI is improved when the organic content in the mobile phase exceeds 20% (v/v) [48].

2.3.6.1. Negative ionization collision-induced dissociation of phenolic acids

Gioacchini and co-workers developed a selective and sensitive negative ionization ESI-MS/MS method for the determination of phenolic acids and aldehydes [63].

Neutral losses of small molecules, such as CO₂ (-44 amu), CO (-28 amu) and H₂O (-18 amu), as well as those of methyl radicals (-15 amu) – generating the formation of [M-CH₃]⁻ ions – commonly occur in negative ion collision-induced dissociation of phenolic acids and their methoxylated derivatives, respectively [66-67].

Product ions at *m/z* 191 and *m/z* 173 refer to quinic acid esters, with the former representing the deprotonated quinic acid and the latter the [quinic acid-H-H₂O]⁻ ion. CID spectra of caffeic acid derivatives contain product ions at *m/z* 179 (deprotonated caffeoyl moiety), *m/z* 161 [caffeic acid-H-H₂O]⁻ and *m/z* 135 [caffeic acid-H-CO₂]⁻, for those of coumaric acid derivatives are fragments at *m/z* 163 (deprotonated coumaroyl moiety) and *m/z* 119 [coumaric acid-H-CO₂]⁻ characteristic [66], while ferulic acid derivatives exhibit product ions at *m/z* 193 (deprotonated feruloyl moiety) and *m/z* 149 [ferulic acid-H-CO₂]⁻ [68-70].

Clifford et al. [71] unambiguously identified caffeoylquinic acid isomers by evaluation of their negative ion CID spectra. According to their substantially different fragmentation behaviour, three compounds showing an $[M-H]^-$ ion at m/z 353 were assigned to 5-caffeoylquinic acid (chlorogenic acid), 3-caffeoylquinic acid (neochlorogenic acid) and 4-caffeoylquinic acid (cryptochlorogenic acid). Results were corroborated also by the elution order of the compounds. The fragmentation of 5-caffeoylquinic acid yielded product ions at m/z 191 and 179, with the quinic acid moiety representing the base peak and the caffeoyl moiety being present only with minor intensities, while that of 3-caffeoylquinic acid resulted in a base peak corresponding to the quinic acid moiety (m/z 191) and a comparatively intense signal from caffeic acid (m/z 179). The $[\text{quinic acid-H-H}_2\text{O}]^-$ ion at m/z 173 as base peak was detected only for 4-caffeoylquinic acid with a prominent signal at m/z 179 resulting from caffeic acid. The 5-caffeoylquinic acid and 3-caffeoylquinic acid isomers were devoid of the $[\text{quinic acid-H-H}_2\text{O}]^-$ ion, which has been explained by their particular stereochemistry, not allowing 1,2-acyl participation during fragmentation.

2.3.6.2. Negative ionization collision-induced dissociation of flavonol aglycones and glycosides

Although negative ionization CID spectra of flavonoids is considered to be more difficult to interpret, negative ionization mode experiments provide better sensitivity for flavonoids [6, 58, 60-61], therefore exclusively results of studies applying negative ionization techniques are discussed in this work dealing primarily with flavonol glycosides. Di Stefano and co-workers [56] concluded that the main fragmentation paths of flavonoids are independent of the ionization mode (ESI, APCI, or MALDI) and the type of analyzer applied (QQQ, IT, or QTOF), therefore, works employing these techniques are discussed. However, relative fragment abundances significantly varied when different instrumentation was used, thus instead of evaluating relative intensities of fragment ions, methods based on detecting the presence or absence of distinctive fragment ions should be preferred.

The nomenclature proposed by Domon and Costello [72] for glycoconjugates is commonly adopted to denote fragment ions deriving from collision-induced dissociation

of flavonoid glycosides (see Fig. 3.). Ions containing the aglycone are labeled $^{k,l}X_j$, Y_j and Z_j , where j is the number of the interglycosidic bond broken, counting from the aglycone, and the superscripts k and l indicate the cleavages within the carbohydrate rings. The glycosidic bond linking the glycan part to the aglycone is numbered 0. For product ions deriving from the fragmentation of the aglycone the nomenclature proposed by Ma and co-workers [73] is usually applied. $^{m,n}A_0$ and $^{m,n}B_0$ labels are used to refer to product ions containing intact A- and B-rings, respectively, where the superscripts m and n denote the C-ring bonds that have been broken. The subscript 0 is used to avoid confusion with the A_i and B_i ($i \geq 1$) labels which are used to designate carbohydrate fragments containing terminal sugar unit.

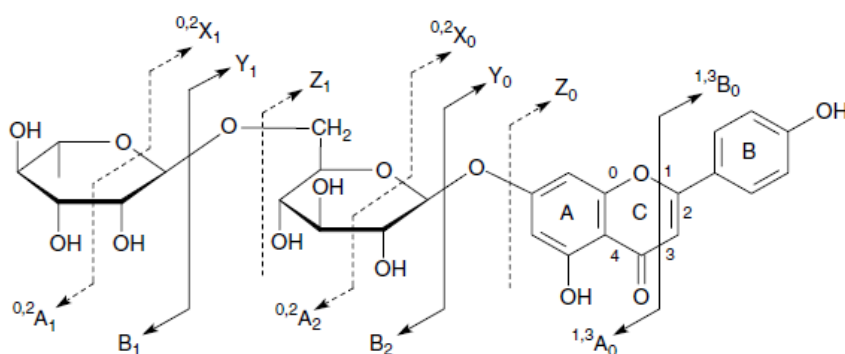


Fig.3. Nomenclature proposed by Domon and Costello [72] and Ma and co-workers [73] to denote fragment ions deriving from CID of flavonoid glycosides.

Tandem MS techniques are useful for structural elucidation in analysis of flavonoids [58]. Careri and co-workers concluded [51] that CID-MS/MS experiments cause the fragmentation of the flavonoid molecules according to fixed pathways. Thus flavonols, flavones and flavanones can be discriminated according to their CID spectra, on the basis of three types of ring cleavages in the pyran ring of the molecules. However, de Rijke and co-workers found in their more recent review work [6] that most retro-Diels-Alder (RDA) C-ring cleavages in negative ion CID experiments (shown in Fig. 4.) were observed for all classes of flavonoids, thus fragments deriving from RDA fragmentation mechanisms can be proposed as diagnostic ions for flavonoid classes only reservedly.

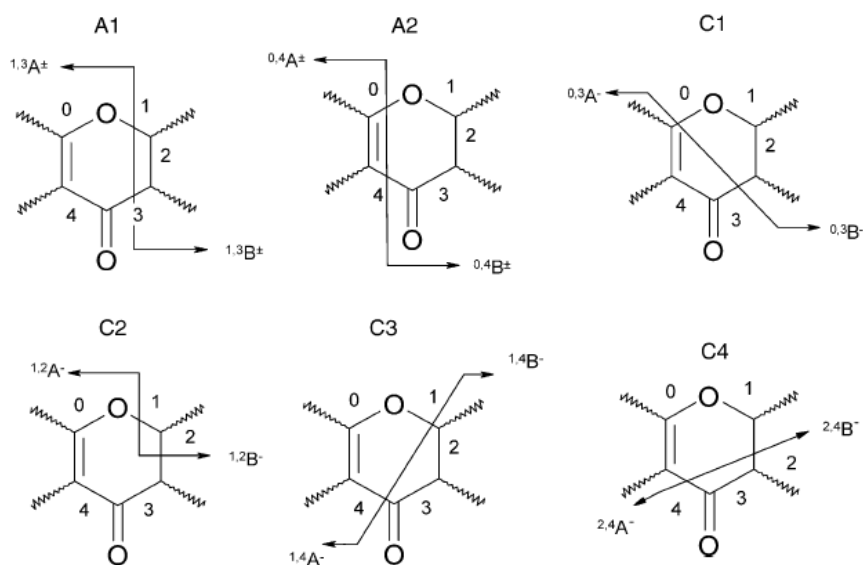


Fig. 4. Fragmentation pathways for flavonoids caused by cleavage of C-ring bonds; (A) in both PI and NI: (A1) 1 and 3, (A2) 0 and 4; (C) in NI: (C1) 0 and 3, (C2) 1 and 2, (C3) 1 and 4, (C4) 2 and 4 [6].

The negative ion ESI-MS/MS behaviour of flavonol aglycones was studied in detail by Fabre and co-workers [74]. The fragments obtained from flavonol aglycone pseudomolecular anions exhibit losses of small neutral molecules, such as CO (-28 amu) and CO₂ (-44 amu) that may be attributed to C-ring. Neutral loss of C₂H₂O (-42 amu) involving A-ring occurs only for flavonol aglycones mono- or unhydroxylated in B-ring. The successive loss of these molecules may also be prominent. Cleavage of C-ring by RDA mechanism leads to ^{m,n}A⁻ and ^{m,n}B⁻ ions, providing information on the number and type of substituents in A- and B-rings [59]. Hydroxylation of B-ring has an impact on the fragmentation: in the CID spectra of flavonols containing two or more hydroxyl groups in B-ring, e.g. quercetin and myricetin, ions corresponding to [^{1,2}A-H]⁻ and [^{1,2}B-H]⁻ can be seen, while to obtain fragmentation of flavonols unsubstituted in B-ring much higher collision energy is required, which leads to numerous product ions [59].

Although ESI-MS/MS is not suitable for the unambiguous structural identification of flavonoid glycosides (e.g. stereochemistry of the glycan part), it provides sufficient information regarding the aglycone structure, the attachment point of substituents and the monosaccharide units of the glycan sequence. Fragmentation pathway of

O-glycosylated flavonoids starts with the cleavage of the glycosidic bonds and elimination of the sugar moieties with charge retention on the aglycone or sugar fragments [55]. In the CID spectra of deprotonated flavonol glycosides, ions corresponding to the deprotonated aglycones, $[Y_0]^-$ at m/z 285, 301 and 317 generated by the loss of sugar units, furthermore the following fragment ions for aglycones are detected: $[Y_0-H]^-$ at m/z 284, 300 and 316, $[Y_0-H-CO-H]^-$ at m/z 255, 271 and 287 for kaempferol, quercetin and myricetin, respectively [75]. Other characteristic ions [76-77] observed in the product spectra of kaempferol, quercetin and myricetin aglycones and glycosides are shown in Table 2.

Table 2. Familiar fragment ions deriving from negative ionization CID of flavonol glycosides [74-77].

Fragment ions (m/z)	Aglycone structure		
	Kaempferol	Quercetin	Myricetin
$[Y_0]^-$	285	301	317
$[Y_0-H]^-$	284	300	316
$[Y_0-2H]^-$	283	299	315
$[Y_0-CO]^-$	257	273	289
$[Y_0-H-CO-H]^-$	255	271	287
$[Y_0-H-CO_2]^-$	241	257	273
$[Y_0-H-CO_2-H]^-$	239	255	271
$[Y_0-H-2CO-H]^-$	227	243	n.d.
$^{1,2}A^-$	179	179	179
$^{1,3}A^-$	151	151	151

n.d. No data

The glycoside moieties attached to flavonoid aglycones and phenolics through an *O*-glycosidic bond can be identified in tandem mass spectrometry, according to the neutral losses of sugar units [78]. Difference of 162 amu indicates a hexose, 146 amu denotes a desoxyhexose, 132 amu represents a pentose and 176 amu refers to a glucuronic acid moiety, while identification of neutral losses of di-, tri- and tetrasaccharides can be achieved by adding the neutral losses of the adequate glycan

units, e.g. neutral loss of a familiar disaccharide, rutinose (α -L-rhamnopyranosyl-(1 \rightarrow 6)- β -D-glucopyranose) is 308 amu (146 amu + 162 amu).

Additionally, the two types of glycosidic bonds, *C*- and *O*-glycosylation can be distinguished easily, as ESI-MS/MS fragmentation patterns of *C*-glycosyl flavonoids is different from those of *O*-glycosyl flavonoids. Neutral losses of 96, 120 and 150 amu were observed for 6-*C*- and 8-*C*-glycosyl flavonoids by Kazuno and co-workers [79].

According to the results of Hvattum and Ekeberg [80], the nature and position of the sugar substitution also affects the fragmentation of flavonol *O*-glycosides rendering radical aglycone product ions. The authors studied formation of the radical aglycone product ion after CID of the deprotonated flavonoid *O*-glycosides. Product ion spectrum of rutin representing product ions from both heterolytic and homolytic cleavages is shown in Fig. 5. The authors remarked that stable flavonoid radicals are also obtained when the compounds are acting as antioxidants by donation of a hydrogen atom to free radicals.

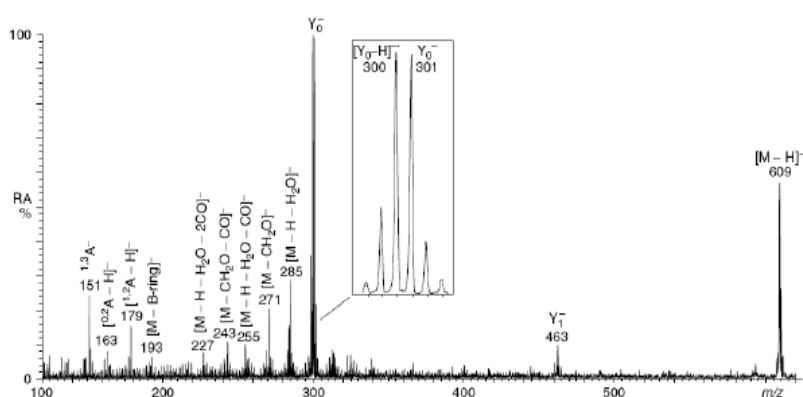


Fig. 5. Product ion spectrum of deprotonated rutin (quercetin 3-*O*-rutinoside). The homolytic cleavage of the 3-*O*-glycosidic bond produces $[Y_0-H]^-$ ion (m/z 300), while the $[Y_0]^-$ ion (m/z 301) derives from heterolytic cleavage [59].

Relative abundance of the stable radical aglycone $[Y_0-H]^-$ obtained by homolytic cleavage of the flavonol 3-*O*-glycosidic bond compared to that of the aglycone product ion $[Y_0]^-$ deriving from the heterolytic cleavage increased with the increase in collision energy, as well as with the increase in the number of OH substituents in B-ring (Fig. 6.).

The opposite behaviour is observed for flavone 7-*O*-glycosides, where less OH substitution in B-ring favours the formation of radical aglycone. However, Davis and Brodbelt [76] drew attention to inconsistency of the correlation between B-ring hydroxylation and the formation of radical aglycone after CID of flavonol 3-*O*-glycosides. Kaempferol 3-*O*-glycosides were the most affected.

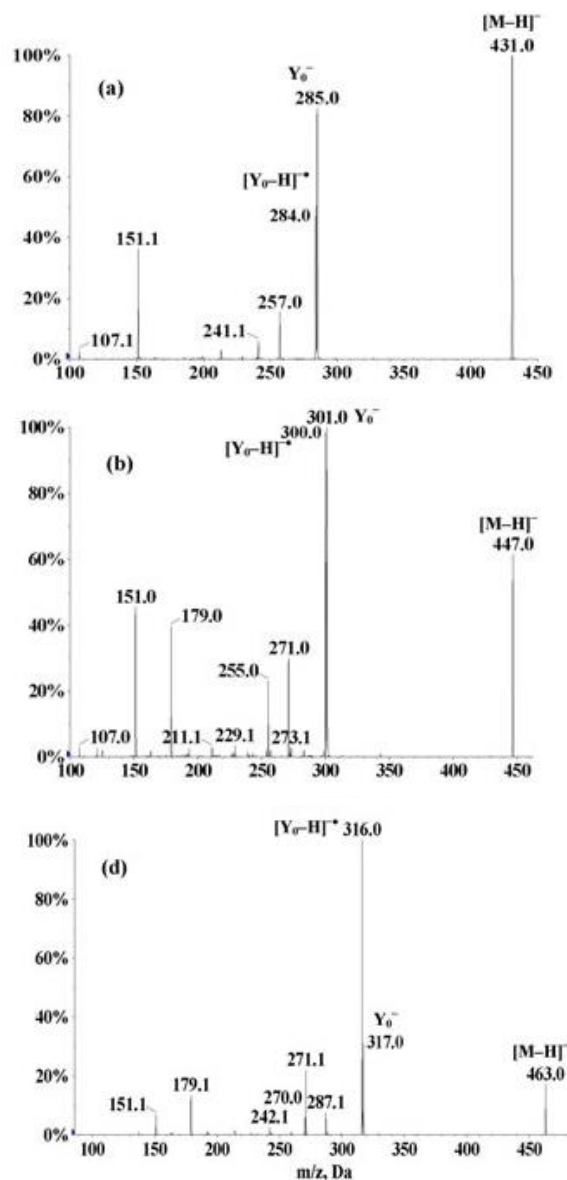


Fig. 6. Product ion spectra of $[M-H]^-$ ions of a) kaempferol 3-*O*-rhamnoside, b) quercetin 3-*O*-rhamnoside and d) myricetin-3-*O*-rhamnoside [77].

Ablajan and co-workers confirmed that relative abundance of the radical aglycone anion $[Y_0-H]^-$, deriving from a homolytic cleavage, compared to that of the aglycone anion $[Y_0]^-$, deriving from a heterolytic cleavage is in correlation with the position of glycosylation [75]. Fragmentation scheme of flavonol 3-*O*-glycosides showed more abundant $[Y^3_0-H]^-$ ions compared to $[Y^3_0]^-$ ions, in contrast to flavonol 7-*O*-glycosides where abundance of $[Y^7_0-H]^-$ ions was lower relative to $[Y^7_0]^-$ ions (Fig. 7.).

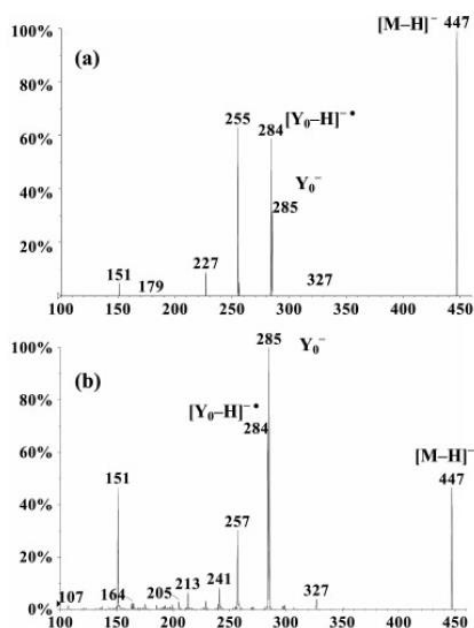


Fig. 7. $[M-H]^-$ product ion spectra of (a) kaempferol 3-*O*-glucoside and (b) kaempferol 7-*O*-glucoside [75].

MS^n evaluation of flavonol 3-*O*- and 7-*O*-glycosides revealed that the second generation product ions of $[Y_0-H]^-$ and $[Y_0]^-$ ions are different, the $[Y_0-H]^-$ ion is the precursor of $[Y_0-H-CO-H]^-$ and $[Y_0-H-CO_2-H]^-$ ions, while the $[Y_0]^-$ ion is the precursor of the $[Y_0-CO]^-$ ion. Consequently, isomeric flavonol 3-*O*- and 7-*O*-glycosides can be differentiated on the basis of formation and relative abundance of $[Y_0-H]^-$ and $[Y_0]^-$ ions and by comparing the diagnostic ions formed by retro-Diels–Alder reactions [75]. However, MS^3 spectra of flavonol 3-*O*-glycosides are to match sagely with MS/MS spectra of the adequate aglycones, the aglycone part of flavonol glycoside compounds can not always be determined by simple comparison with the adequate aglycones (Fig. 8.) [76]. Familiar product ions of kaempferol, quercetin and myricetin glycosides were shown previously, in Table 2.

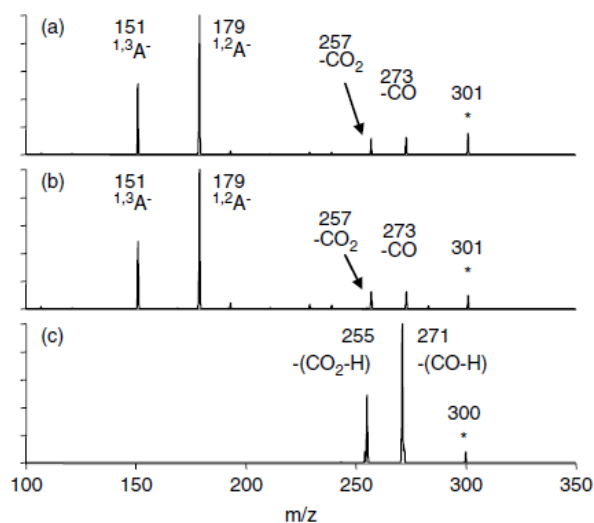


Fig. 8. CID spectra of quercetin and quercetin 3-*O*-glucoside. (a) MS/MS spectrum of deprotonated quercetin, (b) MS³ spectrum of quercetin 3-*O*-glucoside using the [Y₀]⁻ ion as the precursor; (c) MS³ spectrum of quercetin 3-*O*-glucoside using the [Y₀-H]⁻ ion as the precursor [76].

Lu et al. found [81] that relative abundance ratio of [Y₀-H]⁻ and [Y₀]⁻ ions deriving from homolytic and heterolytic cleavages of the sugar moieties from kaempferol 3-*O*-glycosides may be influenced by the length of the saccharide substituent. Negative ion CID of kaempferol 3-*O*-monoglycosides induced a predominant homolytic cleavage, while kaempferol 3-*O*-di- and -triglycosides, similarly to flavonol 7-*O*-glycosides, gave abundant heterolytic cleavage fragments. The authors concluded that the differences in the fragmentation pathways may be attributed to the length of the saccharide chains, as electron-donating effect from the B-ring was reduced by the large steric hydrance caused by the long saccharide chains.

Ablajan and co-workers concluded [75] that product ion spectra of flavonol 3,7-di-*O*-glycosides substantially differ from those of their isomeric flavonol mono-*O*-diglycosides. In order to characterize a flavonoid as a flavonol 3,7-di-*O*-glycoside, both [Y³₀-H]⁻ ion formed by homolytic cleavage of the 3-*O*-glycosidic bond and [Y₀-2H]⁻ ion generated by the elimination of two glycosyl radicals at the 3-*O* and 7-*O* positions successively should be present in the [M-H]⁻ spectrum. Product ion spectra of kaempferol, quercetin and myricetin 3,7-di-*O*-glycosides containing both [Y³₀-H]⁻ and [Y₀-2H]⁻ ions are shown in Fig. 9.

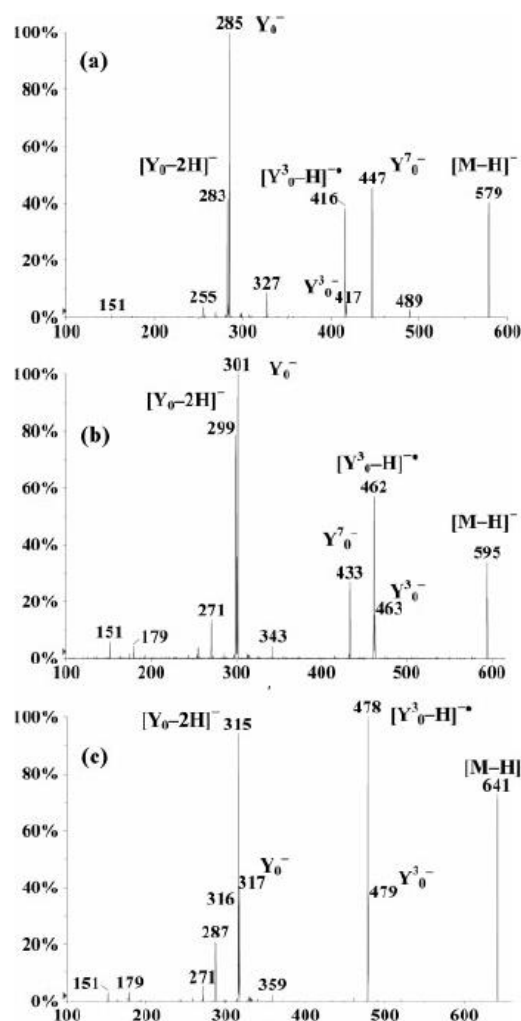


Fig. 9. Product ion spectra of deprotonated (a) kaempferol 3-*O*-glucoside-7-*O*-arabinoside, (b) quercetin 3-*O*-arabinoside-7-*O*-glucoside and (c) myricetin 3,7-di-*O*-glucoside showing $[Y_0-H]^-$ and $[Y_0-2H]^-$ ions formed by homolytic cleavages of the glycosidic bonds [75].

Furthermore, the glycan sequence, which is either (1 → 2) or (1 → 6), also has a significant influence on the relative abundances of $[Y_0-H]^-$ and $[Y_0]^-$ ions. Cuyckens and Claeys [82] found that intensity ratio of $[Y_0-H]^-$ and $[Y_0]^-$ ions deriving from CID of isomeric flavone 7-*O*-diglycosides was distinctly different. Rhoifolin (apigenin 7-*O*-neohesperidoside) and isorhoifolin (apigenin 7-*O*-rutinoside) differ only in the interglycosidic linkage between the terminal rhamnose and the internal glucose residues, which is (1 → 2) and (1 → 6) for rhoifolin and isorhoifolin, respectively.

Ferreres and co-workers could also differentiate the (1 → 2) and (1 → 6) interglycosidic linkages and discern between isomeric di-, tri- and tetraglycosylated flavonoids by evaluation of characteristic product ions in the (–)-ESI-MS/MS spectra of flavonoid isomers [83]. The (1 → 2) linkage was characterized by high relative abundance of the Y_1^- (-162 amu) ion and the Z_1^- (-180 amu) ion, while for compounds with the (1 → 6) linkage Y_1^- was observed at very low abundance and Z_1^- was not detected.

However, because of the numerous factors affecting the formation of radical and non-radical product ions during CID of flavonoid glycosides, Cuyckens and Claeys concluded [82] that the $[Y_0-H]^- : [Y_0]^-$ ion ratio can be used only for suggesting the position of the glycan substitution, especially in the case of flavonol 3-*O*-glycosides, rather than as a diagnostic tool for the characterization of the glycosylation position in unknown flavonoid *O*-glycosides.

2.4. *Sempervivum tectorum* L.

2.4.1. Taxonomic classification

Sempervivum tectorum L., common houseleek is a wild-growing succulent belonging to the family of Crassulaceae. *Sempervivum* is a genus of some 30 species mostly native to Mediterranean and highland areas of Europe. *S. tectorum* is spread from the Pyrenees, through the Alps up to the northern regions of the Balkan Peninsula. It grows under very poor soil conditions, primarily on rocks, roofs, old walls and chimneys [84-87].

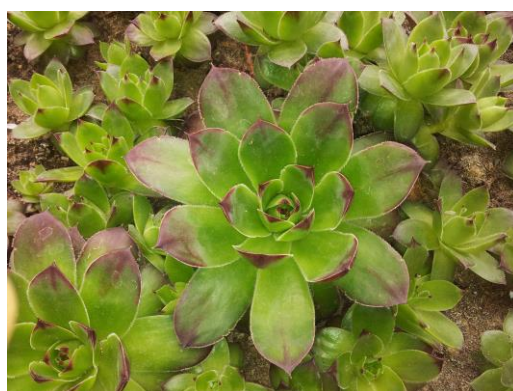


Fig. 10. *Sempervivum tectorum* L.

Name of the plant derives from the Latin words *semper* (= ever), *vivum* (*vivere* = to live) and *tectum* (= roof) and refers to the fact that it is an evergreen perennial plant, as well as to its use in the ancient times. Houseleek was known and cultivated already by the ancient Romans on ridges and copings of houses from clay and straw mortar as protection against wash-out. The observation that fires caused by lightnings hardly damaged houses that were grown houseleek on their roofs, led on the belief that the plant protects from thunderbolts. This persuasion was kept by some of its common names of German origin, e.g. Dimerkraut (Dimer = storm, thunder-shower; Kraut = plant/herb). The name Donnerkrut comes from the name of Donar, a Germanic god associated with thunder, lightning and storms.[84-85].

2.4.2. Morphology

S. tectorum is a robust succulent herb (Fig. 10.). Leaves are crowded in perennial basal rosettes 8-15 cm in diameter. Houseleek is a self-propagating plant, it produces offshoots around its base. Offshoots are 3-6 cm long, chunky, leafy. The succulent basal leaves are obovate-lanceolate, with the apex pointed, green, but tinged intense red on the upper surface. The plant blooms from July to September, the flowering stems are 20-40 cm tall, robust, covered with glandular hair all over. *S. tectorum* is a hapaxanthic plant, after fruiting, the leaves senesce and the plant dies. The flowers are regular, hermaphrodite, up to 3 cm in diameter, arranged in a panicle with more than 30 flowers. Number of sepals, petals and carpels is 12-16, that of stamens is the double. Petals are lanceolate, reddish, fused from the base for a short distance. The fruit is a group of follicles.

S. tectorum has been planted on roofs and stone-walls since the antiquity. It often grows wild, follicles of wild-grown varieties are sterile. The stock species growing wild are to find in Hungary solely in the Villányi-mountain, on Jura-limestone rock lawns of Szársomlyó, consequently, the plant is protected [88-90].

2.4.3. Traditional use

Sempervivum species were already referred to by Theophrastus, Dioscorides and Plinius as remedy against burns and wounds. Charlemagne ordered cultivation of houseleek in his *Capitulare de villis* for the officials of the crown lands [84]. *Sedum* or *Sempervivum* spp. leaves were practical drugs used by medieval (10-14th century) Mediterranean communities according to prescriptions, lists of materia medica and letters of physicians from the Cairo Genizah, a collection of Jewish manuscript fragments found in the genizah (store room) of the Ben Ezra Synagogue in Old Cairo. Indications were: eye diseases, fever, bleeding and kidney pains [91-92]. A *Sempervivum* species, *S. maius* was comprised in the first official German Pharmacopoeia [93]. Internal use of leaf juice was indicated only against raging fever, intense diarrhoea and to free from worms, otherwise external use was preferred for burst of fury, eye-boils, ear-flow, pain by inflammation of the liver and the kidney, podagra and burns. Local use was recommended by erysipelas, inflammation of the eyes, burns, spreading ulcers, swellings / tumidity. In traditional folk medicine smashed leaves and leaf juice of houseleek were used against rash eruptions, open wounds, bee-stings, burns [84], itching of the skin, intermittent fever and scrofulous ulcers [85]. Leaf juice of *S. tectorum* was furthermore applied against corns and freckles, internally against sore throat, dys- and amenorrhoea [84], uterus neuralgia, gastric ulcer, dysentery and haemorrhage [85]. In Denmark it was used internally against bladder complaints, in Poland as mucilaginosum for ulcers, in Hungary against fever, epilepsy and warts. Leaves allowed to infuse for a while in cold water resulted in a cooling drink which was used by fever and catarrhal diseases of the respiratory system. Covering with smashed leaves or rubbing in with the leaf juice was thought to be useful against warts, freckles, burnings, inflammations of the eyes, erysipelas, ulcers and corns [85].

Leaves mixed with fat were used as a goitre ointment, remedy for catarrh of the eyelids, against chapped, rough skin and earache. Infusion of the herb was used against fever, the leaves put into carious tooth relieved the pain. In some regions leaves and young sprouts were consumed as a salad [84]. In Bosnia and Herzegovina *S. tectorum* leaves were applied internally for anxiety by children, insomnia, epilepsy, ear inflammations, stomach ulcers, menstruation ailments and as a tincture for renal ailments, externally for

warts, ulcers, skin rash, corns and sunspots [94-95]. In Serbia houseleek was used for earache as a compress made from leaf juice applied to the sore area, for stomach and intestinal ulcers (eaten one leaf a day). The juice obtained from cutting the leaf longitudinally was applied to the area with *Herpes zoster* [96]. In Danish traditional medicine medication prepared from aerial parts of houseleek with distilled spirits was applied for epilepsy [97].

In Italy fresh leaves of *S. tectorum* were used to insect bites, latex was applied to warts as keratolytic, defoliated, mashed aerial parts were used to haematomas, while beaten aerial parts were placed on the brow with handkerchief for headache [98-99]. In the Italian alpine area, similarly to *S. tectorum*, a number of different medications prepared from *S. montanum* has been applied: infusion from fresh leaves as a diuretic and appetizer drink or as a rinse for sore throat, smashed leaves – besides the usual application purposes, i.e. burns, wounds, insect bites – as a compress or poultice for headache and nose-bleeding [100]. Veterinary use is also known: cattle and sheep were fed leaves of houseleek by gastrointestinal problems, especially by tympanism, when rumination is blocked by overingestion of alfalfa and other fresh plants [101].

Recently the old custom of planting houseleek on roofs can be revived in an up-to-date form by using houseleek or other Crassulaceae species as green roof plants. Green roofs have the potential to function as islands of biodiversity within urban environments. Crassulaceae succulents are highly tolerant of the harsh roof-top environment, e.g. summertime water deficit and heat stress, additionally, they can increase performance of neighbouring plant species [102].

2.4.4. Biological activities

Lyophilized leaf juice of *S. tectorum* displayed dose-dependent $\text{H}_2\text{O}_2/\cdot\text{OH}$ scavenger activity in an *in vitro* chemiluminescence test, moreover its scavenger activity against Fenton type $\cdot\text{OH}$ radicals was proved by electronspinresonance studies [103].

A further study of the research group confirmed superoxide ($\text{O}_2^{\cdot-}$) scavenger activity of lyophilized *S. tectorum* leaf juice. In the same study the leaf juice inhibited *in vitro* non-enzimatically (by ascorbic acid and FeCl_3) induced lipidperoxidation [104].

Houseleek significantly decreased liver enzyme levels elevated due to atherogen diet [105]. Male Wistar rats were fed fat rich diet, while an other group of animals was administered houseleek extract besides the atherogen nutriment. Levels of alkaline phosphatase (ALP), glutamate-pyruvate transaminase (GPT) and gamma-glutamyl transpeptidase (GGT) significantly increased due to atherogen diet in comparison to the control group. Treatment of houseleek extract significantly decreased elevated ALP and GPT levels. Additionally, the beneficial effect of the houseleek extract was observed on activity of cytokines playing an important role in the regulation of lipid metabolism.

Lyophilized leaf juice showed protective activity against diffuse degenerative hepatocellular alterations of the liver due to lipogenic diet [106]. Houseleek leaf juice had livercell protecting effect, decreased the serum triglyceride level, increased HDL cholesterol level. It showed protective activity against harmful effects of lipogenic diet and excessive alcohol consumption by improving vacuolization and necrosis. Liver tissue of animals fed lipogenic diet then administered houseleek leaf juice was similar to that of animals in the control group. Decrease of the serum lipid level and increase of HDL cholesterol level could also contribute to liver cell protecting effect of houseleek leaf juice.

Lyophilized houseleek leaf juice inhibited enzymatically and non-enzymatically induced lipidperoxidation in hyperlipidemic rats [107] and enhanced excretion of toxic Al, Ba, Ni and Ti elements from the liver [108].

Kékesi and co-workers [109] concluded that intraperitoneal and intrathecal administration of *S. tectorum* leaf extract significantly decreased inflammation in the paw-withdrawal test in carrageen induced inflammatory model and had antinociceptive effect in the tail-flick test in healthy rats. However, preparation method and chemical composition of the extract was not specified.

Although a number of homeopathic products containing houseleek are marketed in the USA, the UK, Canada and Germany, while some others in Hungary were notified as cosmetics, *S. tectorum* is not a registered herbal remedy, its thorough chemical and pharmacological characterization is still not available.

2.4.5. Phytochemical characterization

2.4.5.1. Carbohydrates

Modified carbohydrate metabolism is representative for the Crassulaceae family, which takes shape in the accumulation of sedoheptulose. Accordingly, the main sugar compound of *S. tectorum* is sedoheptulose, its content varies between 2-11 % [110]. Moreover it contains fructose, glucose, saccharose, raffinose and verbascose in smaller amounts [110-112].

Carbohydrate metabolism of *S. tectorum* shows annual periodicity: during the summer mainly starch is stored, while in the remaining part of the year sedoheptulose is buffered in the highest quantity [110]. Presumed reason for high content and winter accumulation of sedoheptulose may be that – due to its protective function and osmotic effect – counts sedoheptulose particularly in frost resistance [113].

2.4.5.2. Organic acids, the crassulacean acid metabolism

Similarly to other species of the Crassulaceae family, houseleek is an acid accumulating plant. It contains isocitric (Fig. 11.B), citric, malic (Fig. 11.A) and succinic acid in large quantities, however its organic acid content shows exceeding diurnal fluctuation: organic acids are accumulated during the night, while by day their content is decreased. This fluctuation is in the case of malic acid the most pronounced. Content of the carboxylic acids shows also remarkable annual changes. Isocitric acid always dominates and reaches distinct maxima at the beginning of winter and summer. Quantity of 5.3-9.7 % isocitric acid was observed [111, 114]. The variations in the quantity of total acids are, as a result of the very high proportion of isocitric acid, essentially in accordance with the variations of the latter. The factors temperature, photoperiodism and endogeneous annual rhythm are suggested to be responsible for the fluctuations in contents of the acids. A comparison with the carbohydrate metabolism suggests that acids might take part in the regulation of carbohydrate storage [114]. High quantity of oligosaccharides in the winter months coincides with the elevated malic acid content [87, 111, 114].

Daily fluctuation of the acid content is due to a special carbon fixation pathway, the crassulacean acid metabolism (CAM), that evolved as an adaptation to arid conditions and in which carbon dioxide (CO₂) is fixed at night, when stomata of the plant are open. Chemical fixation of carbon dioxide takes place through its combination to phosphoenolpyruvate creating the four-carbon molecule oxaloacetic acid. This reaction is catalyzed by PEP-carboxylase (PEP-C), however, the enzyme is inhibited by high temperatures or by binding malate [115]. Oxaloacetic acid is subsequently reduced to malate which is transported passively into vacuoles following the actively transported protons. CAM plants store CO₂ in the vacuoles mostly in the form of malic acid, until further converting is enabled by the daylight. β-decarboxylation of malate during the day releases carbon dioxide, thus allowing carbon fixation to 3-phosphoglycerate in the Calvin-cycle. Plants that do not use PEP-carboxylase in carbon fixation are called C₃ plants because the primary carboxylation reaction produces the three-carbon sugar 3-phosphoglyceric acid directly in the Calvin-cycle. Carbon dioxide release during the day conduces to decrease of the malate accumulated during the night which causes significant pH fluctuation: pH 3.8 during the night, pH 5.6 during the day. Refilling of the vacuoles with malate begins only after whole exhaustion, usually the night after. This diurnal fluctuation of the organic acid content shows correlation with fluctuation of the polysaccharide content [116-123].

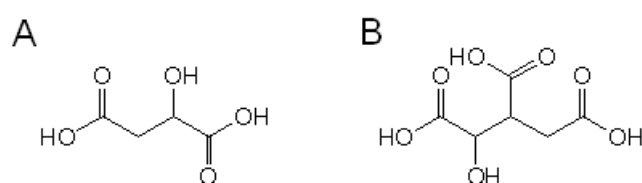


Fig. 11. Characteristic organic acid compounds of *S. tectorum*: A: malic acid and B: isocitric acid.

CAM makes photosynthesis possible at very low concentration of carbon dioxide, due to the high affinity of PEP-carboxylase to carbon dioxide. This type of photosynthesis is favourable for xerophytes, growing in hot and dry conditions, since they can close their stomata during the day to prevent the loss of water [115, 123].

2.4.5.3. Flavonoids

According to the literature, kaempferol glycosides are widely distributed in the *Sempervivum* genus as well as in the Crassulaceae family. Stevens and co-workers [124] analyzed flavonoid aglycone composition of some *Sempervivum* species after acidic hydrolysis. They concluded that kaempferol was the principal flavonoid of all species, in addition presence of herbacetin, quercetin and myricetin glycosides was also proved. However, they studied flavonoid variation of houseleek only at the aglycone level and detailed data on glycosilation pattern of *S. tectorum* flavonols are neither to find in other literature sources. Flavonol and methoxylated flavonol aglycones revealing *S. tectorum* and other species of the Crassulaceae family are shown in Fig. 12.

2.4.5.3.1. Flavonoids in other species of the Crassulaceae family

In the *Sedum* genus flavonol glycosides – kaempferol [125-129], quercetin [127-130] and myricetin [127-128, 131] 3-*O* and 7-*O* substituted mono-, di- and triglycosides with primarily glucose and rhamnose moieties are prevailing. Other flavonoid glycosides and glucuronides with herbacetin and gossypetin aglycones [130, 132], as well as sarmenoside flavonol glycosides [133] were also described. Additionally, a number of methoxylated flavonol (limocitrin and isorhamnetin) mono-, di- and triglycosides glycosylated at the 3-*O* and 7-*O* position mainly with glucose and rhamnose [127, 134], together with acetylated sugar moieties [130, 134] were reported. Furthermore, a quercetin glycoside with feruloyl esterification was described [135].

The species in the *Kalanchoe* genus were characterized by the domination of kaempferol and quercetin 3-*O*- and 7-*O*-monosides and -diglycosides, mainly with rhamnose and glucose sugar units [136-140]. Presence of methoxylated flavonol glycosides with isorhamnetin [141], patuletin [142], and methoxymyricetin [141] aglycone, as well as that of 3-*O* and 7-*O* substituted patuletin with acetylated and diacetylated rhamnose moieties [143] was described.

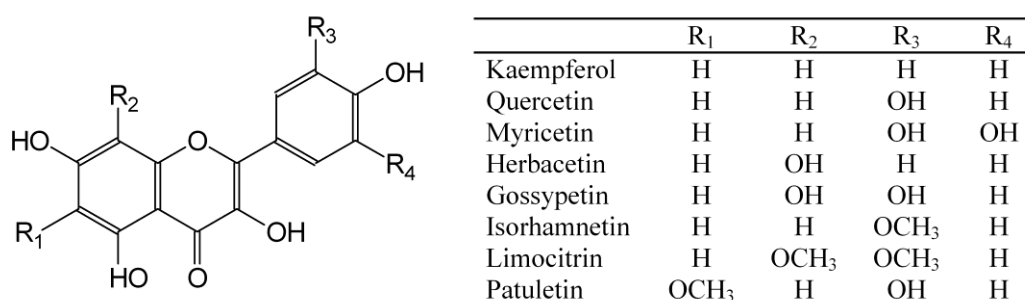


Fig. 12. Flavonol and methoxylated flavonol aglycones detected in *S. tectorum* and other species of the Crassulaceae family.

For further species of the Crassulaceae family were the same flavonoid fingerprints characteristic: kaempferol and quercetin 3,7-*O*-diglycosides, 3-*O*- and 7-*O*-glycosides, additionally kaempferol, quercetin, myricetin and scutellarein methylethers for genera *Orostachys* [144] and *Aeonium* [145], respectively.

2.4.5.4. Procyanidins, anthocyanins and other polyphenols

Abram and Donko detected [146] flavonoids, B2 type procyanidins and anthocyanins in houseleek extract prepared with methanol. Corresponding with the result of Stevens and co-workers [124], aglycone of the flavonoids was kaempferol. Moreover delphinidin was detected after acidic hydrolysis, while structure of procyanidins was of the 4-thiobenzyl(-)-epigallocatechin and the 4-thiobenzyl(-)-epigallocatechin-3-gallate type.

2.4.5.4.1. Polyphenols in the Crassulaceae family

Flowers of *Crassula*, *Cotyledon* and *Tylecodon* species were specified by anthocyanidin 3-*O*-monosides and -diglycosides [147], while those of *Sedum sediforme* contained besides flavonol monosides flavan-gallates and gallic acid [127].

2.4.5.5. Alkaloids

Although alkaloid content of *S. tectorum* was determined by a French research group (0.01-0.03% in the dry plant) [111], neither investigations on structural elucidation were carried out, nor alkaloid content of the plant was confirmed by other studies.

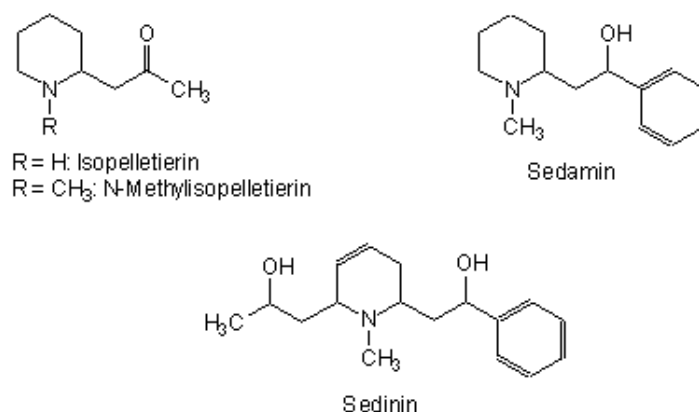


Fig. 13. Alkaloids isolated from species belonging to the Crassulaceae [111].

2.4.5.5.1. Alkaloids in the Crassulaceae family

Alkaloid compounds characteristic of the Crassulaceae family have piperidine structure, some of them are, alike *Lobelia* alkaloids, α,α_1 -disubstituted [111]. Characteristic alkaloids are shown in Fig. 13. Stevens and co-workers [148] compared tannin and piperidine alkaloid composition in 36 species of the Crassulaceae. Species from genera *Crassula*, *Echeveria*, *Bryophyllum*, *Pachyphytum*, *Kalanchoe*, *Sedum*, *Aeonium* and *Sempervivum* were evaluated. Only some *Sedum* and one *Echeveria* species contained alkaloids, while proanthocyanidins and galloyl esters were absent only from some *Sedum* species containing piperidine alkaloids.

2.4.5.6. Amino acids

Amino acids of *S. tectorum* were mentioned only by one paper, free amino acids were studied by Montant, asparagic acid was prevailing [149].

2.5. *Corylus avellana* L.

2.5.1. Taxonomic classification

Corylus avellana L., common hazel is a large shrub belonging to the family of Betulaceae. The genus *Corylus* comprises three species native to Hungary: *C. avellana*, *C. colurna* and *C. maxima*. *Corylus avellana* is widely distributed throughout Europe, reaching as far east as the Ural Mountains in Russia, and from Scandinavia in the north to Spain, Italy and Greece in the south. In Hungary it occurs frequently in Transdanubia. *C. avellana* grows as an understory species in oak and ash woodlands or as a scrub at fringe of deciduous forests [1, 88, 90].



Fig. 14. *Corylus avellana* L.

2.5.2. Morphology

C. avellana is a large shrub, rarely grown as a small tree, with a smooth reddish-brown peeling bark. The twigs are densely covered with reddish glandular hairs. The leaves are alternate, simple, petiolate (the petiole is 0.4–1.1 cm long, covered with glandular hairs), almost globular from a heart-shaped base, with the apex acuminate, shallowly lobed or double-toothed / serrulate, pubescent or glabrous (Fig. 14.). The flowers of each sex are different inflorescences: the male flowers in 1–4 pendulous catkins up to 8 cm long with 4 stamens. There are only a few female flowers, arranged in erect short spikes up to 6 mm long, with red styles. It blooms from January to March. The fruit is a nut, globose or ovoid, up to 2 cm wide, surrounded by a lobed green involucre [88, 90].

2.5.3. Traditional use

The main products of *C. avellana* are kernels, mainly utilized by the confectionary industry, while the leaves have been used for phlebitis, varicose veins and haemorrhoidal symptoms, and also for their slight anti-microbial effect [1, 150-152]. The buds have been applied in gemmotherapy against hepatic, respiratory, circulatory and inflammatory diseases [153]. In traditional folk medicine in Hungary the catkin has been used for reduction of fever, while kernels as a haematogenous remedy [88]. In Britain the dried or fresh leaves have been used for stimulating bile production. Infusion prepared from the leaves has been taken for 3–4 times a day for diarrhoea [90]. In traditional medicine hazel bark has been used as a remedy for intermittent fever. Veterinary use is also known: the pollen has been used against diarrhoea [154].

C. avellana was already cultivated by the ancient Greeks and Romans, remains of hazel were explored at the ruins of Pompeii. Its popularity is announced by the fact that it was referred to by numerous ancient sources, such as Hippocrates, Theophrastus, Dioscurides, Cato, Plinius, Vergilius, Arabic and even Chinese authors [154-155]. In the antiquity it was used as a remedy for sore throat, cronic cough and baldness [156]. In early medieval times tribes living in Northwestern Europe surrounded sacred places where weapons were not to be drawn by a fence made of hazel stakes which formed a magical, as well as a physical barrier [154]. Several habits and believes were attached to hazel. It was a symbol of fertility, furthermore, it was thought that the plant protects from thunderbolts and that an easy touch with a hazel twig would kill a poisonous snake [155]. Hazelnut oil is used in present days not only as food but also in cosmetics, for its astringent and emolient properties [156].

2.5.4. Pharmacological effects

Hazel kernels exhibited concentration-dependent antioxidant activity in different *in vitro* assays: in the reducing power assay, in DPPH scavenging test and in β -carotene linoleate model system [157-158]. The ethanolic extract of the kernels showed significant activity also in total antioxidant and radical scavenging tests, which was

attributed to the relatively high total phenolic content of the extract. Antioxidant capacity of hazel leaves was found to be even higher than that of the kernels [159].

Dietary hazelnut supplementation reduced plasma total cholesterol and VLDL cholesterol, as well as triacylglycerol levels and increased that of HDL cholesterol in both hypercholesterolemic and healthy humans [160-161]. Antimicrobial activity against Gram positive bacteria of *C. avellana* was proved by Oliveira et al. [157]. The leaf of *C. avellana* is official in the 10th edition of the French Pharmacopoea [1].

2.5.5. Phytochemical characterization

2.5.5.1. Flavonoids

Several studies on flavonoid profile of *C. avellana* were published [162-164]. The main flavonoid compounds of hazelnut kernels were identified by Jakopic et al. [158] as myricetin 3-*O*-rhamnoside and quercetin 3-*O*-rhamnoside. Schmitzer and co-workers investigated the effects of skin removal and roasting on flavonol composition of hazelnuts. Quercetin pentoside, quercetin 3-*O*-rhamnoside and myricetin 3-*O*-rhamnoside was detected only in whole kernels [165]. In pollen of hazel flavonoids and quercetin 3-*O*-glucosyl-galactoside as the main compound were detected [166-167]. Amaral and co-workers investigated phenolic constituents of cultivated *C. avellana* leaves. Main flavonoid and caffeic acid derivatives were characterized and influence of environmental conditions, such as cultivar, geographical origin and ripening stage on phenolic composition was studied [168-169]. Presence of flavonol 3-*O*-monoglycosides, i.e. myricetin 3-*O*-rhamnoside, quercetin 3-*O*-hexoside, quercetin 3-*O*-rhamnoside and kaempferol 3-*O*-rhamnoside was shown. Myricetin 3-*O*-rhamnoside (myricitrin) was observed also previously for *C. avellana* leaves [1]. Peev and co-workers found [153] that in the early stages of development the plant tissues of *C. avellana* contained solely glycosides of quercetin, which were later subjected to metabolic transformation (e.g. removal or adding of hydroxyl groups) and also the sugar moieties were diversified in mature organs. In buds, the preferred sugar for glycosylation was galactose, while later flavonol glycosides were mainly rhamnosides.

2.5.5.2. Phenolic compounds: hydroxycinnamic acids, proanthocyanidins

Hydroxycinnamic acid derivatives, such as 3-caffeoylquinic acid, 5-caffeoylquinic acid, caffeoyltartaric acid and *p*-coumaroyltartaric acid were reported for hazelnut leaves [169-170, 162]. Buds contained only esters of quinic acid: chlorogenic and feruloylquinic acid [153].

Proanthocyanidins were also detected [163], and *C. avellana* leaves official in the 10th French Pharmacopoea should contain not less than 2% tannins [1]. The main phenolic compounds of hazelnut kernels were identified as flavan-3-ols: (–)-epicatechin, (+)-catechin, procyanidin dimers and procyanidin trimers; hydroxybenzoic acids: gallic acid, protocatechuic acid; dihydrochalcone: phloretin-2'-*O*-glucoside [158, 165].

2.5.5.3. Fatty acids

Hazel kernels contain 50–60% oil with similar composition to that of almond oil. Main fatty acids are: oleic acid, linoleic acid, palmitic acid, stearic acid and smaller amounts of palmitoleic acid, linolenic acid and margaric acid [1]. Bacchetta et al. evaluated fatty acid and α -tocopherol profile of hazel kernels [171]. Monounsaturated fatty acids were the most abundant, followed by polyunsaturated fatty acids, while saturated fatty acids were only minor components. High portion of favorable monounsaturated fatty acids for hazel kernels was confirmed by some other works [156, 170, 172].

2.5.5.4. Other compounds

Miele et al. [173] reviewed works related to exploring new sources of paclitaxel, included the article of Hoffman et al. [174] in which the authors reported detection of the antitumor drug paclitaxel and other related taxanes in stems and branches of hazel for the first time.

Other nutrients, e.g. vitamins, plant sterols and α -tocopherol were also described for *C. avellana* kernels [158, 170, 172].

3. OBJECTIVES

- Aim of our study was to complete phytochemical characterization of less known herbal remedies, in order to provide explanation to their traditional use and to establish their future therapeutic application. *Sempervivum tectorum* L. and *Corylus avellana* L. have been used in folk medicine primarily for their anti-inflammatory effects, for ear inflammation and for varicose veins, respectively. Flavonoids and other phenolic compounds (hydroxybenzoic and hydroxycinnamic acid derivatives) have been attributed to their pharmacological actions.
- Matrix composition present in plant extracts depends in a large way on the solvents applied for extraction, therefore aim of our work was to compare *S. tectorum* extracts prepared with solvents of different polarity. Orientation of phytochemical analyses was determined by the *in vitro* antioxidant activity of the extracts. We aimed to reveal the correlation between phenolic composition and radical scavenging activity of *S. tectorum* extracts.
- Objective of our work was to analyse phenolic composition of *S. tectorum* extracts showing the highest antioxidant capacity by electrospray ionization tandem mass spectrometry coupled to high-performance liquid chromatography, with particular attention to their flavonoid compounds.
- Mainly kaempferol glycosides have been reported for *S. tectorum* L., however, its flavonoid profile has been studied only at the aglycone level, although glycosyl substitution can have a great impact on bioavailability of compounds. Therefore we aimed to evaluate glycosylation pattern of *S. tectorum*.

- We aimed to develop validated qualitative and quantitative high-performance liquid chromatographic / mass spectrometric methods for comprehensive phytochemical investigation of *S. tectorum* leaf juice, which has been used traditionally as medication against inflammation of the ears.
- Hydroxycinnamic acid derivatives of *S. tectorum* leaf juice have not been studied before, accordingly, we aimed to characterize and determine its hydroxycinnamic acid derivative components.
- Phenolics in kernels of *Corylus avellana* L. have been studied extensively, nevertheless data regarding leaves are less detailed. Therefore we aimed to complement data regarding flavonol glycoside and phenolic acid profile of *C. avellana* leaves. Our further objective was to study influence of solvents used for extraction on phenolic composition of the extracts.

4. MATERIALS AND METHODS

4.1. Plant material

4.1.1. *Sempervivum tectorum* L.

Plant material was cultivated at the Research Station of the Corvinus University of Budapest, in Soroksár, leaf samples were collected in June 2007. Authenticated *S. tectorum* samples and herbarium specimen are deposited at the Herbarium of Semmelweis University, Department of Pharmacognosy, Budapest, Hungary (St.003).

4.1.2. *Corylus avellana* L.

Leaves of *C. avellana* were collected in Nógrád, Pest County, Hungary (June 2010). 50-50 g samples were collected from three trees. Plant samples were authenticated in the Department of Pharmacognosy, where voucher specimen are deposited (Ca.001).

4.2. Solvents and chemicals

Methanol and hydrochloric acid of reagent grade were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and Carlo Erba Reagenti SpA (Rodano, Italy), respectively. Chloroform, ethanol, ethyl acetate and acetone of reagent grade were purchased from Molar Chemicals Kft. (Budapest, Hungary). Butanol, acetic acid and formic acid of reagent grade and spectroscopy grade ethanol were supplied by Reanal (Budapest, Hungary). Acetonitrile and methanol of HPLC supergradient grade were purchased from Sigma-Aldrich. LC grade water was prepared with a Millipore Direct Q5 water purification system (Bedford, MA, USA). All aqueous eluents for HPLC and LC-MS were filtered through MF-Millipore membrane filters (0.45 µm, mixed cellulose esters) (Billerica, MA, USA) and degassed in an ultrasonic bath before use. Hide powder was supplied by Filk gGmbH (Freiburg, Germany). Folin - Ciocalteu's phenol reagent and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were obtained from Fluka Chemie GmbH (Buchs, Switzerland), sodium carbonate was

purchased from Molar, while sodium hydroxide, sodium nitrite, sodium molybdenate, potassium peroxydisulfate, aluminium chloride, hexamethylenetetramine and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were supplied by Sigma-Aldrich. Kaempferol, quercetin, rutin, myricitrin, caffeic acid, chlorogenic acid, rosmarinic acid, syringic acid, gallic acid, L-ascorbic acid and trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) reference compounds were supplied by Sigma-Aldrich, while *p*-coumaric acid was purchased from Fluka.

4.3. Sample preparation

4.3.1. Preparation of *S. tectorum* extracts

10-10 g lyophilized, freshly powdered (mesh size 0.8 mm) leaves were extracted exhaustively (for 10 hours) with 500 mL chloroform, acetone, ethanol, 70% (v/v) ethanol, methanol and 80% (v/v) methanol in a Soxhlet apparatus. Additionally, the extract containing flavonoid *O*-glycosides in the form of aglycones prepared by simultaneous acidic hydrolysis and extraction with acetone, followed by liquid-liquid extraction with ethyl acetate was evaluated. After filtration the extracts were evaporated to dryness at 60 °C under reduced pressure. The residues were redissolved in 3 mL 80% (v/v) HPLC grade methanol and submitted to SPE purification. 500 mg/3 mL Supelco Supelclean LC-18 SPE cartridges (Supelco, Bellefonte, PA, USA) were activated with 3 mL methanol, then with 3 mL water. After the addition of the samples, the analytes were eluted with 6 x 750 µL 50% (v/v) methanol, 6 x 750 µL 75% (v/v) methanol, then with 6 x 750 µL 100% (v/v) methanol. Prior to evaluation the samples were evaporated to dryness at 60 °C under reduced pressure, redissolved in 4.0 mL 70% (v/v) supergradient grade methanol and filtered through Sartorius (Goettingen, Germany) Minisart RC15 (0.2 µm) syringe filters.

4.3.2. Separation of *S. tectorum* 80% (v/v) methanolic extract

A parallel of houseleek extract prepared with 80% (v/v) methanol (conditions see in text, section **4.3.1. Preparation of *S. tectorum* extracts**) was fractionated with Silicagel

column chromatography. The extract was introduced to 5 g Silicagel and during evaporation of the solvent permanently homogenized. Suspension of Silicagel 60 (0.063–0.200 mm) (Merck, Darmstadt, Germany) (100 g) with chloroform (200 mL) was prepared and used as stationary phase. Before transferring the sample, the suspension was allowed to settle, final dimensions of the bed were: length: 315 mm, diameter: 30 mm). The sample adsorbed on Silicagel was layered on the top of the stationary phase and eluted with 100–100 mL of the following eluent mixtures (proportions by volume):

	Chloroform : Methanol : Water		
1.	120	:	6 : 0
2.	90	:	10 : 1
3.	84.5	:	14 : 1.5
4.	73	:	24.5 : 2.5
5.	64.5	:	32 : 3.5
6.	57.5	:	38.5 : 4
7.	53.5	:	42 : 4.5
8.	47.5	:	42 : 10.5
9.	43	:	46 : 11

The collected fractions were evaporated to dryness at 60 °C under reduced pressure and redissolved in 3.0 mL 80% (v/v) methanol. Further sample purification steps were the same as described above (see in section **4.3.1. Preparation of *S. tectorum* extracts**).

4.3.3. Preparation of *S. tectorum* decoction

5 g lyophilized, freshly powdered (mesh size 0.8 mm) *S. tectorum* leaf sample was extracted with 500 mL hot water and allowed to infuse for forty minutes. The extract was filtered hot, allowed to cool down and evaporated to dryness at 60 °C under reduced pressure. The residue was redissolved in 25% (v/v) HPLC grade methanol and submitted to SPE purification. 500 mg/3 mL Supelco Supelclean LC-18 SPE cartridges were activated with 3 mL methanol, then with 3 mL water. After the addition of the samples, the analytes were eluted with 4 x 500 µL water, 4 x 500 µL 25% (v/v)

methanol, then with 4 x 500 μ L 100% (v/v) methanol. Prior to evaluation the collected fractions were evaporated to dryness at 60 °C under reduced pressure, redissolved in 4.0 mL 50% (v/v) supergradient grade methanol and filtered through Minisart RC15 (0.2 μ m) syringe filters.

4.3.4. Preparation of *S. tectorum* leaf juice

The sap of the leaves was squeezed fresh after collection and dried by lyophilization. 0.725 g dried sap was redissolved in 45 mL 80% (v/v) methanol, the removal of precipitated polysaccharides was performed by centrifugation (6000 rpm for 10 min). The supernatant was evaporated to dryness at 60 °C under reduced pressure, redissolved in 3 mL 80% (v/v) methanol, then purified by solid phase extraction. 500 mg/3 mL Supelco Supelclean LC-18 SPE cartridges were activated with 3 mL methanol, then with 3 mL water. After the addition of the redissolved supernatant, analytes were eluted with 6 x 750 μ L 50% (v/v) methanol, 6 x 750 μ L 75% (v/v) methanol, then with 6 x 750 μ L 100% (v/v) methanol. The collected fractions were evaporated to dryness at 60 °C under reduced pressure and redissolved in 4.5 mL 70% (v/v) supergradient grade methanol, finally the extract was filtered through Sartorius Minisart RC15 (0.2 μ m) syringe filter. The same sample was studied both in qualitative and in quantitative analyses.

4.3.5. Preparation of *C. avellana* extracts

Dried and milled (mesh size: 0.8 mm) leaves (10 g) were extracted with 250 mL of *n*-hexane and chloroform for 6 - 6 hours in a Soxhlet apparatus consecutively, following ethyl acetate and ultimately methanol extraction for 6 - 6 hours. The extracts were evaporated to dryness under reduced pressure with a rotary evaporator at 50 °C. The dried extracts were redissolved in 70% (v/v) HPLC grade methanol, SPE purification steps were the same as described above (see in section **4.3.1. Preparation of *S. tectorum* extracts**). Prior to evaluation the collected fractions were evaporated to dryness at 50 °C under reduced pressure, redissolved in 4.0 mL 70% (v/v) supergradient

grade methanol and filtered through Phenex-RC 15 mm (0.2 µm) syringe filters (Gen-Lab Ltd, Budapest, Hungary).

4.4. Quantitative phytochemical analyses

Contents of the main constituents in *S. tectorum* leaves: flavonoids, total polyphenols, tannins, hydroxycinnamic acid derivatives, proanthocyanidins and anthocyanins were determined by spectrophotometry, according to the methods of the Ph. Hg. VIII. [175]. Measurements were carried out in six parallels. The lyophilized plant material, as well as the extracts and fractions of the 80% (v/v) methanolic extract were studied.

4.5. Antioxidant activity assays

4.5.1. ABTS and DPPH scavenging activity

Antioxidant activity of *Sempervivum* samples was determined by spectrophotometry in two *in vitro* decolorization assays using ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] and DPPH [2,2-diphenyl-1-picrylhydrazyl], as free radicals. Detection was carried out at absorption wavelengths characteristic of each radical: at 734 nm and at 515 nm for ABTS and for DPPH, respectively. The method developed by Re et al. [176] and improved by Arts et al. [177], as well as the method described by Brand-Williams et al. [178] with some modifications were applied. Solutions containing the free radicals were prepared as follows: 10 mg ABTS was dissolved in 2.6 mL HPLC grade water and was reacted with 1.72 mg potassium peroxydisulfate to generate ABTS radical cation (ABTS^{•+}). The stock solution was kept at 4 °C in dark before use and was diluted immediately before the examination with spectroscopy grade ethanol until absorbance of 0.900 (± 0.005) was reached (detection wavelength: 734 nm). 10 mg DPPH was dissolved in 25.0 mL HPLC grade methanol, the stock solution was kept at 4 °C in dark for a maximum of one week before use. The solution was diluted immediately before the measurement with HPLC grade methanol until absorbance of 0.900 (± 0.005) was reached (detected at 515 nm). The assays were carried out with a HITACHI U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan).

S. tectorum extracts prepared with solvents of different polarity (chloroform, acetone, ethanol, 70% (v/v) ethanol, methanol, 80% (v/v) methanol), a solution prepared by simultaneous acidic hydrolysis and extraction with acetone, followed by liquid-liquid extraction with ethyl acetate, houseleek decoction and leaf juice, as well as fractions of the 80% methanolic extract were studied. For comparison solutions of trolox, ascorbic acid, gallic acid, caffeic acid, chlorogenic acid, kaempferol, quercetin, rutin and myricitrin standards were evaluated with both free radicals. 20 μ L of each sample in five different concentrations, in three parallels were introduced to 2.5 mL of the solution containing the free radicals. The mixture was vortexed and allowed to react for six minutes. Absorbance changes were recorded at 30, 40, 50, 60, 90, 120, 150, 180, 210, 240, 300 and 360 seconds after combination. The decrease in absorbance was recorded against a blank sample (ethanol and methanol for ABTS and DPPH, respectively). The final absorbance (at $t = 360$ sec) was extrapolated by numerically solving the simplest possible reaction kinetics model. The percentage of radical scavenging activity was calculated as: Inhibition % = $(A_b - A_f) / A_f \times 100$, where A_b = absorbance of the blank solution containing solely the free radical and A_f = absorbance of the free radical solution after reacting with the sample. Percentage of the free radicals reduced against concentration (μ g/mL) of each sample was plotted, and half maximal inhibitory concentration (IC_{50} value as μ g/mL), that is the concentration of antioxidants required to decrease the initial free radical concentration by 50% was determined.

Data were analyzed at significance level $p < 0.05$. The results are expressed as mean values and standard deviation (S.D.). For *S. tectorum* extracts antiradical activity data were plotted as IC_{50}^{-1} (mL/ μ g) against total polyphenol contents (g/100 g). In order to evaluate correlation, Student's t-test was applied. Comparison between *S. tectorum* 80% (v/v) methanolic extract and its fractions was made by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc HSD test. Differences with $p < 0.05$ and lower between experimental groups were considered statistically significant.

4.5.2. Peroxynitrite scavenging activity

Peroxynitrite scavenging activity was determined in collaboration with Compound Profiling Laboratory, Gedeon Richter Plc., in pyrogallol red bleaching test, an *in vitro*

colorimetric assay developed by Bavaloine and Geletii [179] and adopted by Könczöl et al. [180]. Test solutions and samples were prepared in 96-well polypropylene plates with a total volume of 0.4 mL. Sample mixtures were prepared by mixing 200 μ L solution of pyrogallol red in glycine buffer (100 mM) pH 7.00, 50 μ L peroxyntirite solution (2 mM) and 150 μ L sample solution in five different concentrations. Control solutions with no antioxidants (200 μ L pyrogallol red solution, 50 μ L peroxyntirite solution, 150 μ L methanol; 0 % inhibition) and blank mixtures without ONOO⁻ (200 μ L pyrogallol red solution, 50 μ L water, 150 μ L methanol; 100% inhibition) were also evaluated. Peroxyntirite was synthesized daily by preparing and cooling on ice a solution containing 0.5 M NaNO₂ and 0.5 M H₂O₂, then 1 mL of pre-cooled 1 M HCl and 1 mL of pre-cooled NaOH was injected simultaneously into a rapidly stirred solution. The concentration of the resulting ONOO⁻ solution was determined by measuring the absorbance at 302 nm. The stock solution was diluted with 0.5% NaOH and kept frozen in dark before use.

The mixture plate containing test, control and blank solutions in triplicate was mixed thoroughly and analyzed in a Thermo Multiskan Microplate spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) with a 96-well plate reader, detection was performed at 542 nm. Inhibition percentage was calculated as follows: Inhibition % = $PR / PR_0 \times 100$, where PR = the measured pyrogallol red concentration of the sample after reaction with ONOO⁻ and PR₀ = initial pyrogallol red concentration (blank). Peroxyntirite scavenging activity was characterized by IC₅₀ values (μ g/mL) calculated as the concentration of antioxidants required to protect 50% pyrogallol red against 500 μ M ONOO⁻ by Prism 4 for Windows (Graph Pad Software, San Diego, USA) after sigmoidal dose-response curve fitting. *S. tectorum* leaf juice, as well as extracts prepared with 70% (v/v) ethanol and 80% (v/v) methanol were studied. For comparison solutions of gallic acid, caffeic acid, kaempferol, quercetin and rutin standards were evaluated.

4.6. HPLC and LC-MS/MS experiments – Instrumentation

Chromatographic separation was carried out with an ABL&E-Jasco system (ABL&E-Jasco Kft., Budapest, Hungary) equipped with a Jasco PU-980 pump, Jasco LG-980-02

gradient unit, ERC-3113 degasser, Jasco-UV-975 UV-VIS detector and Rheodyne 7725t (20 μ L) injector. Identification of the components was achieved by the comparison of their retention times and UV spectra with those of reference compounds. The Borwin 1.21.04. (JMBS Developpements, Grenoble, France) software was used for data acquisition, qualitative and quantitative analyses.

Chromatographic separation and on-line mass spectral analyses were performed with an Agilent 6410B triple quadrupole equipped with an electrospray ionization source (ESI) (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent 1100 HPLC system (G1379A degasser, G1312A binary gradient pump, G1329A autosampler, G1316A column thermostat and G1315C diode array detector) (Agilent Technologies, Waldbronn, Germany). The Masshunter B.01.03 software was used for data acquisition, for qualitative and quantitative analyses. For unambiguous identification retention times, UV and mass spectral data of compounds were compared to literature data and to those of authentic standards, where available.

4.7. HPLC and LC-MS/MS experiments – Qualitative analyses

4.7.1. HPLC evaluation of *S. tectorum* extracts

Houseleek extracts were separated on a Supelcosil LC18 (Supelco, Bellefonte, PA, USA) (250 x 4.6 mm I.D., 5.0 μ m particle size) column, maintained at ambient temperature. Eluent A was acetic acid in water (2.5%, v/v), eluent B was methanol. For the investigation of the different extracts, the following eluent mixtures were used at a flow rate of 1.0 mL/min:

80% (v/v) methanolic extract:

0-20 min 80:20 \rightarrow 30:70 (A:B, v/v)

20-22 min 30:70 \rightarrow 0:100 (A:B, v/v)

22-24 min 0:100 (A:B, v/v)

24-25 min 0:100 \rightarrow 80:20 (A:B, v/v)

Fractions of the 80% (v/v) methanolic extract:

0-30 min 85:15 → 0:100 (A:B, v/v)

30-34 min 0:100 (A:B, v/v)

34-35 min 0:100 → 85:15 (A:B, v/v)

Detection wavelengths were 260 and 340 nm, UV spectra of characteristic compounds were recorded between 200 and 600 nm.

4.7.2. LC-MS/MS evaluation of *S. tectorum* 80% (v/v) methanolic extract

For the chromatographic separation the same column maintained at 25 °C, as well as the same eluents and gradient program at a flow rate of 1.0 mL/min were used as for LC evaluation of the extract (see section **4.7.1. HPLC evaluation of *S. tectorum* extracts**). For the reduction of the flow rate a postcolumn splitter (60-40%, waste-MS) was applied. Chromatograms were acquired at 260 and 340 nm, UV spectra of characteristic compounds were recorded between 200 and 450 nm. Injection volume was 5 µL.

ESI conditions were as follows:

- Ionization mode: negative
- Temperature: 350 °C
- Nebulizer pressure: 45 psi (N₂)
- Drying gas flow rate: 9 L/min (N₂)
- Fragmentor voltage: 120 V
- Capillary voltage: 4000 V
- Scan range: *m/z* 50–1000 dalton (1 scan / sec)
- Collision energy: was changed between 8–45 eV, according to differences in molecule structures (high purity nitrogen was used as collision gas)

4.7.3. LC-MS/MS evaluation of fractions of *S. tectorum* 80% (v/v) methanolic extract

For the chromatographic separation the same column maintained at 25 °C, as well as the same eluents and gradient program at a flow rate of 1.0 mL/min were used as for HPLC

evaluation of the *S. tectorum* 80% (v/v) methanolic extract (see section **4.7.1. HPLC evaluation of *S. tectorum* extracts**). For the reduction of the flow rate a postcolumn splitter (70-30%, waste-MS) was applied. Chromatograms were acquired at 260 and 340 nm, UV spectra of characteristic compounds were recorded between 200 and 600 nm. Injection volume was 15 μ L.

ESI conditions were as follows:

- Ionization mode: negative
- Temperature: 350 °C
- Nebulizer pressure: 40 psi (N₂)
- Drying gas flow rate: 9 L/min (N₂)
- Fragmentor voltage: 135 V
- Capillary voltage: 4000 V
- Scan range: *m/z* 50–1000 dalton (1 scan / sec)
- Collision energy: was changed between 10–50 eV, according to differences in molecule structures (high purity nitrogen was used as collision gas)

4.7.4. LC-MS/MS evaluation of *S. tectorum* leaf juice

The houseleek leaf juice was separated on a Zorbax SB C18 (Agilent Technologies, Santa Clara, CA, USA) (150 x 3.0 mm I.D., 3.5 μ m particle size) column, maintained at 25 °C. Eluent A was formic acid in water (1%, v/v), eluent B was acetonitrile. The following gradient program was applied, at a flow rate of 0.4 mL/min:

- 0-15 min 90:10 → 45:55 (A:B, v/v)
- 15-16 min 45:55 → 0:100 (A:B, v/v)
- 16-19 min 0:100 (A:B, v/v)
- 19-20 min 0:100 → 90:10 (A:B, v/v)

Chromatograms were acquired at 266 and 350 nm, UV spectra of characteristic compounds were recorded between 200 and 600 nm. Injection volume was 5 μ L.

ESI conditions were as follows:

- Ionization mode: negative
- Temperature: 350 °C

- Nebulizer pressure: 45 psi (N₂)
- Drying gas flow rate: 9 L/min (N₂)
- Fragmentor voltage: 250 V
- Capillary voltage: 3500 V
- Scan range: m/z 50–1000 dalton (1 scan / sec)
- Collision energy: was changed between 5–45 eV, according to the differences in molecule structures (high purity nitrogen was used as collision gas)

4.7.5. LC-MS/MS evaluation of *S. tectorum* ethanolic extract

The houseleek ethanolic extract was separated on a Zorbax SB C18 (Agilent Technologies, Santa Clara, CA, USA) (150 x 3.0 mm I.D., 3.5 μ m particle size) column, maintained at 25 °C. Eluent A was formic acid in water (0.5%, v/v), eluent B was acetonitrile. The following gradient program was applied, at a flow rate of 0.5 mL/min:

- 0-15 min 90:10 → 50:50 (A:B, v/v)
- 15-17 min 50:50 → 0:100 (A:B, v/v)
- 17-18 min 0:100 (A:B, v/v)
- 18-19 min 0:100 → 90:10 (A:B, v/v)

Chromatograms were acquired at 260 and 340 nm, UV spectra of characteristic compounds were recorded between 200 and 600 nm. Injection volume was 4 μ L.

ESI conditions were as follows:

- Ionization mode: negative
- Temperature: 350 °C
- Nebulizer pressure: 40 psi (N₂)
- Drying gas flow rate: 9 L/min (N₂)
- Fragmentor voltage: 120 V
- Capillary voltage: 4000 V
- Scan range: m/z 50–1000 dalton (1 scan / sec)
- Collision energy: was changed between 15–50 eV, according to the differences in molecule structures (high purity nitrogen was used as collision gas)

4.7.6. LC-MS/MS evaluation of *C. avellana* extracts

C. avellana extracts were separated on a Zorbax SB C18 (150 x 3.0 mm I.D., 3.5 µm particle size) column, maintained at 25 °C.

Eluent A was acetic acid in water (0.2%, v/v), eluent B was methanol. The following gradient program was applied, at a flow rate of 0.3 mL/min:

0-20 min 70:30 → 0:100 (A:B, v/v)

20-22 min 0:100 (A:B, v/v)

22-23 min 0:100 → 70:30 (A:B, v/v)

Chromatograms were acquired at 280 and 340 nm, UV spectra of characteristic compounds were recorded between 200 and 600 nm. Injection volume was 10 µL.

ESI conditions were as follows:

- Ionization mode: negative
- Temperature: 350 °C
- Nebulizer pressure: 40 psi (N₂)
- Drying gas flow rate: 9 L/min (N₂)
- Fragmentor voltage: 120 V
- Capillary voltage: 3500 V
- Scan range: *m/z* 50–1000 dalton (1 scan / sec)
- Collision energy: was changed between 10–35 eV, according to differences in molecule structures (high purity nitrogen was used as collision gas)

4.8. HPLC and LC-MS/MS experiments – Quantitative analyses

4.8.1. Determination of hydroxycinnamic acid derivatives in *S. tectorum* leaf juice by an HPLC-UV method

Hydroxycinnamic acids in houseleek leaf juice were separated on a Supelcosil LC18 (250 x 4.6 mm I.D., 5.0 µm particle size) column, maintained at ambient temperature. Analyses were performed at detection wavelength of 320 nm. Eluent A was formic acid

in water (0.1%, v/v), eluent B was methanol. The following gradient program was applied, at a flow rate of 1.0 mL/min:

0-14 min 90:10 → 85:15 (A:B, v/v)
14.0-14.1 min 85:15 → 60:40 (A:B, v/v)
14.1-20.0 min 60:40 → 40:60 (A:B, v/v)
20.0-21.0 min 40:60 → 0:100 (A:B, v/v)
21.0-24.0 min 0:100 (A:B, v/v)
24.0-25.0 min 0:100 → 90:10 (A:B, v/v).

4.8.1.1. Validation - Calibration plot, precision and quality control samples

For the quantitative determination the external standard calibration method was applied. Standard solutions for the calibration were prepared by use of authentic reference compounds and 70% (v/v) HPLC grade methanol at 1, 5, 25, 50 and 150 µg/mL for caffeic acid and rosmarinic acid and at 2, 10, 50, 100 and 300 µg/mL for chlorogenic acid. All analyses were performed in triplicate. Calibration plot was constructed by plotting peak areas against corresponding concentrations. Slope, intercept and correlation coefficient were determined by least squares polynomial regression analysis. Quality control samples were prepared at concentrations of 1, 25 and 150 µg/mL for caffeic acid and rosmarinic acid, and at concentrations of 2, 50 and 300 µg/mL for chlorogenic acid. These were used to determine both the intra-day and inter-day precision (low, mid and high concentrations of the standard in three parallel runs on the same day and on three successive days, respectively). Retention time repeatability was checked with six successive runs of *S. tectorum* extract. Blank sample (pure solvent) was analyzed to check the occurrence of any impurity or co-elution.

4.8.1.2. Method recovery test

0.215 g lyophilized *S. tectorum* extract was spiked with 260 µL of the standard solutions (caffeic acid concentration: 150 µg/mL; chlorogenic acid concentration: 300 µg/mL; rosmarinic acid concentration: 150 µg/mL) and dissolved in 15.0 mL 80% (v/v) super

gradient grade methanol, in three parallels. Further sample preparation steps were the same as described above (see in section **4.3.4. Preparation of *S. tectorum* leaf juice**). Prior to injection the purified samples were evaporated to dryness at 60 °C under reduced pressure and redissolved in 1.0 mL 70% (v/v) supergradient grade methanol. Method recovery was tested in a concentration range to match with that of the target analyte in the plant sample. Recovery (R) was calculated for each compound as $R = 100 (C_{\text{found}} - C_{\text{initial}}) / C_{\text{added}}$, where C_{found} = measured concentration in the fortified sample, C_{initial} = initial concentration in the sample, C_{added} = concentration in the standard solution used.

4.8.2. Determination of kaempferol glycosides in *S. tectorum* leaf juice by HPLC-DAD and LC-ESI-MS/MS methods

4.8.2.1. Isolation of the flavonoid compounds of interest

Quantitation was performed with the external standard calibration method using the three isolated flavonol glycoside compounds as standards. Isolation was carried out by separation of 80% (v/v) methanolic *S. tectorum* extract with consecutive column chromatographic methods. 50 g lyophilized leaf sample was extracted with 1000 mL 80% (v/v) methanol in a Soxhlet apparatus. The extract was evaporated to dryness at 60 °C in a rotary evaporator and redissolved in 15 mL methanol. The concentrated extract was introduced to 5 g Silicagel and during evaporation of the solvent permanently homogenized. Suspension of Silicagel 60 (0.063–0.200 mm) (132 g) with chloroform (250 mL) was prepared and used as stationary phase. Before transferring the sample, the suspension was allowed to settle, final dimensions of the bed were: length: 335 mm, diameter: 31 mm. The sample adsorbed on Silicagel was layered on the top of the stationary phase and eluted with 100–100 mL of the following eluent mixtures (proportions by volume):

	Chlorofom	Methanol	Water
1.	200.0	10.0	0.0
2.	90.0	10.0	1.0
3.	84.5	14.0	1.5
4.	73.0	24.5	2.5
5.	64.5	32.0	3.5
6.	57.5	38.5	4.0
7.	53.5	42.0	4.5
8.	47.5	42.0	10.5
9.	47.5	42.0	10.5
10.	43.0	45.0	12.0
11.	43.0	45.0	12.0
12.	0.0	100.0	0.0

Fractions of 100 mL volume were collected. Fraction 12 contained kaempferol 3-*O*-rhamnosyl-glucoside-7-*O*-rhamnoside in the highest concentration, hence for isolation, it was further separated by Sephadex column chromatography. Suspension of Sephadex LH20 (25–100 μm) (Sigma-Aldrich) with 25% (v/v) methanol was prepared, filled in a glass column and allowed to settle (final dimensions were: length: 310 mm, diameter: 16 mm). Concentrated solution of fraction 12 was carried on top of the Sephadex column and successively eluted with the following eluent mixtures:

- 20 mL 25% (v/v) methanol
- 40 mL 40% (v/v) methanol
- 50 mL 60% (v/v) methanol
- 50 mL 75% (v/v) methanol

Fractions of 2.0 mL were collected. Fractions 42–45 contained exclusively kaempferol 3-*O*-rhamnosyl-glucoside-7-*O*-rhamnoside, the main flavonoid compound of *S. tectorum*. In order to separate the analyte from polysaccharides, water soluble organic acids and other matrix constituents, the fractions were purified by solid phase extraction. The Supelco DSC Phenyl SPE cartridges (500 mg/3 mL) (Supelco) were activated with 3 mL methanol, then with 3 mL water. After the addition of the united

and concentrated fractions 42–45, matrix compounds were washed from the SPE cartridge with 4 x 750 μ L water, then the analyte was eluted with 8 x 750 μ L super gradient grade methanol.

Fractions 8–10 of the Silicagel column chromatography contained kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside and kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside among other flavonoids, thus fractions were combined and further separated by Sephadex column chromatography as referred to above. Fractions 62–69 contained kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside in high concentration, however it was not the sole constituent, hence for isolation the fractions were further separated by polyamide column chromatography. The concentrated fractions 62–69 from Sephadex LH20 column chromatography were introduced to 0.15 g polyamide and during evaporation of the solvent permanently homogenized. Suspension of MN polyamide SC-9 (ICN Biomedicals GmbH, Eschwege, Germany) (6.8 g) with 25% (v/v) methanol (55 mL) was prepared and filled in a glass column. Before transferring the sample, the suspension was allowed to settle, final dimensions were: length: 200 mm, diameter: 8 mm. The combined fractions adsorbed on polyamide were layered on the top of the stationary phase and successively eluted with the following eluent mixtures:

- 25 mL 25% (v/v) methanol,
- 30 mL 30% (v/v) methanol,
- 10 mL 35% (v/v) methanol
- 20 mL 40% (v/v) methanol

Fractions of 1.0 mL were collected. Fractions 62–64 contained exclusively kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside. In order to separate the analyte from matrix constituents, the fractions were purified by solid phase extraction using Supelco DSC Phenyl SPE cartridges, as previously described.

Fractions 54–58 from Sephadex column chromatography contained kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside in high concentration, however it was not the sole constituent, thus for isolation the fractions were combined and further separated by polyamide column chromatography, as described above. The combined fractions were eluted with the following eluent mixtures:

- 15 mL 25% (v/v) methanol,
- 10 mL 35% (v/v) methanol
- 15 mL 45% (v/v) methanol

Kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside was present in fractions 29–34 in the highest concentration, thus those were combined and purified by solid phase extraction using phenyl SPE cartridges, as referred to above.

4.8.2.2. NMR spectroscopy

Structural identification of the isolated kaempferol triglycoside was performed by NMR spectroscopy. NMR experiments were carried out in collaboration with the Department of Pharmaceutical Chemistry, Semmelweis University, on a 600 MHz Varian DDR NMR spectrometer (Palo Alto, CA, USA) equipped with a dual 5 mm inverse-detection gradient (IDPFG) probehead. Standard pulse sequences and processing routines available in VnmrJ 2.2C / Chempack 4.0 were used for structure identification. The probe temperature was maintained at 298 K and standard 5 mm NMR tubes were used. 0.9 mg sample was dissolved in 600 μ L CD₃OD (Sigma-Aldrich).

4.8.2.3. HPLC-MS/MS conditions

The same LC-MS conditions were applied as described above in section **4.7.4. LC-MS/MS evaluation of *S. tectorum* leaf juice**. Before quantitation both fragmentor voltage and collision energy were optimized with the use of the sample extract by parameter ramping, from 70 to 250 V, with steps of 10 V and from 5 eV up to 50 eV, with steps of 5 eV, respectively. Optimal settings for quantitation of kaempferol 3-*O*-rhamnosyl-glucoside-7-*O*-rhamnoside, kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside and kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside for fragmentor voltage were 250 V, 210 V and 240 V, respectively and for collision energy 35 eV, 30 eV and 30 eV, respectively. Quantitation was achieved in selected reaction monitoring (SRM) mode. The fragment ion m/z 593 (transition m/z 739 \rightarrow m/z 593) was chosen as quantifier ion for kaempferol 3-*O*-rhamnosyl-glucoside-7-*O*-rhamnoside, the fragment

ion m/z 285 (transition m/z 577 \rightarrow m/z 285) was chosen as quantifier ion for kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside and fragment ion m/z 285 (transition m/z 593 \rightarrow m/z 285) was chosen for kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside.

4.8.2.4. Validation - Calibration plot, precision and quality control samples

Quantitation was performed with the external standard calibration method. Quantity of the three characteristic flavonol glycosides from *S. tectorum* leaf juice was determined by SRM chromatograms, while that of the main flavonoid compound was determined also by UV chromatograms detected at 350 nm. Standard solutions for the calibration were prepared by use of the isolated kaempferol 3-*O*-rhamnosyl-glucoside-7-*O*-rhamnoside, kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside and kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside. Purity of the isolated compounds was surveyed thoroughly by HPLC-DAD-MS/MS, identity of the main flavonol compound was assayed by NMR spectroscopy. Standard solutions were prepared for kaempferol 3-*O*-rhamnosyl-glucoside-7-*O*-rhamnoside and kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside at 5, 25, 100, 250 and 500 $\mu\text{g/mL}$ with 70% (v/v) methanol of supergradient grade. Since purity of the isolated kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside was 54.7%, by preparing standard solutions a 0.547-fold correction to the concentration values was suited. Standard solutions were prepared at 2, 10, 40, 100 and 200 $\mu\text{g/mL}$ with 70% (v/v) methanol of supergradient grade. Each standard solution was prepared in triplicate and injected once. Calibration plot was constructed by plotting peak areas against corresponding concentrations. Slope, intercept and correlation coefficient were determined by least squares polynomial regression analysis. Quality control samples were prepared at concentrations of 5, 100 and 500 $\mu\text{g/mL}$ for kaempferol 3-*O*-rhamnosyl-glucoside-7-*O*-rhamnoside and kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside, and at concentrations of 2, 40 and 200 $\mu\text{g/mL}$ for kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside. These were used to determine both the intra-day and inter-day precision (low, mid and high concentrations of the standard in three parallel runs on the same day and on three successive days, respectively). Retention time repeatability was checked with six successive runs of the

houseleek extract. Blank sample (pure solvent) was analyzed to check the occurrence of any impurity or co-elution.

4.8.2.5. Method recovery test

0.135 g lyophilized *S. tectorum* leaf juice was spiked with solutions of the isolated standards (kaempferol 3-*O*-rhamnosyl-glucoside-7-*O*-rhamnoside: 120 μ L, concentration: 833.3 μ g/mL; kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside: 100 μ L, concentration: 1000 μ g/mL; kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside: 50 μ L, concentration: 800 μ g/mL) and dissolved in 15.0 mL 80% (v/v) super gradient grade methanol, in three parallels. Further sample preparation steps were the same as described above (see in text, section **4.3.4. Preparation of *S.tectorum* leaf juice**). Prior to injection, the purified samples were evaporated to dryness at 60 °C under reduced pressure and redissolved in 2.5 mL 70% (v/v) supergradient grade methanol. Method recovery was tested in a concentration range to match with that of the target analyte in the plant sample. Recovery (R) was calculated for each compound as $R = 100 (C_{\text{found}} - C_{\text{initial}}) / C_{\text{added}}$, where C_{found} = measured concentration in the fortified sample, C_{initial} = initial concentration in the sample, C_{added} = concentration in the standard solution used.

5. RESULTS

5.1. Quantitative phytochemical analyses

In order to obtain preliminary information on phytochemical constitution of *S. tectorum*, flavonoid, total polyphenol, tannin, proanthocyanidin and anthocyanin content was determined from lyophilized leaf samples according to the methods of the Ph. Hg. VIII. [175]. Results (expressed as mean values and standard deviation) are as follows: flavonoids: 0.94 ± 0.07 g/100 g (expressed as hyperoside), total polyphenols: 1.56 ± 0.08 g/100 g (expressed as pyrogallol), tannins: 0.18 ± 0.03 g/100 g (expressed as pyrogallol), proanthocyanidins: 0.26 ± 0.03 g/100 g (expressed as cyanidin chloride) and anthocyanins: 0.10 ± 0.01 g/100 g (expressed as cyanidin-3-glucoside chloride). Related to radical scavenging assays total polyphenol contents (expressed as pyrogallol) of *S. tectorum* extracts prepared with solvents of different polarity were determined (results are shown in Table 3.).

Table 3. Total polyphenol content of *S. tectorum* extracts prepared with solvents of different polarity (Data are mean \pm S.D.).

	Total polyphenol content (expressed as pyrogallol) (g/100 g dried extract \pm S.D.)
Houseleek flavonoid aglycone extract	74.36 ± 3.91
Houseleek ethanol extract	51.40 ± 3.68
Houseleek 80% (v/v) methanol extract	27.42 ± 1.44
Houseleek 70% (v/v) ethanol extract	19.55 ± 1.01
Houseleek methanol extract	14.10 ± 0.78
Houseleek decoction	14.07 ± 0.72
Houseleek acetone extract	12.98 ± 0.67
Houseleek chloroform extract	4.98 ± 0.31
Houseleek leaf juice	4.01 ± 0.29

In addition, flavonoid and total hydroxycinnamic acid derivative content of lyophilized *S. tectorum* leaf juice was evaluated. Results (expressed as mean values and standard deviation) are as follows: flavonoids: 0.19 ± 0.01 g/100 g dried sample (expressed as hyperoside) and total hydroxycinnamic acid derivatives: 0.56 ± 0.03 g/100 g dried sample (expressed as rosmarinic acid).

5.2. Antioxidant activity assays

5.2.1. DPPH and ABTS scavenging activity

Antioxidant activity of *S. tectorum* extracts prepared with solvents of different polarity was determined. Two different *in vitro* tests, ABTS^{•+} radical scavenging capacity and DPPH[•] radical test were used. Radical scavenger activities were compared to that of reference compounds revealing the samples, result are shown in Table 4. and Table 5.

Table 4. DPPH and ABTS scavenging activity of *S. tectorum* extracts prepared with solvents of different polarity (Data are mean \pm S.D.).

	ABTS IC ₅₀ ($\mu\text{g/mL}$) \pm S.D.	DPPH IC ₅₀ ($\mu\text{g/mL}$) \pm S.D.
Houseleek flavonoid aglycone extract	7.5 ± 0.5	12.9 ± 0.7
Houseleek ethanol extract	12.9 ± 1.4	21.2 ± 1.9
Houseleek 80% (v/v) methanol extract	14.6 ± 0.6	34.9 ± 1.8
Houseleek 70% (v/v) ethanol extract	15.3 ± 0.9	74.5 ± 3.6
Houseleek methanol extract	37.3 ± 3.1	78.6 ± 6.4
Houseleek water extract	35.5 ± 1.7	159.3 ± 10.2
Houseleek chloroform extract	176.3 ± 14.1	2073.0 ± 200.0
Houseleek acetone extract	670.3 ± 63.0	109.5 ± 9.6
Houseleek leaf juice	71.4 ± 3.1	438.0 ± 16.2

Table 5. ABTS and DPPH scavenging activity of reference compounds

(Data are mean \pm S.D.).

	ABTS	DPPH
	IC ₅₀ ($\mu\text{g/mL}$) \pm S.D.	IC ₅₀ ($\mu\text{g/mL}$) \pm S.D.
Gallic acid	0.9 \pm 0.1	2.7 \pm 0.2
Quercetin	1.2 \pm 0.1	3.4 \pm 0.2
Ascorbic acid	4.7 \pm 0.2	3.9 \pm 0.2
Myricitrin	4.7 \pm 0.3	4.8 \pm 0.2
Caffeic acid	1.9 \pm 0.1	5.1 \pm 0.2
Trolox	2.1 \pm 0.1	5.3 \pm 0.2
Chlorogenic acid	4.7 \pm 0.2	7.4 \pm 0.3
Rutin	3.2 \pm 0.1	7.3 \pm 0.3
Kaempferol	8.4 \pm 0.2	19.1 \pm 0.7

Antioxidant activity of houseleek extracts was higher in the assay containing ABTS^{•+} radical as compared to that containing DPPH[•]. In addition, total polyphenol contents of *S. tectorum* extracts were determined (results are shown in Table 3.). Antioxidant activity data were plotted as IC₅₀⁻¹ (mL/ μg) against total polyphenol contents and correlation was evaluated between the variables. The correlation of total polyphenol content and radical scavenging activity was considered as highly significant for the assay containing DPPH[•] as well as ABTS^{•+} radicals.

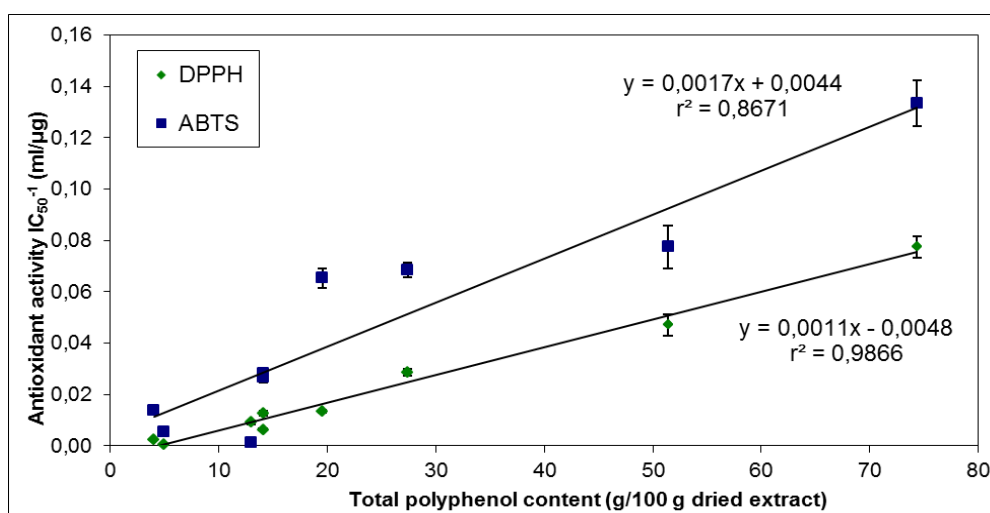


Fig. 15. Correlation between total polyphenol content (g/100 g) and radical (DPPH[•] and ABTS^{•+}) scavenging activity (IC₅₀⁻¹; mL/ μg) of *S. tectorum* extracts.

Regression coefficient, p -value and linear regression equation were as follows: $r^2 = 0.8671$, $p < 0.005$, $y = 0.00017x + 0.0044$ and $r^2 = 0.9866$, $p < 0.005$, $y = 0.0011x - 0.0048$ for DPPH and ABTS, respectively. Results are shown in Fig. 15.

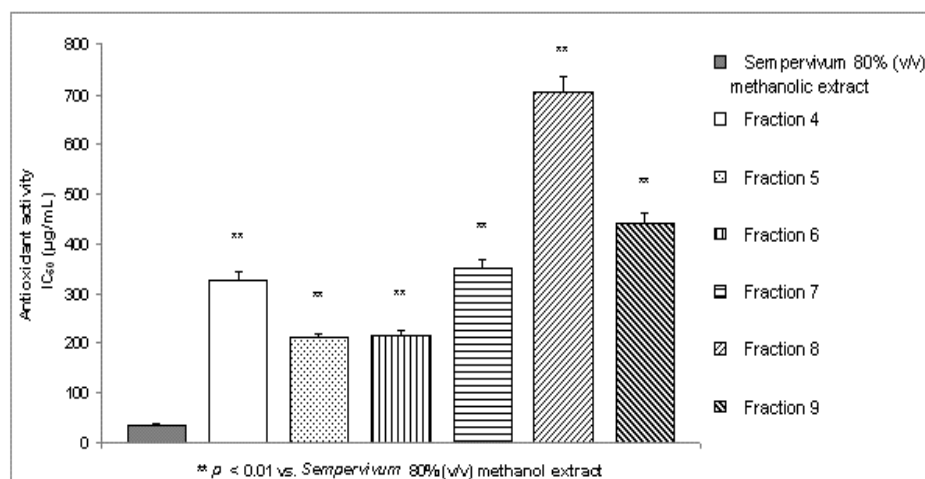


Fig. 16. DPPH scavenging activity of *S. tectorum* 80% (v/v) methanolic extract and its fractions. Values are means \pm S.D. ** $p < 0.01$ compared with the whole extract.

Table 6. DPPH scavenging activity (as IC₅₀, µg/mL) of *S. tectorum* 80% (v/v) methanolic extract and its fractions. Values are means \pm S.D.

Sample	DPPH
	IC ₅₀ (µg/mL) \pm S.D.
Fraction 1	> 5000
Fraction 2	> 5000
Fraction 3	> 5000
Fraction 4	327.7 \pm 14.6
Fraction 5	209.3 \pm 8.9
Fraction 6	215.0 \pm 10.4
Fraction 7	351.4 \pm 14.8
Fraction 8	704.9 \pm 30.4
Fraction 9	439.4 \pm 21.0
Houseleek 80% (v/v) methanol extract	34.9 \pm 1.8

Antioxidant activity of fractionated *S. tectorum* 80% (v/v) methanolic extract in DPPH scavenging assay was also determined, comparison between fractions and the whole extract was made. Antioxidant activity for fractions 1-3 was: IC₅₀ > 5000 µg/mL, while

fractions 4-9 exhibited DPPH scavenging effect. Antioxidant activity (as IC_{50} , $\mu\text{g/mL}$) of all fractions was significantly lower as compared to the whole 80% (v/v) methanolic extract, results are shown in Fig. 16. and Table 6.

Furthermore, an estimated total radical scavenger value (as IC_{50}^{-1} , $\text{mL}/\mu\text{g}$) of all fractions was calculated and compared to that of the whole extract. The fractions, when they were present together in the same system, had stronger antioxidant activity as compared to the effect expected from a simple addition of the individual effects of the fractions (results are not shown), accordingly, a synergistic antioxidative effect between the fractions was presumed [182].

5.2.2. Peroxynitrite scavenging activity

Peroxynitrite scavenging activity of houseleek leaf juice and extracts prepared with 70% (v/v) ethanol and 80% (v/v) methanol was determined by a colorimetric assay based on pyrogallol red bleaching [179]. Peroxynitrite scavenging activity of the extracts was compared to that of reference compounds, result expressed as IC_{50} ($\mu\text{g/mL}$) values are shown in Table 7.

Table 7. Peroxynitrite scavenging activity (as IC_{50} , $\mu\text{g/mL}$) of *S. tectorum* extracts and reference compounds. Values are means \pm S.D.

Extracts and reference compounds	IC_{50} ($\mu\text{g/mL}$) \pm S.D.
Houseleek leaf juice	> 15000
Houseleek 70% (v/v) ethanolic extract	10708.7 \pm 49.8
Houseleek 80% (v/v) methanolic extract	7985.8 \pm 40.1
Rutin	3467.4 \pm 283.9
Caffeic acid	269.4 \pm 21.3
Kaempferol	108.3 \pm 6.9
Gallic acid	99.6 \pm 201
Quercetin	55.9 \pm 10.0

Peroxynitrite scavenging activity of houseleek leaf juice and that of extracts prepared with solvents of better selectivity for phenolics was notably lower as compared to the standard solutions. As the only exception, the scavenging activity of the 80% (v/v) methanolic extract was of the same order as that of rutin.

5.3. Qualitative HPLC and LC-MS/MS analyses

5.3.1. *S. tectorum* 80% (v/v) methanolic extract

Our further objective was high-performance liquid chromatographic evaluation of *S. tectorum* 80% (v/v) methanolic extract. Absorption maxima of distinctive UV spectra of flavonol compounds were characteristic of kaempferol and quercetin *O*-glycosides [49, 183]. LC-DAD profile of *S. tectorum* 80% (v/v) methanolic extract is shown in Fig. 17. Detailed structural survey of flavonol glycosides required tandem mass spectrometric analyses.

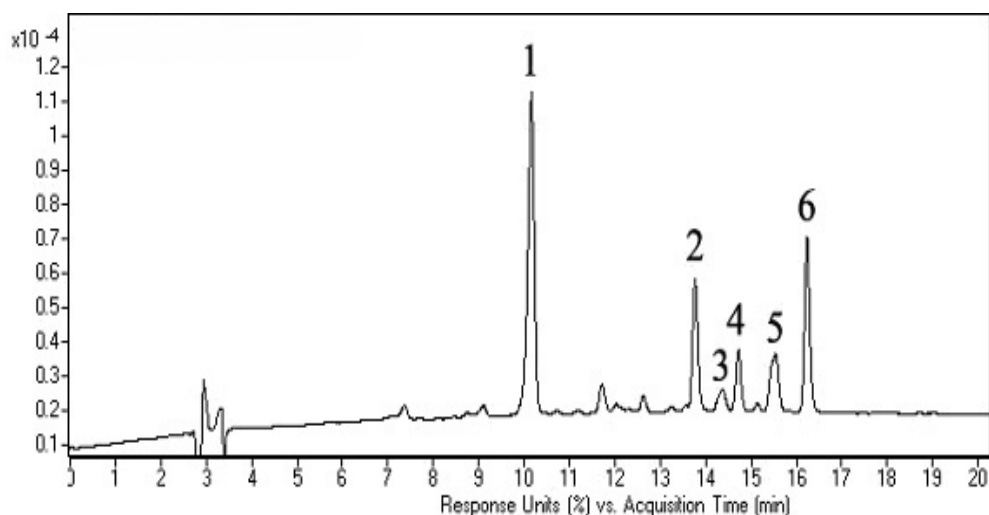


Fig. 17. HPLC-DAD profile of *S. tectorum* 80% (v/v) methanolic extract. [Absorbance vs. time (min)]. Numbering of peaks refers to data shown in Table 8. Chromatographic conditions see in section **4.7.1. HPLC evaluation of *S. tectorum* extracts.**

ESI-MS full scan mode analyses were performed, in order to identify the deprotonated molecular ions $[M-H]^-$, followed by ESI-MS/MS product ion experiments in negative ionization mode using the deprotonated molecular ion as precursor to study the fragmentation of the compounds. Different collision energy values were applied to each constituent, in order to acquire mass spectra with various fragmentation degrees from the precursor ion thus, obtain as much structural information as it was possible.

In the 80% (v/v) methanolic extract of *S. tectorum* rutin and six kaempferol glycosides of different sugar composition have been described or tentatively characterized by LC-MS/MS. For unambiguous identification retention times, UV spectra and fragmentation patterns of kaempferol and quercetin were compared to those of standard compounds and literature data [80]. Results are shown in Table 8.

Table 8. Tentative identification of flavonol *O*-glycosides in *S. tectorum* leaf 80% (v/v) methanolic extract.

No.	t_R (min)	λ_{max} (nm)	[M-H] ⁻ (<i>m/z</i>)	MS/MS (<i>m/z</i>)	Tentative identification of compounds ^a
1	9.6	266, 350	739	593, 430, 285, 284, 283, 255	K 3- <i>O</i> -desoxyhexosyl-hexoside- 7- <i>O</i> -desoxyhexoside
2	12.1	266, 350	593	430, 285, 284, 283	K 3- <i>O</i> -hexosyl- 7- <i>O</i> -desoxyhexoside
3	12.4	268, 352	609	300, 271, 255	Q 3- <i>O</i> -rutinoside
4	12.8	266	871	725, 563, 430, 284, 283	K 3- <i>O</i> -desoxyhexosyl-pentoside- 7- <i>O</i> -desoxyhexosyl-hexoside
5a	13.6	266, 346	709	563, 430, 284, 283	K 3- <i>O</i> -desoxyhexosyl-pentoside- 7- <i>O</i> -desoxyhexoside
5b	13.6	266, 346	577	430, 285, 284, 283	K 3- <i>O</i> -desoxyhexoside- 7- <i>O</i> -desoxyhexoside
6	14.1	266, 350	447	284, 255, 227	K 3- <i>O</i> -hexoside

^a Peak numbers and retention times (t_R) refer to HPLC-DAD chromatogram shown in Fig. 17. Abbreviations: K: kaempferol, Q: quercetin.

5.3.2. Fractions of *S. tectorum* 80% (v/v) methanolic extract

Fractions 4–9 of houseleek 80% (v/v) methanolic extract, obtained by Silicagel column chromatography were evaluated by HPLC. LC-DAD profile of fractions is shown in Fig. 18., chromatograms were detected at 260 nm, characteristic of hydroxybenzoic acid derivatives and at 340 nm, representative for flavonoids. According to UV spectral data,

in fractions eluted later from Silicagel column were several flavonoid compounds detected, while fractions eluted foremost contained numerous hydroxybenzoic and hydroxycinnamic acid derivatives. Structural characterization of the compounds required tandem mass spectrometric analyses.

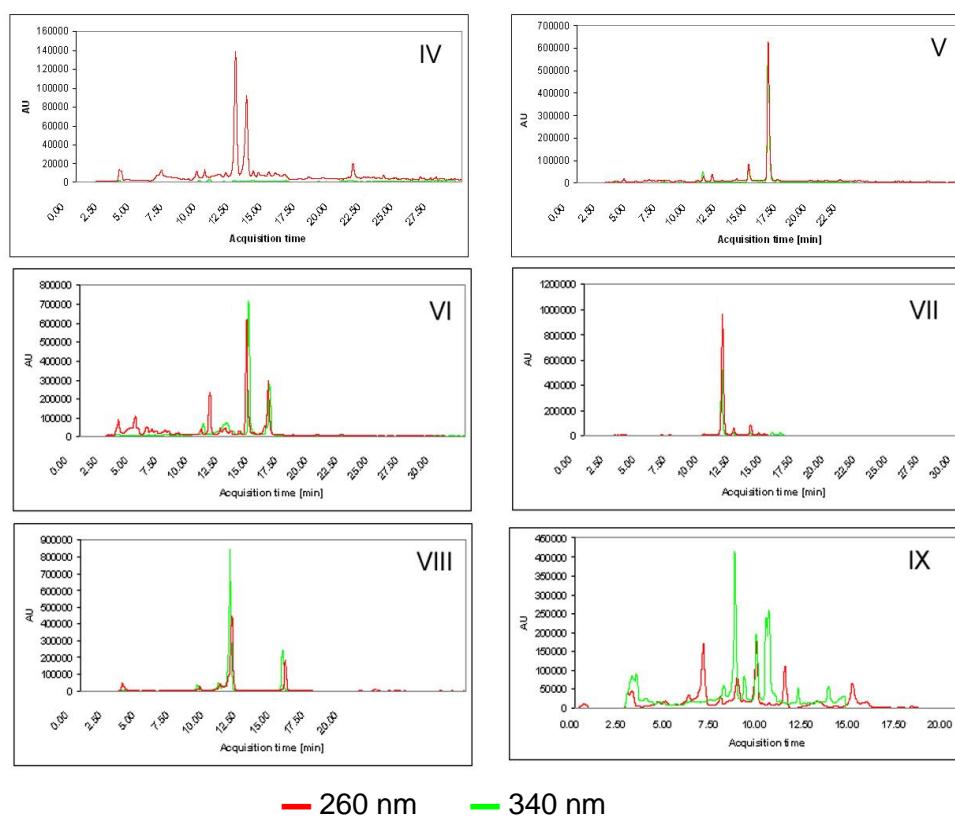


Fig. 18. LC-DAD profile of fractions 4-9 from *S. tectorum* 80% (v/v) methanolic extract, chromatograms were detected at 260 and 340 nm. [Absorbance vs. time (min)].

Chromatographic conditions see in section **4.7.1. HPLC evaluation of *S. tectorum* extracts.**

In fractions of 80% (v/v) methanolic extract of *S. tectorum* fifteen flavonol glycosides and seven phenolic acid derivatives were detected and tentatively characterized by LC-MS/MS. For unambiguous identification retention times, UV spectra and fragmentation patterns of compounds were compared with those of available standard compounds and those from the literature. Results are shown in Table 9., base peak chromatograms of fractions are shown in Fig. 19.

Table 9. Tentatively characterized compounds from fractions 5–9 of *S. tectorum*
80% (v/v) methanolic extract.

No.	t _R (min)	[M-H] ⁻ (m/z)	Fragment ions (m/z)	Tentative identification ^a	Fr.
1	3.0	341	179, 161	Caffeoyl hexoside	6
2	3.2	331	211, 169, 125	Galloyl hexoside	6
3	4.6	331	169, 151, 125	Galloyl hexoside	6
4	6.1	225	149, 119, 75	Not identified	5, 6
5	8.5	755	609, 447, 300	Q 3- <i>O</i> -desoxyhexoside- 7- <i>O</i> -desoxyhexosyl-hexoside	5
6	11.2	739	593, 430, 284	K 3- <i>O</i> -desoxyhexosyl-hexoside- 7- <i>O</i> -desoxyhexoside	5, 6
		775	593, 284	K 3- <i>O</i> -desoxyhexosyl-hexoside- 7- <i>O</i> -desoxyhexoside derivative	5, 6
		821	593, 284	K 3- <i>O</i> -desoxyhexosyl-hexoside- 7- <i>O</i> -desoxyhexoside derivative	5, 6
7	11.3	337	111, 85	Coumaroylquinic acid	7
8	11.5	377	225, 169, 149, 125	Galloyl hexoside derivative	7, 8
9	12.5	609	463, 300, 299, 271	Q 3- <i>O</i> -hexoside- 7- <i>O</i> -desoxyhexoside	6
				K 3- <i>O</i> -desoxyhexosyl-pentoside- 7- <i>O</i> -desoxyhexoside	6
10	13.5	709	563, 430, 284	K 3- <i>O</i> -desoxyhexosyl-pentoside- 7- <i>O</i> -desoxyhexoside	6
11	14.4	609	301	Q 3- <i>O</i> -rutinoside	5
12	14.5	593	430, 285, 283	K 3- <i>O</i> -hexoside- 7- <i>O</i> -desoxyhexoside	6, 7, 8
				Q 3- <i>O</i> -hexoside	8, 9
13	14.9	463	300, 271, 255, 211	Quercetin derivative	8
		615	301, 151	Quercetin derivative	8
14	15.7	871	725, 563, 430, 284	K 3- <i>O</i> -desoxyhexosyl-pentoside- 7- <i>O</i> -desoxyhexosyl-hexoside	5, 6
				K 7- <i>O</i> -desoxyhexosyl-pentoside	5, 6, 9
15	16.3	563	285	K 7- <i>O</i> -desoxyhexosyl-pentoside	5, 6, 9
16	16.4	577	283	K 3- <i>O</i> -desoxyhexoside- 7- <i>O</i> -desoxyhexoside	6, 7, 8
				K 3- <i>O</i> -desoxyhexoside- 7- <i>O</i> -desoxyhexoside	6, 7, 8

Table 9. continued

16	16.4	709	284	K 3- <i>O</i> -desoxyhexosyl-pentoside-7- <i>O</i> -desoxyhexoside	6, 7, 8
17	16.8	447	284, 255, 227, 151	K 3- <i>O</i> -hexoside	7, 8, 9
		463	301, 300, 151	Q 3- <i>O</i> -hexoside	8

^a Peak numbers and retention times (t_R) refer to BPC chromatograms shown in Fig. 19.

Abbreviations: K: kaempferol, Q: quercetin, Fr: Fractions containing certain compounds

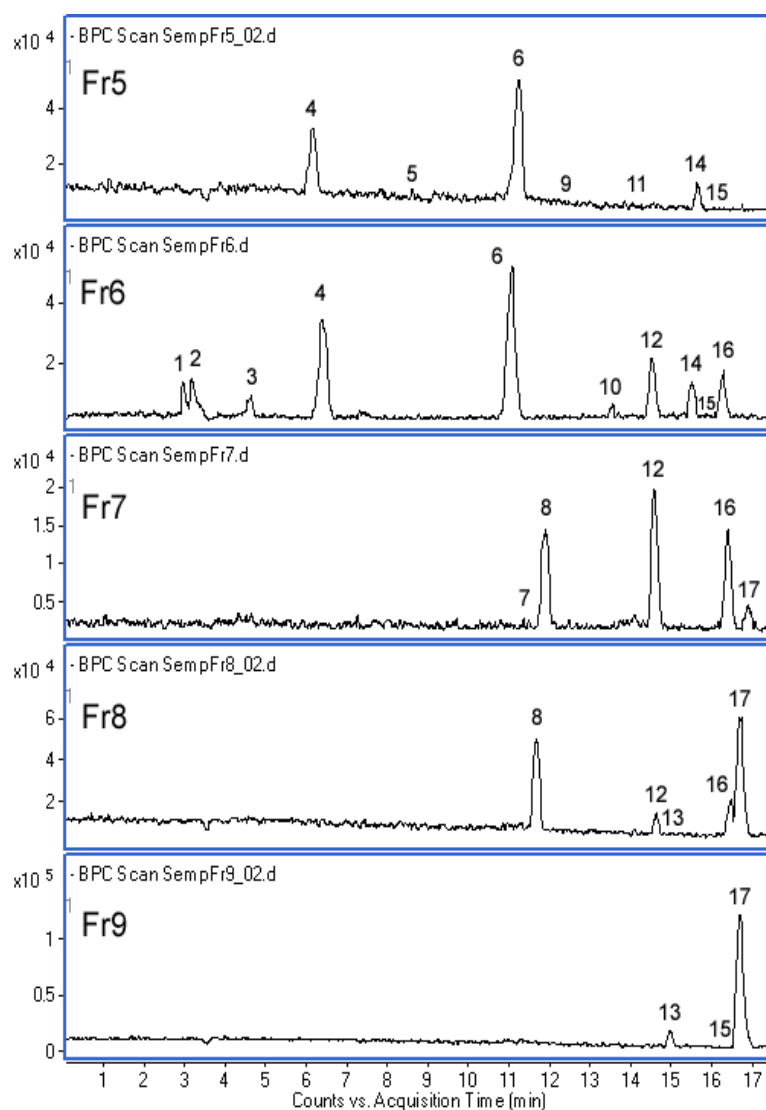


Fig.19. Base peak chromatograms of fractions 5–9 from *S. tectorum* 80% (v/v) methanolic extract. [Response unit vs. time (min)]. Numbering of peaks refers to data shown in Table 9. Chromatographic conditions see in text, section 4.7.1. **HPLC evaluation of *S. tectorum* extracts.**

5.3.3. *S. tectorum* leaf juice

S. tectorum leaf juice was studied using LC-DAD-MS/MS methods. The applied gradient elution system consisting of 1.0% (v/v) formic acid in water and acetonitrile allowed adequate separation for a number of compounds and good resolution for the main compound of interest regarding quantitation ($t_R = 7.01$ min) (Fig. 20.).

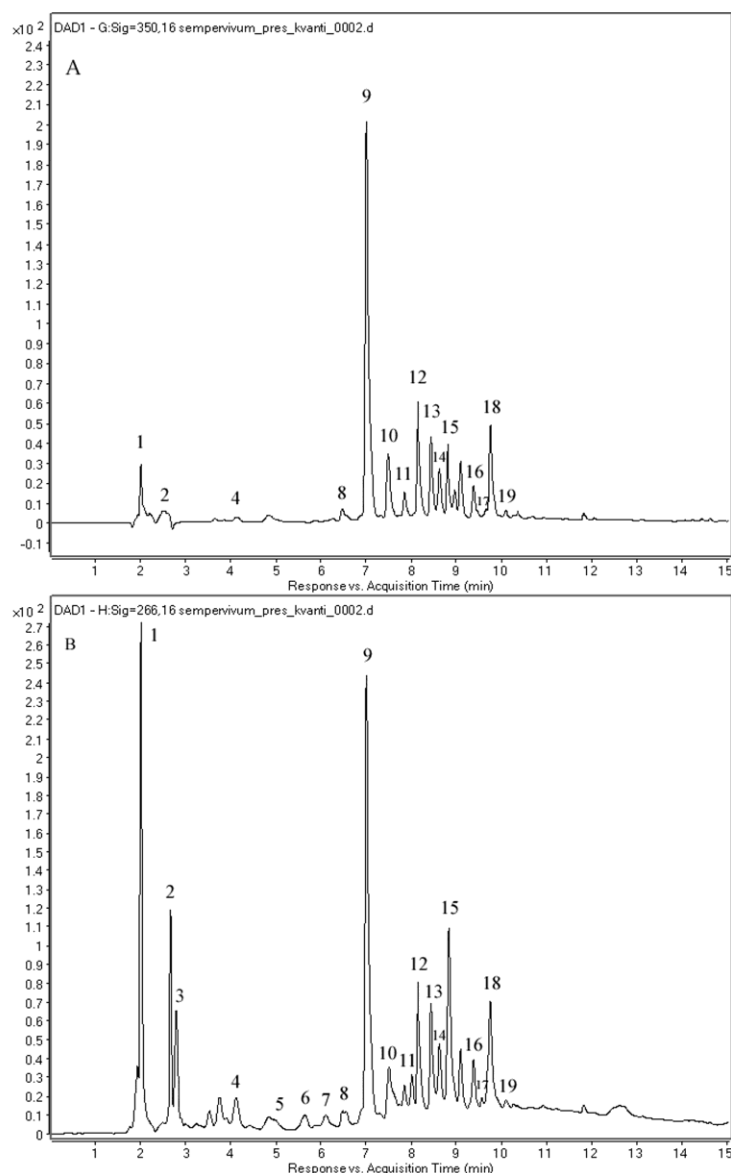


Fig. 20. HPLC-DAD chromatograms of *S. tectorum* leaf juice (A) Detection wavelength: 350 nm (B) Detection wavelength: 266 nm. [Absorbance vs. time (min)]. Numbering of peaks refers to data shown in Table 10. Chromatographic conditions see in text, section 4.7.4. LC-MS/MS evaluation of *S. tectorum* leaf juice.

Ten flavonol glycosides and sixteen phenolic acids were detected in the leaf juice. Based on comparison of their retention time, UV and mass spectral data with those of available reference compounds and those from the literature, simple phenolics were principally characterized as glycosides and other derivatives of hydroxybenzoic (gallic acid) and hydroxycinnamic acids (coumaric and caffeic acid). Results are shown in Table 10.

Table 10. LC-MS/MS data of identified and tentatively characterized compounds in *S. tectorum* leaf juice.

No.	t _R (min)	[M-H] ⁻ (m/z)	Fragment ions (m/z)	Tentative identification ^a	
1	2.01	353	191, 85	Chlorogenic acid	
			191	173, 155, 129,	Isocitric acid
				111, 85, 73, 57	
			133	115, 89, 73, 71	Malic acid
2	2.67	173	155, 129, 111, 85	Aconitic acid	
			331	211, 169, 151,	Galloyl hexoside
				125, 89, 59	
3	2.79	205	169	125, 79, 69, 67	Gallic acid
				173, 155, 111, 99,	Shikimic acid derivative
4	4.13	359	83, 73, 55		
			299, 239, 197,	Syringic acid hexoside	
5	5.03	225	153, 89, 59		
			149, 119, 75, 57	Not identified	
6	5.65	426	246, 202, 175,	Gallic acid derivative	
				169, 125, 107	
7	6.12	325	163, 119	Coumaroyl hexoside	
8	6.47	499	173, 155, 111, 85	Feruloylcoumaroylquinic acid	
9	7.01	739	593, 430, 285,	K 3- <i>O</i> -rhamnosyl-glucoside-7- <i>O</i> -rhamnoside ^b	
				284, 283, 255	
10	7.50	539	377, 331, 225,	Galloyl dihexoside derivative	
				169, 125	
			609	300	Rutin (Q 3- <i>O</i> -rhamnosyl-glucoside)

Table 10. continued

11	7.85	267	225, 207, 163, 118, 93, 59	Coumaric acid derivative
12	8.15	593	430, 285	K 3- <i>O</i> -hexoside-7- <i>O</i> -desoxyhexoside
13	8.44	871	725, 563, 430, 284	K 3- <i>O</i> -desoxyhexosyl-pentoside- 7- <i>O</i> -desoxyhexosyl-hexoside
		969	871, 725	K 3- <i>O</i> -desoxyhexosyl-pentoside-7- <i>O</i> - desoxyhexosyl-hexoside fumaric acid derivative
14	8.63	709	563, 430, 284	K 3- <i>O</i> -desoxyhexosyl-pentoside- 7- <i>O</i> -desoxyhexoside
		807	709, 563, 284	K 3- <i>O</i> -desoxyhexosyl-pentoside-7- <i>O</i> - desoxyhexoside fumaric acid derivative
15	8.81	577	431, 285, 283, 249	K 3- <i>O</i> -desoxyhexoside-7- <i>O</i> -desoxyhexoside
		377	225, 169, 125, 119, 107, 75	Galloyl hexoside derivative
16	9.38	337	173, 155, 129, 111, 85, 67	Coumaroylquinic acid
17	9.62	415	179, 173, 111, 101	Caffeoylquinic acid derivative
18	9.77	463	301, 300	Q 3- <i>O</i> -hexoside
19	10.09	447	285, 284, 255,	K 3- <i>O</i> -hexoside

^a Peak numbers and retention times (t_R) refer to LC chromatograms shown in Fig. 20.

^b Identified by NMR spectroscopy; Abbreviations: K: kaempferol, Q: quercetin.

5.3.4. *S. tectorum* ethanolic extract

UV spectra were characteristic of kaempferol and quercetin *O*-glycosides [49, 183], as well as of hydroxybenzoic and hydroxycinnamic acid derivatives [8]. However, detailed structural survey of flavonol glycosides required tandem mass spectrometric analyses. BPC chromatogram and LC-DAD profile of *S. tectorum* ethanolic extract is shown in Fig. 21.

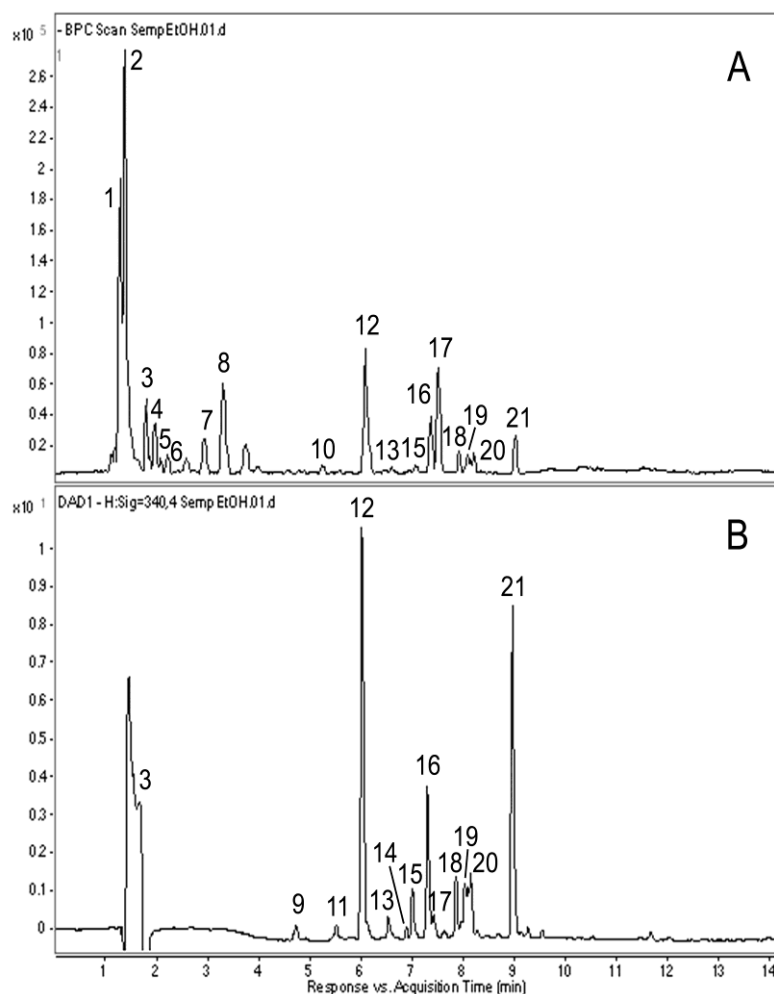


Fig. 21. Base peak (A) and HPLC-DAD (B) chromatograms of *S. tectorum* ethanolic extract, detection wavelength: 340 nm. [Absorbance / Response units vs. time (min)]. Numbering of peaks refers to data shown in Table 11. Chromatographic conditions see in section **4.7.5. LC-MS/MS evaluation of *S. tectorum* ethanolic extract.**

Twelve flavonol glycosides and twelve phenolic acid derivatives were detected in the ethanolic extract. Based on comparison of their retention times and mass spectral data with those of available reference compounds and those from the literature, similarly to the leaf juice, simple phenolics were principally characterized as glycosides and other derivatives of hydroxybenzoic (gallic acid) and hydroxycinnamic acids (coumaric and caffeic acid), while among flavonols kaempferol glycosides prevailed and quercetin glycosides were less frequent. Results are shown in Table 11.

Table 11. LC-MS/MS data of identified and tentatively characterized compounds from *S. tectorum* ethanolic extract.

Peak	t _R (min)	[M-H] ⁻ (m/z)	MS/MS (m/z)	Tentative identification ^a
1	1.30	245	119, 101, 85, 71	Coumaric acid derivative
2	1.38	191	173, 155, 129, 111, 85	Isocitric acid
		133	115, 89, 73, 71	Malic acid
3	1.80	327	89	Not identified
		237	113, 85, 71	Not identified
4	1.98	169	125, 79, 69	Gallic acid
5	2.09	331	211, 169, 151, 125, 89	Galloyl hexoside
6	2.22	173	155, 129, 111, 85, 83	Aconitic acid
7	2.93	345	-	Not identified
8	3.31	219	-	Not identified
9	4.79	755	447, 301, 300	Q 3- <i>O</i> -desoxyhexosyl- 7- <i>O</i> -desoxyhexosyl-hexoside
		325	163, 119	Coumaroyl hexoside
10	5.25	267	225, 207, 163, 118, 93	Coumaric acid derivative
11	5.58	755	446, 301, 300	Q 3- <i>O</i> -desoxyhexosyl- 7- <i>O</i> -desoxyhexosyl-hexoside
12	6.09	739	593, 430, 285, 284	K 3- <i>O</i> -rhamnosyl-glucoside- 7- <i>O</i> -rhamnoside
13	6.60	609	463, 300, 299, 255	Q 3- <i>O</i> -hexoside-7- <i>O</i> -desoxyhexoside
14	6.95	463	300, 271, 255, 151	Q 3- <i>O</i> -hexoside
15	7.07	337	173, 155, 129, 111, 85	Coumaroylquinic acid
		479	317, 316, 271	M 3- <i>O</i> -hexoside
		709	563, 430, 284	K 3- <i>O</i> -desoxyhexosyl-pentoside- 7- <i>O</i> -desoxyhexoside
16	7.37	593	430, 285	K 3- <i>O</i> -hexoside-7- <i>O</i> -desoxyhexoside

Table 11. continued

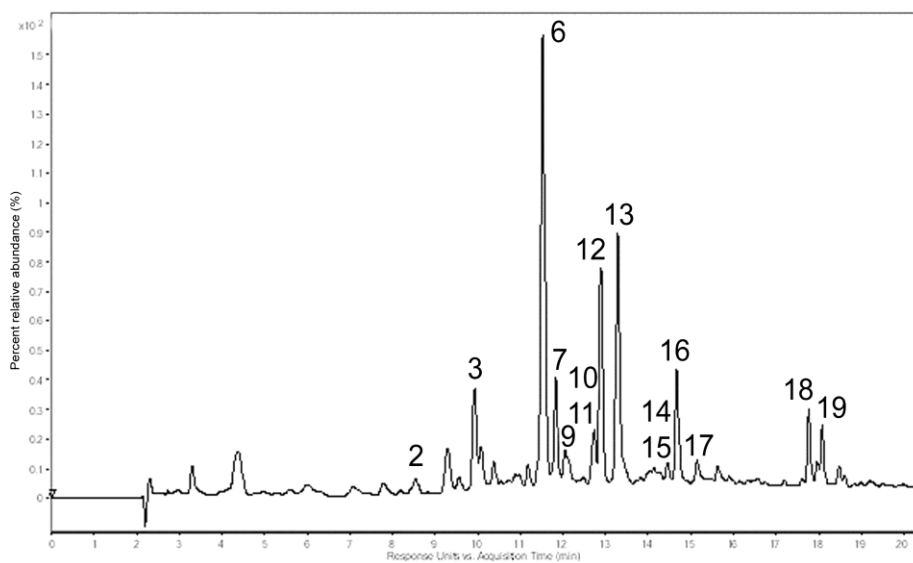
17	7.51	377	225, 169, 125, 119	Galloyl hexoside derivative
18	7.93	871	725, 563, 430, 284	K 3- <i>O</i> -desoxyhexosyl-pentoside- 7- <i>O</i> -desoxyhexosyl-hexoside
19	8.09	709	563, 430, 284	K 3- <i>O</i> -desoxyhexosyl-pentoside- 7- <i>O</i> -desoxyhexoside
20	8.20	577	431, 285, 283, 249	K 3- <i>O</i> -desoxyhexoside- 7- <i>O</i> -desoxyhexoside
21	9.02	447	285, 284, 255, 227	K 3- <i>O</i> -hexoside

^a Peak numbers and retention times (t_R) refer to BPC and LC-DAD chromatograms shown in Fig. 21. Abbreviations: K: kaempferol, Q: quercetin, M: myricetin.

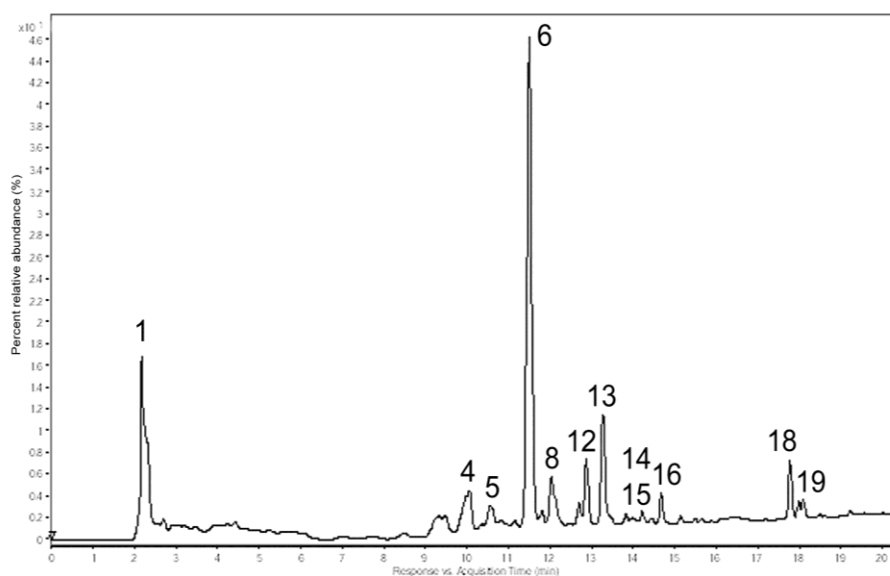
5.3.5. *C. avellana* ethyl acetate and methanolic extracts

For the characterization of phenolic compounds in *C. avellana* ethyl acetate and methanolic extracts UV spectral data, obtained by LC-DAD and fragmentation pattern, acquired by LC-ESI-MS/MS analyses were compared with those of authentic standards and with literature data [73, 75]. LC-DAD chromatograms were acquired at 280 and 350 nm, as the most selective wavelengths for the detection of flavonoids. LC-DAD profile of hazel extracts is shown in Fig. 22.

Studies on the extracts of *C. avellana* leaves revealed that flavonoids were the major constituents of the samples. In the methanolic extract six flavonoid glycosides (compound 5, 6, 8, 13, 16, 18), one caffeic acid derivative and rosmarinic acid (compound 1 and 3) were detected. Results are shown in Table 12. Based on chromatographic data, the main compound of the extract was myricetin 3-*O*-desoxyhexoside (6). In the ethyl acetate extract, in addition to five flavonoids (compound 6, 8, 13, 16, 18), rosmarinic acid (compound 3) was detected. The main compound (6) of this extract was also identified as myricetin 3-*O*-desoxyhexoside.



a



b

Fig. 22. LC-DAD chromatograms of *C. avellana* ethyl acetate (a) and methanolic (b) extracts. Detection wavelength was 280 nm. [Absorbance vs. time (min)]. Numbering of peaks refers to data shown in Table 12. Chromatographic conditions see in section

4.7.6. LC-MS/MS evaluation of *C. avellana* extracts

Table 12. Phenolic acid and flavonol glycoside compounds detected in *C. avellana* leaves methanolic and ethyl-acetate extracts.

No.	t_R (min)	[M-H] ⁻ (m/z)	Fragment ions (m/z)	Tentative characterization ^a	Extract	
					M	EA
1	2.2	377	341, 215, 179, 161	Caffeic acid derivative	X	
2	8.4	445	385, 205, 161, 153	Not identified		X
3	10.0	359	329, 269, 191, 161	Rosmarinic acid	X	X
5	10.6	479	317, 316	M 3- <i>O</i> -hexoside	X	
6	11.5	463	317, 316, 271	M 3- <i>O</i> -desoxyhexoside	X	X
8	12.2	463	301, 300, 271, 255	Q 3- <i>O</i> -hexoside	X	X
13	13.3	447	301, 300, 271, 151	Q 3- <i>O</i> -desoxyhexoside	X	X
16	14.8	431	285, 284, 257, 227	K 3- <i>O</i> -desoxyhexoside	X	X
17	15.2	301	179, 151, 121	Not identified		X
18	17.9	577	514, 285, 284	K di(desoxyhexoside)	X	X

^a Peak numbers and retention times refer to LC-DAD chromatograms shown in Fig. 22.

Abbreviations: K: kaempferol, Q: quercetin, M: myricetin; M: methanolic extract, EA: ethyl acetate extract.

5.4. Quantitative analyses

5.4.1. Determination of hydroxycinnamic acid derivatives in *S. tectorum* leaf juice

The HPLC method applied provided good separation for each compound. Caffeic acid (Fig. 23.A), chlorogenic acid (Fig. 23.B) and rosmarinic acid (Fig. 23.C) reference compounds were detected at retention times of 12.90 min, 14.48 min and 20.47 min, respectively. HPLC-UV chromatogram of the compounds is shown in Fig.24.

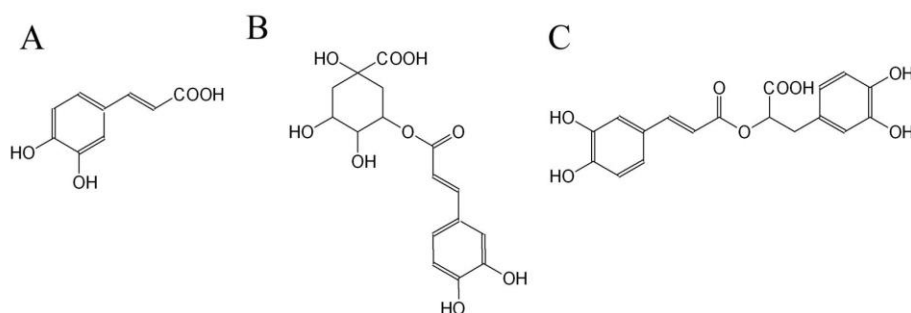


Fig. 23. A: Caffeic acid, B: Chlorogenic acid, C: Rosmarinic acid.

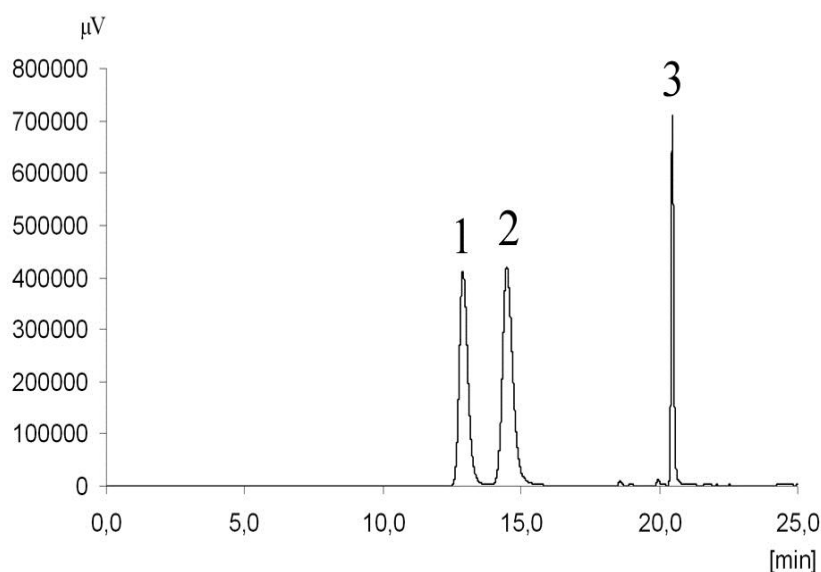


Fig. 24. HPLC-UV chromatogram of hydroxycinnamic acid reference compounds evaluated in *S. tectorum* leaf juice. 1: Caffeic acid, 2: Chlorogenic acid, 3: Rosmarinic acid. Detection wavelength was 320 nm. [Absorbance vs. time (min)]. Chromatographic conditions see in section **4.8.1. Determination of hydroxycinnamic acid derivatives in *S. tectorum* leaf juice by an HPLC-UV method.**

5.4.1.1. Quantitative analysis and method validation

Quantity of caffeic, chlorogenic and rosmarinic acid in *S. tectorum* leaf juice was determined according to results from DAD (320 nm) experiments. Detection wavelength was designated by evaluation of the UV spectra of the reference compounds. The applied gradient elution ensured good resolution, thus proper selectivity for all hydroxycinnamic acid derivatives.

Retention time repeatability was suitable for all compounds, relative standard deviation ranged from 0.26% to 1.16% ($n = 6$). Specificity of the method was checked by injecting pure solvent (70%, v/v methanol). No co-elution was observed near by the retention time of the analytes of interest.

Table 13. Method validation: Precision and accuracy of the quantitative method.

Nominal conc. ($\mu\text{g/mL}$)	Precision (RSD%)		Accuracy (%)	
	<i>Intra-day</i>	<i>Inter-day</i>	<i>Intra-day</i>	<i>Inter-day</i>
Caffeic acid				
1	0.41	10.77	118.4	125.4
25	1.70	2.94	97.8	96.1
150	5.11	9.08	100.0	110.1
Chlorogenic acid				
2	1.04	13.17	117.8	79.1
150	1.27	2.10	96.9	103.8
300	5.02	10.29	103.8	95.4
Rosmarinic acid				
1	3.16	8.19	92.9	118.8
25	2.02	4.13	80.6	97.2
150	6.10	7.66	73.0	106.7

Precision of the method was tested by performing intra- and inter-day evaluation of solutions containing the target analytes 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 < 15% for all quantitative methods, while intra- and inter-day accuracy ranged from 73.0% to 125.4%. Precision and accuracy results are shown in Table 13.

For the target analytes, linear regression analyses were performed by the use of external calibration. Linearity was determined by analyzing the reference compounds at five concentrations (1–150 $\mu\text{g/mL}$ for caffeic acid and rosmarinic acid and 2–300 $\mu\text{g/mL}$ for chlorogenic acid), each in triplicate. Regression was quadratic polynomial for all analytes (see Table 14.). Caffeic acid and chlorogenic acid content of *S. tectorum* leaf juice is shown in Table 14., quantity of rosmarinic acid was < LOD. Method recovery was evaluated using the fortified sample recovery test, tests were performed in triplicate. Results for method recovery are shown in Table 14.

Table 14. Method validation: Quantitative results, regression and method recovery.

Compound	Quantity (mean ± S.D.) ^a	Regression equation	r ²	Regression range ^b	Method recovery
Caffeic acid	2.69 ± 0.06 (RSD%: 2.08)	$y = -86.129x^2 + 77452x - 11313$	1.0000	1 – 150	87.19% (RSD%: 1.06)
Chlorogenic acid	3.54 ± 0.27 (RSD%: 7.68)	$y = -17.743x^2 + 47370x - 1902.4$	1.0000	2 – 300	82.41% (RSD%: 1.11)
Rosmarinic acid	< LOD	$y = -46.198x^2 + 40146x - 4196.3$	1.0000	1 – 150	88.26% (RSD%: 1.16)

^a Values are expressed in mg/100 g dried *S. tectorum* leaf juice. ^b Values are expressed as µg/mL.

Table 15. Method validation: Quantitative results, regression and method recovery.

Compound	Quantity (mean ± S.D.) ^a	Regression equation	r ²	Regression range (µg/mL)	Method recovery
K 3-O-rhamnosyl-glucoside-7-O-rhamnoside (DAD 350nm)	0.116 ± 0.002 (RSD%: 2.06)	$y = 6.4014x + 8.0003$	0.9999	5 – 500	71.61% (RSD%: 6.70)
K 3-O-rhamnosyl-glucoside-7-O-rhamnoside (TIC SRM)	0.097 ± 0.001 (RSD%: 1.21)	$y = -0.77x^2 + 1339.2x + 3141.4$	0.9998	5 – 500	102.73% (RSD%: 2.76)
K 3-O-desoxyhexoside-7-O-desoxyhexoside	0.044 ± 0.001 (RSD%: 0.56)	$y = -1.9154x^2 + 2294.5x + 16435$	0.9998	5 – 500	75.56% (RSD%: 3.75)
K 3-O-hexoside-7-O-desoxyhexoside	0.080 ± 0.002 (RSD%: 2.29)	$y = -0.5971x^2 + 626.93x - 29.451$	1.0000	2 – 200	110.43% (RSD%: 12.08)

^a Values are expressed in g/100 g dried *S. tectorum* leaf juice.

5.4.2. Determination of kaempferol glycosides in *S. tectorum* leaf juice

5.4.2.1. Isolation with column chromatography

Fraction 12 of Silicagel column chromatography was proved to contain the main flavonoid compound in the highest concentration, hence for isolation, the fraction was further separated by Sephadex column chromatography. Although the kaempferol triglycoside was present in fractions 42–51 from Sephadex column chromatography, appropriate purity was achieved only in fractions 42–45. These fractions were pooled and used as external standard for quantitation. Purity of the isolated compound was 95.3% (TIC) and 99.2% (DAD). Structural evaluation of the isolate was carried out by NMR spectroscopy, and it was identified as kaempferol 3-*O*-β-[α-rhamnopyranosyl-(1→2)-glucopyranoside]-7-*O*-α-rhamnopyranoside (Fig. 25.). Isolation process yielded 2.5 mg substance.

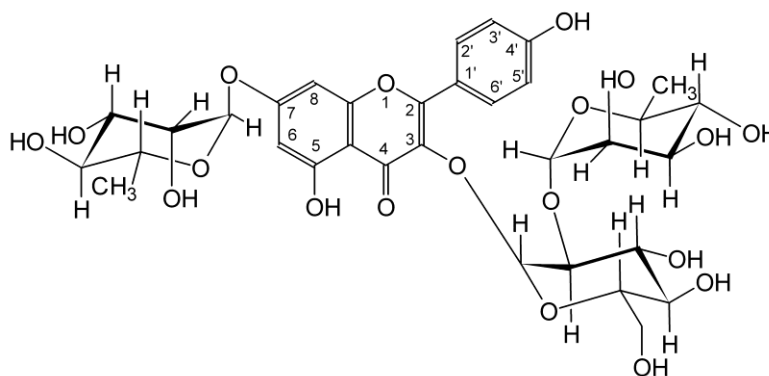


Fig. 25. Structure and numbering of kaempferol 3-*O*-β-[α-rhamnopyranosyl-(1→2)-glucopyranoside]-7-*O*-α-rhamnopyranoside, the main flavonol glycoside compound of *S. tectorum* leaf juice

Isolation of further characteristic flavonol glycosides from *S. tectorum* leaf juice was carried out by successive Silicagel, Sephadex LH20 and polyamide column chromatography. The isolates were used as external standard for quantitation. Appropriate purity was achieved by isolation of kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside (89.7%, TIC), isolation process yielded 1.4 mg substance. Purity

of the isolated kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside however, was lower (54.7%, TIC). Isolation process yielded 2.95 mg substance.

5.4.2.2. Quantitative analysis and method validation

Quantity of kaempferol 3-*O*- β -[α -rhamnopyranosyl-(1 \rightarrow 2)-glucopyranoside]-7-*O*- α -rhamnopyranoside in *S. tectorum* leaf juice was determined according to results from SRM (single reaction monitoring mode) and DAD (350 nm) experiments. For the target analyte, linear regression analyses were performed by the use of external calibration. Linearity was determined by analyzing the isolated standards at five concentrations (5–500 μ g/mL), each in triplicate. Linear regression was achieved only by the LC-DAD method, while regression of the SRM method was quadratic polynomial (see Table 15.). Kaempferol 3-*O*-rhamnosyl-glucoside-7-*O*-rhamnoside content of *S. tectorum* leaf juice was 0.097 g/100 g dried juice (RSD% = 1.21) and 0.116 g/100 g dried juice (RSD% = 2.06) for TIC SRM and for DAD 350 nm method, respectively.

Quantity of kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside and kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside in *S. tectorum* leaf juice was determined according to the results from SRM experiments. Linearity was determined by analyzing the isolated standards at five concentrations (5–500 μ g/mL for kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside and 2–200 μ g/mL for kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside), each in triplicate. Regression for both quantitations was quadratic polynomial (see Table 15.). Kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside and kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside content of *S. tectorum* leaf juice was 0.044 g/100 g dried juice (RSD% = 0.56) and 0.080 g/100 g dried juice (RSD% = 2.29), respectively. Retention time repeatability was suitable for all compounds, relative standard deviation was < 0.15% (n = 6). Specificity of the method was checked by injecting pure solvent (70%, v/v methanol). No co-elution was observed near by the retention time of the analytes of interest. Method recovery was evaluated using the fortified sample recovery test, tests were performed in triplicate. Results for method recovery are shown in Table 15.

Precision of the methods applied was tested by performing intra- and inter-day evaluation of solutions containing the target analytes 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 < 15% for all quantitative methods, while intra- and inter-day accuracy ranged from 78.3% to 113.3%. Precision and accuracy results are shown in Table 16.

Table 16. Method validation: Precision and accuracy of the quantitative methods.

Nominal conc. ($\mu\text{g/mL}$)	Precision (RSD%)		Accuracy (%)	
	<i>Intra-day</i>	<i>Inter-day</i>	<i>Intra-day</i>	<i>Inter-day</i>
Kaempferol 3-<i>O</i>-rhamnosyl-glucoside-7-<i>O</i>-rhamnoside (DAD 350nm)				
5	2.69	13.50	80.4	81.8
100	1.62	5.04	92.5	99.8
500	1.85	4.46	92.2	97.6
Kaempferol 3-<i>O</i>-rhamnosyl-glucoside-7-<i>O</i>-rhamnoside (TIC SRM)				
5	3.54	11.38	78.3	78.5
100	1.74	11.84	103.1	93.1
500	7.64	11.11	100.1	92.9
Kaempferol 3-<i>O</i>-desoxyhexoside-7-<i>O</i>-desoxyhexoside				
5	3.88	5.55	80.5	81.0
100	1.88	8.50	97.5	108.5
500	3.12	10.65	99.8	91.2
Kaempferol 3-<i>O</i>-hexoside-7-<i>O</i>-desoxyhexoside				
2	6.28	6.49	113.3	107.4
40	4.37	13.90	98.5	100.4
200	7.16	8.93	99.9	97.4

6. DISCUSSION

6.1. Antioxidant activity assays

6.1.1. DPPH and ABTS scavenging activity

Phenolics form a vast group of secondary metabolites in plants and are present in raw plant material in various forms, mainly as glycosides. The number of glycoside moieties modifies the antioxidant activity of the phenolics by influencing the number of free functional phenolic groups or by altering hydrophobicity of the molecules [184].

Plant phenolics act as antioxidants by scavenging free radicals, chelating metals and quenching singlet oxygen [182, 185]. Beside substitution of free hydroxyl groups, antioxidant activity of plant phenolics depends on the presence of other antioxidants [186], as well as on the methodology of the antioxidant assay used [187]. Accordingly, a simple method is insufficient to measure and characterize antioxidant capacity of single compounds or mixtures [187-188].

Antioxidant activity of *S. tectorum* extracts was determined in two different *in vitro* tests. ABTS^{•+} radical scavenging capacity and DPPH[•] radical test were used, with the former merely based on single electron transfer and the latter on hydrogen atom transfer reactions [188]. There are some limitations and shortcomings of ABTS and DPPH scavenging assays. First of all, ABTS^{•+} and DPPH[•] radicals are not representative of biomolecules. Additionally, steric accessibility of DPPH[•] is a major determinant of the reaction, small molecules that have better access to the radical site have relatively higher antioxidant capacity [189]. Scavenging reaction of DPPH[•] may take 20 minutes up to 6 hours [17], therefore to save time, absorbance changes were recorded for six minutes after combination of DPPH and the sample solution, and the final absorbance was extrapolated according to the simplest possible reaction kinetics model calculated from absorbance change data. Results obtained from ABTS scavenging assay are usually expressed as trolox equivalents and are therefore related to an antioxidant standard that shows different kinetic behaviour [177, 189]. In order to eliminate dependence of results provided by the study on time of analysis, similarly to DPPH scavenging assay, results were expressed as IC₅₀ values (µg/mL).

Antioxidant activity of *S. tectorum* extracts was higher in the assay containing ABTS^{•+} radical as compared to that containing DPPH[•], as shown in Table 4. Antioxidant activity data of houseleek extracts were plotted against total polyphenol contents and Student's t-test was applied to evaluate correlation between the variables. The correlation of total polyphenol content and radical scavenging activity was considered as highly significant for the assay containing DPPH[•], as well as ABTS^{•+} radicals. Regression coefficient, *p*-value and linear regression equation were as follows: $r^2 = 0.9668$, $p < 0.005$, $y = 0.0005x - 0.0037$ and $r^2 = 0.6929$, $p < 0.005$, $y = 0.0008x + 0.0053$ for DPPH and ABTS, respectively. This strong association accounted for the observation that extracts prepared with solvents of better selectivity for phenolics had the highest antioxidant activities in the applied assays.

Hvattum and co-workers studied the reaction products of flavonols with DPPH[•] by LC-ESI-MS/MS [190]. Two reaction products of oxidized quercetin were identified, which were produced by transfer of two H-atoms followed by subsequent incorporation of either two CH₃OH molecules or that of one CH₃OH and one H₂O molecule. Kaempferol and myricetin yielded methoxylated oxidation products similar to those of quercetin, while kaempferol exhibited additional products, obtained by transferring one H-atom to DPPH[•] and subsequently reacting with CH₃O[•], forming two isomeric products.

S. tectorum extracts, prepared with ethanol and 80% (v/v) methanol, those that have the highest antioxidant activities contained mainly kaempferol and quercetin di- and triglycosides. In the ethanolic extract three organic acids, four gallic acid derivatives, three hydroxycinnamic acid derivatives, one myricetin and two quercetin glycosides were detected, furthermore the presence of six kaempferol mono-, di- and triglycosides and that of a kaempferol tetraglycoside was confirmed. Results are shown in Fig. 21. and Table 11. For the 80% (v/v) methanolic extract predominance of kaempferol glycosides and occurrence of a quercetin diglycoside was characteristic, as described previously (see section **5.3.1. *S. tectorum* 80% (v/v) methanolic extract**). It was presumed that the differences in the chemical composition account for the variances in antioxidant activity between *S. tectorum* extracts, although the explanation was neither quantitative nor accurate.

Differences between radical scavenging activities of *S. tectorum* extracts and reference compounds may be attributed to the glycosylation pattern of houseleek flavonols and especially to the length of the saccharide chains, as according to literature sources, electron-donating effect from the B-ring is reduced by the large steric hydrance caused by the long saccharide chains [81]. Flavonol compounds present in *S. tectorum* are glycosylated primarily by di-, tri- and even tetrasaccharides, consequently antioxidant activity of *S. tectorum* extracts is notably lower than that of standard compounds without sugar substitution or with short saccharide chains.

In order to understand antioxidant action of plant phenolics, screening of scavenger activity of extracts prepared with solvents of different polarity, accordingly selective for distinct groups of phenolics is required. However, it should be considered that antioxidant activity of houseleek leaf juice applied in traditional medicine - principally locally – in inflamed complaints is not indicative of the pharmacological action. It was presumed that other mechanisms are involved in its anti-inflammatory effects.

Antioxidant activity of fractionated *S. tectorum* 80% (v/v) methanolic extract in DPPH scavenging assay was also determined. Antioxidant activity for fractions 1-3 could not be detected, while fractions 4-9 exhibited DPPH scavenging effect. Antioxidant activity of all fractions was significantly lower as compared to the whole 80% (v/v) methanolic extract (Fig. 16.). Furthermore, an estimated total radical scavenger value of all fractions was calculated and compared to that of the whole extract. The fractions, when they were present together in the same system, had stronger antioxidant activity as compared to the effect expected from a simple addition of the individual effects of the fractions (results are not shown), accordingly a synergistic antioxidative effect between the fractions was presumed [182]. Antioxidants, such as plant phenolics show different interactions, these can be synergistic, antagonistic or additive [182, 186]. Several mechanisms are involved in synergism among antioxidants: a) one antioxidant is regenerated by others, b) one antioxidant is protected by an other one and c) antioxidants show different mechanisms of action [191]. The combination of various antioxidant phenolics present in the whole extract, however, absent from the individual fractions may be the reason for the evaluated synergistic effect.

It should be mentioned, that protection of tissues from free radical oxidative damage by phenolic antioxidants present in food or medicinal plants highly depends on

bioavailability and metabolism of the antioxidants [192]. When evaluating, whether a given compound acts as an antioxidant *in vivo*, it should be elucidated among other things, whether it exerts its antioxidant activity against biologically-important reactive species (e.g. scavenges superoxide or hydroxyl radicals, inhibits peroxydation). An other important question is whether the compound is present *in vivo* at sufficient concentration. Dietary flavonoids and phenolic compounds can exert potent antioxidant activity *in vitro*, however, their concentration *in vivo* is influenced and limited by their absorption and metabolism [193].

Crozier and co-workers concluded in their review paper on the bioavailability of dietary flavonoids and phenolic compounds [194], that flavonoids can be absorbed whether in the small or in the large intestine. In the first case, during passage through the small intestine flavonoid aglycones released from glycosides after hydrolysis are subjected to sulfation, glucuronidation or methylation in the enterocytes prior to absorption of the metabolites to the circulatory system. Some metabolites are treated as xenobiotics and are rapidly removed from the blood stream. In the second case, substantial amount of flavonoid glycosides undergo deglycosylation followed by ring fission through the action of colonic bacterial enzymes releasing phenylacetic acid derivatives. Literature data on dietary chlorogenic acid derivatives are contradictory. Chlorogenic acids (conjugated structures formed by hydroxycinnamates linked to a quinic acid) undergo extensive metabolism prior to absorption, whether in the small intestine or in the colon. However, presence of unmetabolized chlorogenic acids in human plasma was also reported.

6.1.2. Peroxynitrite scavenging activity

Peroxynitrite anion (ONOO^-) formed *in vivo* primarily by the reaction of nitric oxide (NO^*) and superoxide radical ($\text{O}_2^{\bullet-}$) is an unstable non-radical reactive species and an important oxidizing and nitrating agent [195]. Peroxynitrite anion, once protonated under physiological conditions to give peroxynitrous acid (HOONO) can produce very reactive „hydroxyl-radical like” species and contribute to tissue injuries, various forms of cardiovascular, inflammatory and neurodegenerative diseases [196-199].

Peroxynitrite scavenging activity of *S. tectorum* extracts was determined according to the method described by Bavaloiné and Geletii [179]. Principles of the method are briefly: in mixtures containing peroxynitrite anion pyrogallol red is decolourized by free radicals deriving from peroxynitrite, decrease in absorbance of pyrogallol red at its absorption maximum ($\lambda_{\max} = 542 \text{ nm}$) can be detected. Compounds and plant extracts containing antioxidants can prevent decolorization of pyrogallol red by scavenging peroxynitrite anion. Peroxynitrite scavenging activity of houseleek leaf juice and that of extracts prepared by solvents of better selectivity for phenolics was notably lower as compared to the standard solutions (see Table 7.). As the only exception, the scavenging activity of the 80% (v/v) methanolic extract was of the same order as that of rutin.

Haenen and co-workers found that flavonoids are excellent scavengers of peroxynitrite anion [200]. Catechol group in B-ring was confirmed to give the highest contribution to the peroxynitrite scavenging effect, that is flavonoids with two free aromatic hydroxyl groups in *ortho* and *para* position had higher activity compared with flavonoids with the OH groups in the *meta* position. When all hydroxyl groups in B-ring were substituted, substitution of hydroxyl group in position 3 seemed to influence the scavenging effect. Thus, quercetin bearing two unsubstituted OH groups in *ortho* position in B-ring and one at position C3 was the most potent scavenger of peroxynitrite among the compounds evaluated. The catechol group dependent structure – peroxynitrite scavenging activity relationship of phenolics has been confirmed by Heijnen and co-workers [201] and Könczöl and co-workers [180]. *S. tectorum* samples were characterized by the presence of flavonoids with kaempferol aglycones glycosylated at the 3-*O* position, resulting in a lower peroxynitrite scavenging capacity, since neither catechol group in B-ring, nor unsubstituted OH group at the C3 position was present.

6.2. Qualitative analyses

6.2.1. *S. tectorum* 80% (v/v) methanolic extract

Higher activity of polar extracts abundant in phenolics was indicated in the applied *in vitro* antioxidant action assays. Accordingly, our further objective was high-performance liquid chromatographic evaluation of *S. tectorum* 80% (v/v) methanolic

extract. Studying distinctive UV spectra of flavonol compounds yielded a preliminary structural description. Absorption maxima were characteristic of kaempferol and quercetin *O*-glycosides [49, 183]. LC-DAD profile of houseleek 80% (v/v) methanolic extract is shown in Fig. 17. Detailed structural survey of flavonol glycosides required tandem mass spectrometric analyses. Table 8. shows the most frequent ions which characterize the fragmentation of flavonol *O*-glycosides present in *S. tectorum* leaf 80% (v/v) methanolic extract.

Although ESI-MS/MS is not capable for the unambiguous structural identification of flavonoid glycosides, it provides sufficient information regarding the aglycone structure and the glycan sequence. In the CID spectra of deprotonated flavonol glycosides ions corresponding to the deprotonated aglycones, $[Y_0]^-$ at m/z 285 and 301 generated by the loss of sugar units, furthermore the following fragment ions for aglycones were observed: $[Y^3_0-H]^-$ at m/z 284 and 300, $[Y_0-H-CO-H]^-$ at m/z 255 and 271 for kaempferol and quercetin, respectively [75]. The glycosides attached were identified according to the neutral losses of sugar units: difference of 162 amu indicated a hexose, 146 amu denoted a desoxyhexose, supposedly rhamnose and 132 amu pointed to a pentose, presumably xylose moiety [78].

Ablajan et al. [75] found that relative abundance of radical aglycone anion $[Y_0-H]^-$, deriving from a homolytic cleavage, compared to that of aglycone anion $[Y_0]^-$, deriving from a heterolytic cleavage, is in correlation with the position of glycosylation. Relative abundance of radical aglycone anion $[Y_0-H]^-$ is higher in the case of flavonol 3-*O*-glycosides. Relative abundance of $[Y_0-H]^-$ ion compared to that of $[Y]^-$ ion was higher in mass spectra of all compounds, than that of $[Y_0]^-$ ion, therefore we presumed, that they are 3-*O*-glycosylated. Further observed was $[Y_0-2H]^-$ ion (m/z 283) with high abundance in the product ion spectra of compounds 1, 2, 4, 5a and 5b. Formation of $[Y_0-2H]^-$ ion is explained by the elimination of two glycosyl radicals at the 3-*O* and 7-*O* positions successively [75], thus we assumed, that these compounds are glycosylated both at 3-*O* and at 7-*O* position. Fragmentation scheme of kaempferol *O*-glycosides showed correspondence also to the results of March et al. [202].

Peak 1 ($t_R = 9.6$ min) was identified as a kaempferol triglycoside: kaempferol 3-*O*-desoxyhexosyl-hexoside-7-*O*-desoxyhexoside, it provided a $[M-H]^-$ ion at m/z 739

and fragment ions at m/z 593, 431/430 and 284/283, resulting from the loss of a desoxyhexose, a hexose and a desoxyhexose moiety, respectively.

Product ion at m/z 255 represents the $[Y_0-H-CO-H]^-$ ion of kaempferol [75]. Product ions at m/z 430 and 284 suggested two homolytic cleavages resulting in a radical fragment ion $[M-H-desoxyhexose-hexose-H]^-$ and a radical aglycone anion $[Y_0-H]^-$, respectively [80]. Presence of both $[Y_0-H]^-$ ion (m/z 284) and $[Y_0-2H]^-$ ion (m/z 283) suggested cleavage of two glycosyl radicals at the 3-*O* and 7-*O* positions successively [75]. Fragment ions revealing fragmentation of compound 1 are shown in Fig. 27.

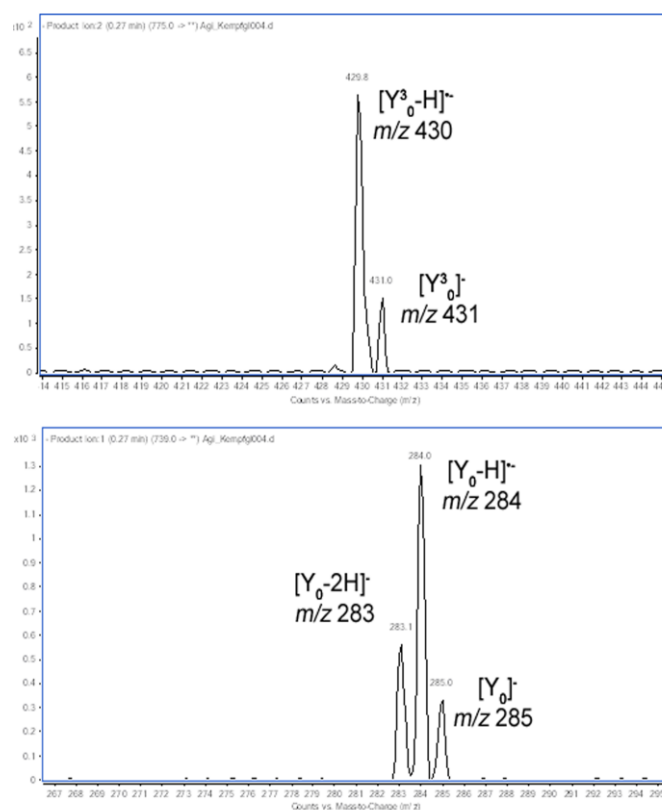


Fig. 27. Fragment ions deriving from negative ionization CID of the main flavonol glycoside compound detected in *S. tectorum* leaf 80% (v/v) methanolic extract.

[Response units vs. time (min)].

Thus, analogously to March et al. [203], the following fragmentation scheme was proposed for compound 1: The heterolytic cleavage of a desoxyhexose moiety at position 7-*O* was supposed to produce $[Y^7_0]^-$ ion (m/z 593), as heterolytic cleavages yield more abundant $[Y^7_0]^-$ ions compared to $[Y^7_0-H]^-$ ions occurring for

7-*O*-glycosylated flavonols. On the contrary, scission of the desoxyhexosyl-hexoside glycan residue from the pseudomolecular ion was assumed to form radical anion $[Y^3-H]^-$ (m/z 430), since fragmentation of flavonol 3-*O*-glycosides show more abundant $[Y^3_0-H]^-$ ions compared to $[Y^3_0]^-$ ions. Homolytic elimination of the desoxyhexosyl-hexose glycan residue from the $[Y^7_0]^-$ fragment ion was assumed to yield radical aglycone anion $[Y^7_0][Y^3_0-H]^-$ or $[Y_0-H]^-$ at m/z 284, while heterolytic cleavage of the desoxyhexose glycan residue from the $[Y^3_0-H]^-$ ion (m/z 430) was presumed to produce the same $[Y^7_0][Y^3_0-H]^-$ ion (m/z 284). The cleavage of the two glycosyl radicals at the 3-*O* and 7-*O* positions successively to generate the $[Y_0-2H]^-$ ion at m/z 283 was also suggested [75]. According to our results, which were consistent with other studies [75-76, 80, 82], and assuming that flavonol 3-*O*-glycosides favoured the homolytic cleavage and that glycosylation site could be deduced from the relative abundance ratio: $[Y_0]^- : [Y_0-H]^-$, the following structure was proposed for compound 1: kaempferol 3-*O*-desoxyhexosyl-hexoside-7-*O*-desoxyhexoside. The presumed structure, confirmed by the results of NMR investigations, as well as (-)-ESI-MS/MS fragmentation scheme of compound 1 are shown in Fig. 28.

Peak 2 ($t_R = 12.1$ min) gave a $[M-H]^-$ ion at m/z 593, producing fragment ions at m/z 430 and 285, which corresponded to the homolytic cleavage of a hexose and to the heterolytic cleavage of a desoxyhexose moiety, respectively, therefore it was identified as kaempferol 3-*O*-hexosyl-7-*O*-desoxyhexoside. Peak 3 ($t_R = 12.4$ min) exhibited a $[M-H]^-$ ion at m/z 609 and a fragment ion at m/z 300, resulting from the loss of a desoxyhexose and a hexose moiety. The aglycone fragment at m/z 300 suggested a homolytic cleavage. Furthermore were product ions $[Y_0-H-CO-H]^-$ and $[Y_0-H-CO_2-H]^-$ at m/z 271 and 255 characteristic of quercetin [75, 80], thus peak 3 was identified as rutin (quercetin 3-*O*-rutinoside). Comparison of data with mass spectra of standard substances confirmed these results. Peak 4 ($t_R = 12.8$ min) was assumed to be kaempferol 3-*O*-desoxyhexosyl-pentoside-7-*O*-desoxyhexosyl-hexoside by its $[M-H]^-$ ion at m/z 871 and fragment ions at m/z 725, 563, 430, 284. Loss of 146 amu between m/z 871 and 725 and between m/z 430 and 284 indicates two desoxyhexosyl units, loss of 162 amu between m/z 725 and 563 points to a hexose, while loss of 132 amu refers to a pentose moiety.

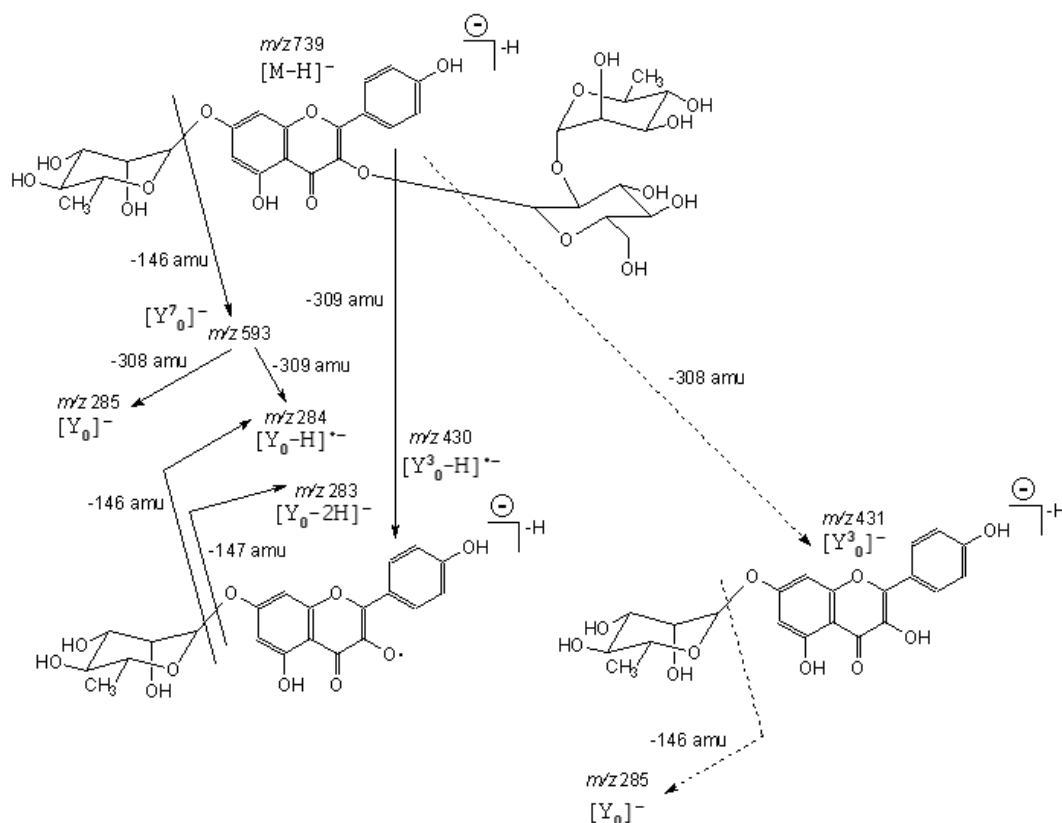


Fig. 28. Proposed fragmentation mechanism of $[M-H]^-$ of compound 1 (m/z 739), kaempferol 3-*O*-desoxyhexosyl-hexoside-7-*O*-desoxyhexoside.

Peak 5 ($t_R = 13.6$ min) consisted of two compounds, which could be characterized with two different mass spectra. Peak 5a provided $[M-H]^-$ ion at m/z 709 and fragment ions at m/z 563, 430 and 284, indicating the heterolytic cleavage of a desoxyhexose and the homolytic cleavage of a pentose and a desoxyhexose sugar substituent, respectively. Thus peak 5a was assumed to be kaempferol 3-*O*-desoxyhexosyl-pentoside-7-*O*-desoxyhexoside. Peak 5b presented a $[M-H]^-$ ion at m/z 577 and fragments at m/z 430 and 284, with losses of 147 and 146 amu, respectively, hence it was identified as kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside. Fragment ion at m/z 430 denotes a homolytic cleavage, while fragment at m/z 284 indicates a heterolytic cleavage [80]. Peak 6 ($t_R = 14.1$ min) exhibited $[M-H]^-$ ion at m/z 447 and fragments at m/z 284, 255 and 227 and was, therefore, identified as kaempferol 3-*O*-hexoside [75, 80]. Aglycone fragment at m/z 284 marks a homolytic cleavage, as described previously.

6.2.2. Fractions of *S. tectorum* 80% (v/v) methanolic extract

In order to enrich concentration of the constituents and to gain more information on contribution of distinct compound groups to antioxidant activity, houseleek 80% (v/v) methanolic extract was fractionated by Silicagel column chromatography. Fractions exhibiting DPPH scavenging activity were evaluated by HPLC. According to UV spectral data, in fractions eluted later from Silicagel column flavonoid compounds were detected, while fractions eluted foremost contained hydroxybenzoic and hydroxycinnamic acid derivatives.

Structural characterization of the compounds required tandem mass spectrometric analyses, results are shown in Table 9., while Fig. 19. presents BPC chromatograms of the fractions. Besides the six flavonol glycosides already described for the crude extract, nine additional kaempferol and quercetin glycosides were observed. While flavonols were present in almost all fractions, the five gallic acid derivatives and a caffeic acid derivative were observed in fractions eluted foremost from Silicagel column, which was in agreement with our results from HPLC studies. Enrichment of constituents by Silicagel column chromatography enabled detection of compounds in the fractions which were not observed for *S. tectorum* 80% (v/v) methanolic extract. Interpretation of (–)-ESI-MS/MS spectra was performed in a similar manner as by *S. tectorum* 80% (v/v) methanolic extract, *S. tectorum* leaf juice and *S. tectorum* ethanolic extract (see in sections 6.2.1. *S. tectorum* 80% (v/v) methanolic extract, 6.2.3. *S. tectorum* leaf juice and 6.2.4. *S. tectorum* ethanolic extract).

6.2.3. *S. tectorum* leaf juice

Houseleek leaf juice has been used extensively in traditional medicine, however data on its phytochemical composition are incomplete, therefore our purpose was to characterize the phenolic profile of the extract by LC-DAD-MS/MS methods. Phenolic acid and flavonoid glycoside profile of *S. tectorum* leaf juice was characterized for the first time. LC-DAD chromatograms detected at 266 and 350 nm are shown in Fig. 20. Table 10. shows the most frequent ions which characterize the fragmentation of flavonol *O*-glycosides and phenolic acids present in *S. tectorum* leaf juice.

6.2.3.1. Characterization of phenolic acids

Compounds 1a, 1b, 1c and 2a were identified as chlorogenic acid, isocitric acid, malic acid and aconitic acid, respectively, by comparison with literature data and mass spectra of reference compounds [204-205].

Compound 1a exhibited $[M-H]^-$ ion at m/z 353 and product ions at m/z 191 and 179, with the latter being present only with vanishing intensities. Fragment ion at m/z 173 was not observed, thus compound 1 was identified as 5-*O*-caffeoylquinic acid (chlorogenic acid) [71]. Fragmentation of compound 1b showing $[M-H]^-$ ion at m/z 191 yielded product ions at m/z 111, as the base peak and at m/z 155 and 85 with comparatively intense signals, therefore according to Bylund et al. [204], compound 1b could be distinguished from citric acid and was identified as isocitric acid. Compound 2a, representing $[M-H]^-$ ion at m/z 173 and fragment ions at m/z 155, 129, 111 and 85 with similar relative intensities, was characterized as aconitic acid, however, neither the *trans*, nor the *cis* isomeric form could be confirmed [204]. Presence of these simple organic acids, characteristic products of CAM (crassulacean acid metabolism) has been previously reported for *S. tectorum* [87], but it was confirmed for the first time by LC-MS/MS experiments. Peak 2c, presenting $[M-H]^-$ ion at m/z 169 and fragment ion at m/z 125, was identified as gallic acid, according to mass spectra of authentic standards, while peak 6 presenting the same fragment ions was characterized as a gallic acid derivative. Peaks 2b, 10a and 15b showed neutral loss of 162 amu and fragment ions at m/z 169 and 125, therefore those were tentatively characterized as galloyl hexoside, a derivative of galloyl dihexoside and a derivative of galloyl hexoside, respectively [206]. Compound 3, displaying $[M-H]^-$ ion at m/z 205 and fragment ions characteristic of shikimic acid at m/z 173, 83 and 73, was tentatively described as a derivative of shikimic acid [204]. For peak 4 were $[M-H]^-$ ion at m/z 359, fragment ion due to the neutral loss of 162 amu at m/z 197, and a fragment at m/z 153 observed. It was identified as syringic acid hexoside, according to mass spectrum of an authentic standard and literature data [206-207]. Compounds 7 and 11 showed fragment ions at m/z 163, 119 and 93 characteristic for coumaric acid, according to mass spectrum of reference compound and literature data, thus they were identified as coumaroyl hexoside and a derivative of coumaric acid [8, 206].

Peaks 8, 16 and 17, displaying fragment ions at m/z 173, 155, 129 and 111 characteristic of hydroxycinnamoyl esters of quinic acid, were identified as feruloylcoumaroylquinic acid, coumaroylquinic acid and a caffeoylquinic acid derivative, respectively [66, 68, 208].

6.2.3.2. Characterization of flavonol glycosides

According to data obtained by our HPLC-DAD-MS/MS experiments, solely flavonol glycosides were detected in *S. tectorum* leaf juice. Kaempferol glycosides prevail, while quercetin glycosides are less characteristic. For unambiguous identification retention times, UV and mass spectra of kaempferol and quercetin were compared to those of reference compounds. Stevens et al. [124] analyzed flavonoid aglycone composition of some *Sempervivum* species after acidic hydrolysis. They concluded that kaempferol was the principal flavonoid of all species, which is in accordance with our results. However, they studied flavonoid variation of houseleek only at the aglycone level and detailed data on glycosylation pattern of *S. tectorum* flavonols are neither to find in other literature sources.

In *S. tectorum* leaf juice ten flavonol glycosides were detected, among which seven compounds (9: kaempferol 3-*O*-rhamnosyl-glucoside-7-*O*-rhamnoside, 10b: rutin, 12: kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside, 13a: kaempferol 3-*O*-desoxyhexosyl-pentoside-7-*O*-desoxyhexosyl-hexoside, 14a: kaempferol 3-*O*-desoxyhexosyl-pentoside-7-*O*-desoxyhexoside, 15a: kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside and 19: kaempferol 3-*O*-hexoside) were reported by our research group for the extract of *S. tectorum* leaves prepared with 80% (v/v) methanol, while three additional flavonol glycosides were described for *S. tectorum* for the first time. Product ions $[Y_0-H]^-$ were present in mass spectra of compounds 9, 10b, 13a, 14a, 14b, 15a, 18 and 19 with high abundances, therefore we presumed, that these compounds are 3-*O*-glycosylated. Further observed was $[Y_0-2H]^-$ ion with high abundance in the product ion spectra of compounds 9, 13a, 14a, 14b and 15a, thus we assumed, that these compounds are glycosylated both at 3-*O* and at 7-*O* positions [75].

Compound 18 exhibited product ions $[Y_0]^-$, $[Y_0-H-CO-H]^-$ and $[Y_0-H-CO_2-H]^-$ at m/z 301, 271 and 255, respectively, characteristic of quercetin. $[Y^3_0-H]^-$ product ion at

m/z 300 with high abundance was observed, suggesting a homolytic cleavage of the 3-*O*-glycosidic bond [75, 80], thus this compound was identified as a glycoside of quercetin. Compound 18 exhibited $[M-H]^-$ ion at m/z 463 and fragments at m/z 301, 300, 271 and 255. Aglycone fragment at m/z 300 marks a homolytic cleavage of a hexose moiety, as described previously, therefore this component was identified as quercetin 3-*O*-hexoside.

Compounds 13b and 14b presented $[M-H]^-$ ions a m/z 969 and 807, respectively, 98 amu higher, than those of compounds 13a and 14a and a fragment ion at m/z 97, therefore they were tentatively characterized as fumaric acid esthers of compounds 13a and 14a.

6.2.4. *S. tectorum* ethanolic extract

Antioxidant activity of *S. tectorum* ethanolic extract was the highest in the applied *in vitro* antioxidant action assays, accordingly, our objective was its phytochemical screening, in order to reveal constituents attributing to radical scavenging effect. LC-DAD profile of *S. tectorum* ethanolic extract is shown in Fig. 21.A, while chromatographic and mass spectral data of compounds detected in the extract are shown in Table 11. Studying UV spectra of compounds yielded a preliminary structural description. Absorption maxima were characteristic of kaempferol and quercetin *O*-glycosides [49, 183], as well as of hydroxybenzoic and hydroxycinnamic acid derivatives [8]. Compound 1 and 8 showed absorption maxima at around 260 nm and at 290-300 nm, respectively, while peak 3 had its maximum at both wavelengths, thus they were preliminary described as hydroxybenzoic and / or hydroxycinnamic acid derivatives [8]. However, detailed structural survey of flavonol glycosides required tandem mass spectrometric analyses.

6.2.4.1. Characterization of phenolic acids

In *S. tectorum* ethanolic extract twelve phenolic acid derivatives were detected, among which nine compounds (2a: isocitric acid, 2b: malic acid, 4: gallic acid, 5: galloyl hexoside, 6: aconitic acid, 9b: coumaroyl hexoside, 10: coumaric acid derivative, 15a

coumaroylquinic acid and 17: galloyl hexoside derivative) were described for *S. tectorum* leaf juice (see section **6.2.3.1. Characterization of phenolic acids**). Additionally, compound 1 ($t_R = 1.30$ min) displaying $[M-H]^-$ ion at m/z 245 and fragment ion [coumaric acid- $H-CO_2$] $^-$ at m/z 119, characteristic of coumaric acid [66], was tentatively described as a derivative of coumaric acid.

6.2.4.2. Characterization of flavonol glycosides

In *S. tectorum* ethanolic extract twelve flavonol glycosides were detected, among which seven compounds (12: kaempferol 3-*O*-rhamnosyl-glucoside-7-*O*-rhamnoside, 14: quercetin 3-*O*-hexoside, 15c: kaempferol 3-*O*-desoxyhexosyl-pentoside-7-*O*-desoxyhexoside, 16: kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside, 18: kaempferol 3-*O*-desoxyhexosyl-pentoside-7-*O*-desoxyhexosyl-hexoside, 20: kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside and 21: kaempferol 3-*O*-hexoside) were described by our research group for the extract of *S. tectorum* leaves prepared with 80% (v/v) methanol and for *S. tectorum* leaf juice (see sections **6.2.1. *S. tectorum* 80% (v/v) methanolic extract** and **6.2.3.2. Characterization of flavonol glycosides**).

Relative abundance of $[Y_0-H]^-$ ion was higher in mass spectra of all flavonol compounds than that of $[Y_0]^-$ ion, therefore we presumed, that they are 3-*O*-glycosylated [80]. Further observed was $[Y_0-2H]^-$ ion with high abundance in the product ion spectra of compound 12, 13, 15c, 18 and 19 which is explained by the elimination of two glycosyl radicals at the 3-*O* and 7-*O* positions successively [75, 78], thus we assumed, that these compounds are glycosylated both at 3-*O* and at 7-*O* positions.

In the product ion spectra of compound 9, 11 and 13 were fragment ions $[Y_0]^-$ at m/z 301, $[Y_0^3-H]^-$ at m/z 300 and $[Y_0-H-CO-H]^-$ at m/z 271 observed and assigned to quercetin aglycone [75], thus these were characterized as quercetin glycosides. Compound 13 ($t_R = 6.60$ min) exhibited $[M-H]^-$ ion at m/z 609 and fragments at m/z 463, 300, 299, 271 and 255. Heterolytic cleavage of a desoxyhexose moiety was assumed at 7-*O* position to generate fragment ion at m/z 463, while aglycone fragment $[Y_0-H]^-$ at m/z 300 marked a homolytic cleavage of a hexose moiety. Presence of the $[Y_0-2H]^-$ ion together with the former one suggested that this component is a quercetin

3,7-*O*-diglycoside, therefore compound 13 was identified as quercetin 3-*O*-hexoside-7-*O*-desoxyhexoside. Compound 9 and 11 with retention times of 4.79 and 5.58 min, respectively provided $[M-H]^-$ ions at m/z 755 and fragment ions at m/z 447/446 and 301/300, indicating heterolytic and homolytic cleavages of a desoxyhexosyl-hexose and a desoxyhexose sugar substituent, respectively, thus compound 9 and 11 were assumed to be quercetin 3-*O*-desoxyhexosyl-7-*O*-desoxyhexosyl-hexoside isomers. Compounds 15c and 19 exhibiting $[M-H]^-$ ions at m/z 709 and fragment ions at m/z 563, 430, 284 and 283 were characterized as kaempferol 3-*O*-desoxyhexosyl-pentoside-7-*O*-desoxyhexoside isomers. Presence of one of the isomers was described for *S. tectorum* leaf juice (see in section **6.2.3.2. Characterization of flavonol glycosides**). Collision induced dissociation of the $[M-H]^-$ ion at m/z 479 of compound 15b resulted in product ions at m/z 317, 316 and 271, corresponding to $[Y_0]^-$, $[Y^3_0-H]^-$ and $[Y_0-H-CO_2-H]^-$ ions, respectively. These were characteristic of myricetin, additionally, product ion at m/z 316 with high abundance suggested a homolytic cleavage of a hexose moiety [75, 80], thus compound 15b was identified as myricetin 3-*O*-hexoside, the only myricetin compound detected in our samples and described by LC-MS/MS for *S. tectorum*.

6.2.5. *C. avellana* ethyl acetate and methanolic extract

6.2.5.1. Characterization of flavonol glycosides

Using complementary information given by DAD and MS/MS detectors, the characterization of eight phenolic compounds in *C. avellana* extracts was completed. LC-DAD chromatograms are shown in Fig. 22., while detected compounds are in displayed Table 12. Flavonol glycosides with three different aglycones were detected in *C. avellana* extracts (Fig. 29.). In the CID spectra of deprotonated flavonol glycosides fragment ions corresponding to the deprotonated aglycones, $[Y_0]^-$ at m/z 285, 301 and 317 generated by the loss of sugar units, furthermore the following fragment ions for aglycones were detected: $[Y_0-H]^-$ at m/z 284, 300 and 316, $[Y_0-H-CO-H]^-$ at m/z 255, 271 and 287 for kaempferol, quercetin and myricetin, respectively [75].

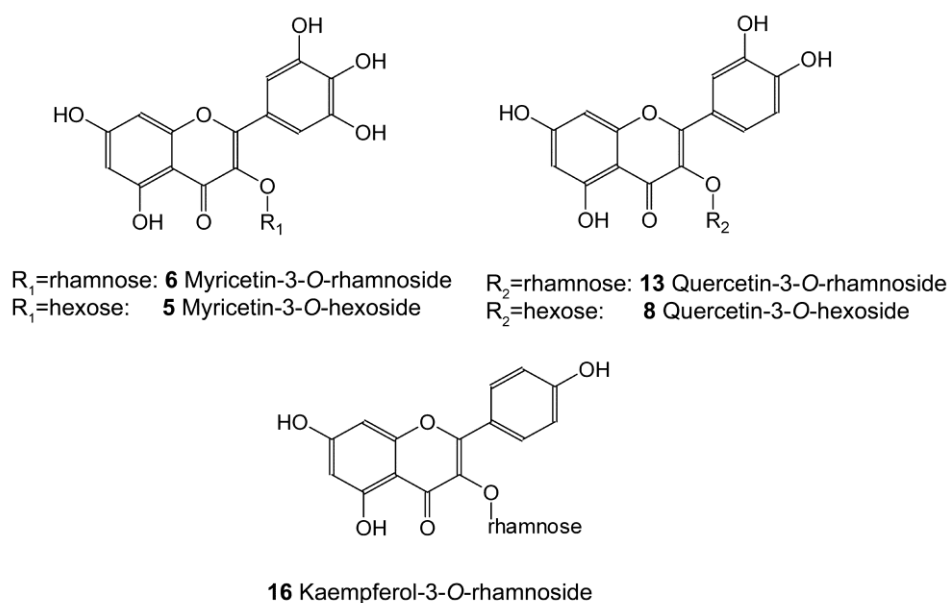


Fig. 29. Flavonol 3-*O*-monoglycosides identified in *Corylus avellana* extracts by LC-ESI-MS/MS.

Substitution position of sugar moieties were tentatively characterized, according to fragmentation pattern of the flavonol glycosides. The cleavage of the glycosidic bond in deprotonated myricetin, quercetin and kaempferol glycosides provided besides deprotonated flavonol aglycone [Y_0] $^-$ product ions (m/z 317, 301 and 285, respectively), radical aglycone ions [Y_0-H] $^{\bullet}$ (m/z 316, 300 and 284, respectively), deriving from homolytical cleavage of the sugar moieties. According to Hvattum and Ekeberg [80], presence of the abundant [$Y-H$] $^{\bullet}$ fragment ions in the mass spectra of flavonol glycosides suggests loss of sugar moieties from the 3-*O* position, therefore all flavonoid compounds were characterized as flavonol 3-*O*-glycosides. Relative intensity of [$Y-H$] $^{\bullet}$ fragments increased with the increasing number of hydroxyl groups attached to B-ring of flavonols. Relative intensity of [$Y-H$] $^{\bullet}$ fragment was the highest in the case of myricetin glycosides with three OH groups on B-ring (compound 6, Fig. 30.A), while it was approximately equal to that of the [$Y-H$] $^-$ fragment in the case of quercetin glycosides (compound 13, Fig. 30.B) and it was the lowest with kaempferol glycosides containing only one OH group in B-ring (compound 16, Fig. 30.C).

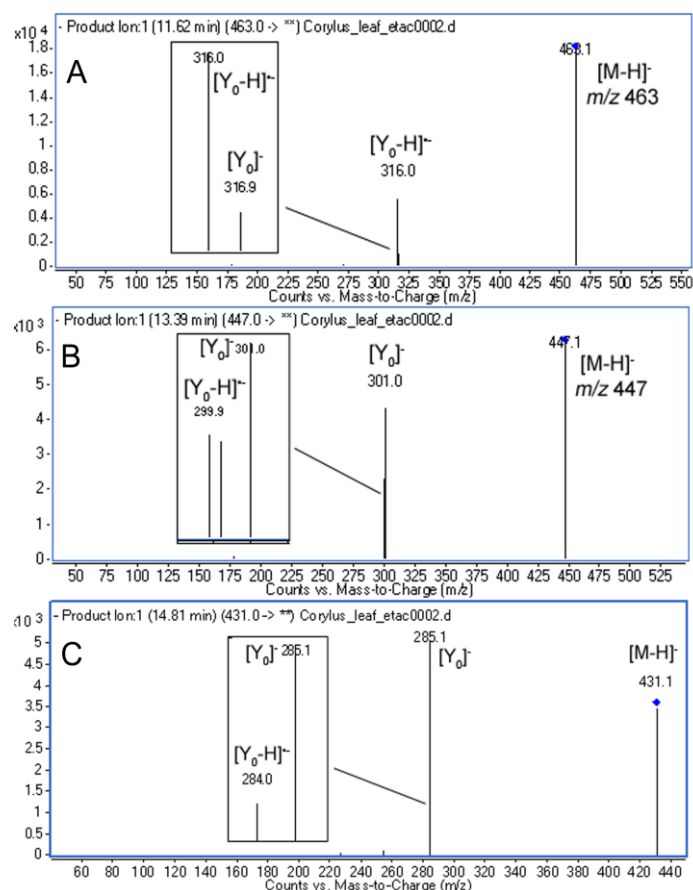


Fig. 30. (–)-CID spectra of A: myricetin 3-*O*-rhamnoside, B: quercetin 3-*O*-rhamnoside, C: kaempferol 3-*O*-rhamnoside.

[Response units vs. time (min)]. Collision energy of -15 eV was applied.

Compound 6 (t_R : 11.5 min) provided molecular ion $[M-H]^-$ at m/z 463 and fragments: $[Y_0]^-$ at m/z 317 and $[Y_0-H]^-$ at m/z 316, with neutral loss of 147 amu indicating the homolytic cleavage of a desoxyhexose moiety at 3-*O* position. Compound 6 was tentatively identified as myricitrin (myricetin 3-*O*-rhamnoside), since it was previously described for *C. avellana* leaves [168-169]. Compound 5 (t_R : 10.6 min), exhibiting pseudomolecular ion $[M-H]^-$ at m/z 479 and fragments $[Y_0]^-$ at m/z 317 and $[Y_0-H]^-$ at m/z 316 (with neutral loss of 163 amu), was analogously characterized as myricetin 3-*O*-hexoside. This compound was reported for *C. avellana* for the first time.

Compound 8 (t_R : 12.2 min) provided molecular ion $[M-H]^-$ at m/z 463 and fragments $[Y_0]^-$ at m/z 301 and $[Y_0-H]^-$ at m/z 300 and other pronounced fragments at m/z 271, 255 and 151. The neutral loss of 163 amu between m/z 463 and 300 denoted a homolytic cleavage of a hexose moiety from the 3-*O* position, thus compound 8 was identified as

quercetin 3-*O*-hexoside. The fragmentation of the pseudomolecular ion $[M-H]^-$ of compound 13 (t_R : 13.3 min) at m/z 447 yielded fragment ions $[Y_0]^-$ at m/z 301 and $[Y_0-H]^-$ at m/z 300, and other abundant fragments at m/z 271 and 151, therefore according to the considerations previously described for other hazel flavonol glycosides and to literature data [168-169], compound 13 was suggested to be quercetin 3-*O*-rhamnoside.

Compound 16 (t_R : 14.8 min) provided molecular ion $[M-H]^-$ at m/z 431, and fragment ions $[Y_0]^-$ at m/z 285 and $[Y_0-H]^-$ at m/z 284. The neutral loss of 146 amu indicated the elimination of a desoxyhexose moiety. Kaempferol 3-*O*-rhamnoside has been reported previously for hazel leaves [168], consequently compound 16 was tentatively identified as kaempferol 3-*O*-rhamnoside. Compound 18 (t_R : 17.9 min) exhibited molecular ion $[M-H]^-$ at m/z 577 with fragment ions at m/z 514, 285. The aglycone anion fragment $[Y_0]^-$ at m/z 285 pointed to kaempferol aglycone, while the neutral loss of 292 amu indicated a di(desoxyhexose) glycan part, therefore compound 18 was tentatively identified as kaempferol di(desoxyhexoside).

6.2.5.2. Characterization of hydroxycinnamic acid derivatives

Compound 1 (t_R : 2.2 min) exhibited molecular ion $[M-H]^-$ at m/z 377 and fragment ions at m/z 341, 215, 179 and 161. Based on the presence of characteristic product ions at m/z 179 and 161, compound 1 was tentatively identified as a caffeoyl hexoside derivative [66]. Compound 3 (t_R : 10.0 min) provided pseudomolecular ion $[M-H]^-$ at m/z 359 and product ions at m/z 329, 269, 191 and 161. Compound 3 was identified as rosmarinic acid by comparison of chromatographic, UV and mass spectral data with those of an authentic reference compound.

6.3. Quantitative analyses

6.3.1. Determination of hydroxycinnamic acid derivatives in *S. tectorum* leaf juice

The applied gradient elution ensured good resolution, thus proper selectivity for all hydroxycinnamic acid derivatives. Rosmarinic acid was not detected, however besides

caffeic and chlorogenic acid, presence of other caffeic acid derivatives was proved. LC-MS/MS studies also confirmed the results of HPLC-UV detection (see Table 10.).

This work presents selective LC-DAD quantitation method for two characteristic hydroxycinnamic acid derivatives of *S. tectorum*, providing good linearity, precision and accuracy. According to the literature, quantitative data regarding hydroxycinnamic acid derivative compounds of *S. tectorum* were not reported before.

6.3.2. Determination of kaempferol glycosides in *S. tectorum* leaf juice

6.3.2.1. Isolation with column chromatography

Successive Silicagel, Sephadex LH20 and polyamide column chromatography allowed effective isolation of the main flavonol glycoside compound and further characteristic flavonol glycosides of *S. tectorum* leaf juice. Purity of the isolated kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside was lower. In spite of the application of multiple column chromatographic steps, separation from the main contaminant kaempferol 3-*O*-desoxyhexosyl-pentoside-7-*O*-desoxyhexoside (30.3%, TIC) could not be carried out. Isolation process yielded 2.95 mg substance.

6.3.2.2. Quantitative analysis and method validation

Quantity of kaempferol 3-*O*- β -[α -rhamnopyranosyl-(1 \rightarrow 2)-glucopyranoside]-7-*O*- α -rhamnopyranoside in *S. tectorum* leaf juice was determined according to results from SRM (single reaction monitoring) and DAD (350 nm) experiments. Detection wavelength and the quantifier ion were designated by evaluation of the UV and mass spectra of the compound. The SRM mode provided high selectivity, the fragment ion m/z 593 (transition m/z 739 \rightarrow m/z 593) was chosen as quantifier ion. Kaempferol 3-*O*-rhamnosyl-glucoside-7-*O*-rhamnoside possessed absorption maxima at two wavelenghtes ($\lambda_{\max 1} = 266$ nm, $\lambda_{\max 2} = 350$ nm), nevertheless due to higher selectivity, detection wavelength of 350 nm was chosen for the LC-DAD quantitation method. The applied gradient elution ensured a good resolution for the main flavonoid compound, thus the selectivity was proper also for the DAD method.

Linear regression was achieved only by the LC-DAD method, while regression of the SRM method was quadratic polynomial (see Table 15). Thus DAD 350 nm method is recommended to evaluate samples with good chromatographic separation, while SRM method is to apply when high selectivity is required, e.g. in complex herbal or biofluid matrices or when kaempferol triglycoside occurs as a minor component. Linearity range was also evaluated at lower concentrations (0.025, 0.100, 0.500 and 1 $\mu\text{g/mL}$), appropriate accuracy at that range, however, was achieved neither by SRM, nor by DAD results.

Quantity of kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside and kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside in *S. tectorum* leaf juice was determined according to the results from SRM experiments. Quantifier ions were indicated by evaluation of the mass spectra of the compounds. Fragment ion m/z 285 (transition m/z 577 \rightarrow m/z 285) was chosen as quantifier ion for kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside and fragment ion m/z 285 (transition m/z 593 \rightarrow m/z 285) was chosen for kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside. The selected transition for kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside provided high selectivity, presence of the contaminant kaempferol 3-*O*-desoxyhexosyl-pentoside-7-*O*-desoxyhexoside (m/z 709) did not affect quantitation, since it exhibited fragment ions at distinct m/z values (see Table 10.).

Regression for both quantitations was quadratic polynomial (see Table 15.). Though the SRM mode provided high selectivity, the results should be treated carefully, since they could be influenced by ion suppression due to co-elution of an other compound with kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside and contamination of the isolated kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside with a similar flavonoid component. In order to eliminate of ion suppression effects deriving from other compounds present in the extract to the greatest possible extent, fragmentation optimization of target analytes was performed by the use of the whole extract, rather than that of the isolated compounds.

This work presents selective LC-MS/MS and LC-DAD quantitation methods for three characteristic flavonol glycosides of *S. tectorum* providing good linearity, precision and accuracy. According to the literature, quantitative data regarding flavonol glycoside compounds of *S. tectorum* had not been reported before.

7. CONCLUSIONS

- Flavonoid, total polyphenol, tannin, proanthocyanidin, anthocyanin and total hydroxycinnamic acid derivative contents of our samples were determined by spectrophotometric methods. In order to set the course of our phytochemical investigations, *in vitro* antioxidant activity of *Sempervivum tectorum* extracts prepared with solvents of different polarity, thus having distinct phenolic constitution was determined and compared. The methods applied (DPPH, ABTS and peroxy nitrite scavenging capacity) provided valuable information regarding varied phenolic composition of the extracts.
- Phytochemical characterization in phenolic content and composition of the extracts revealed correlations between antioxidant activity and phenolic compounds present in the extracts. Significant differences between *S. tectorum* 80% (v/v) methanolic extract and its fractions were observed. In addition, a synergistic antioxidative effect between the fractions – when they were present together in the whole 80% (v/v) methanolic extract – was presumed.
- LC–ESI–MS/MS in the negative ionization mode was used for the first time for analysis of flavonol *O*-glycosides, simple organic acids, hydroxybenzoic and hydroxycinnamic acid derivatives of houseleek. According to the results of radical scavenging assays, phytochemical investigation of *S. tectorum* extracts prepared with solvents of better selectivity for phenolics, i.e. ethanol and 80% (v/v) methanol, as well as that of the leaf juice, that is used in traditional medicine, was performed. Additionally, a Silicagel column chromatographic method was applied for fractionation of 80% (v/v) methanolic extract, in order to enrich minor compounds and to separate constituents belonging to distinct classes of phenolics.
- Six kaempferol mono-, di-, tri- and tetraglycosides and rutin were identified and tentatively characterized in *S. tectorum* 80% (v/v) methanolic extract. Glycosylation structure of *S. tectorum* flavonols was studied and described for the first time. In fractions of the 80% (v/v) methanolic extract nine further kaempferol and quercetin

glycosides, together with gallic acid and hydroxybenzoic acid derivatives were detected. Additionally, presence of a myricetin monohexoside and that of simple organic acids deriving from the crassulacean acid metabolism was proved for the ethanolic extract.

- This work also presents a fast and excessive qualitative HPLC–DAD–ESI–MS/MS method to characterize phenolic acid and flavonol glycoside composition of *S. tectorum* leaf juice. Selective LC–MS/MS and LC–DAD quantitation methods for its main flavonoid compound were developed, validated and compared, in order to apply them in quality control of *S. tectorum*. Furthermore, LC–MS/MS methods for quantitative determination of additional two characteristic kaempferol glycosides were developed. To complement phytochemical characterization of the leaf juice, quantitation of some of its hydroxycinnamic acid derivatives was performed by an HPLC-UV method.
- For comprehensive characterization of phenolic profile of *Corylus avellana* leaves, samples prepared by successive extraction with different solvents were studied. The applied HPLC–DAD–ESI–MS/MS method was utilized successfully for the investigation of flavonol glycoside compounds in *C. avellana* extracts. Besides flavonol 3-*O*-rhamnosides that have been previously described in the literature for *C. avellana* leaves cultivated in Portugal, flavonol 3-*O*-hexosides were detected in the extracts of *C. avellana* grown wild in Hungary.
- Our results confirmed that negative ionization tandem mass spectrometry coupled to high-performance liquid chromatography provided an appropriate, selective and sensitive tool for qualitative, as well as for quantitative evaluation of phenolic compounds present in extracts of traditional herbal remedies. Although it did not allowed unambiguous structural identification regarding isomers, stereochemistry of glycan substituents, etc., evaluation of presence and / or relative intensity of diagnostic fragment ions deriving from collision-induced dissociation of compounds enabled tentative characterization of flavonol glycosides, as well as that of phenolic acids.

8. SUMMARY

Quality control is essential for innovation of standardized herb extracts. However, it can be difficult, since medicinal plant extracts – and particularly those containing phenolics – are complex mixtures of numerous compounds of great structural variability.

Sempervivum tectorum L. and *Corylus avellana* L. have been used in traditional medicine primarily for their anti-inflammatory effects. Flavonoids and other phenolic acids have been attributed to their pharmacological actions. Aim of our work was comprehensive characterization of the phenolic profile of *S. tectorum* and *C. avellana* by electrospray ionization tandem mass spectrometry coupled to high-performance liquid chromatography, with particular attention to their flavonoid compounds.

We revealed correlation between polyphenol content and radical scavenging activity of *S. tectorum*, and analyzed phenolic composition of extracts showing the highest antioxidant capacity. Simple and fast HPLC methods were developed for separation of flavonoids and phenolic acids in *S. tectorum* 80% (v/v) methanolic and ethanolic extract, as well as in leaf juice. LC–ESI–MS/MS in the negative ionization mode was used for the first time for structural analysis of flavonol *O*-glycosides, simple acids, hydroxybenzoic and hydroxycinnamic acid derivatives of houseleek. In addition, we developed selective and validated HPLC-UV and HPLC-DAD-ESI-MS/MS methods for quantitation of the main kaempferol 3-*O*-glycoside and caffeic acid derivative components in *S. tectorum* leaf juice, which has been used traditionally as a medication against inflammation of the ears.

Data concerning flavonol glycoside profile of *C. avellana* leaves have been complemented by the use of an HPLC-DAD-ESI-MS/MS method. Besides flavonol 3-*O*-rhamnosides, previously described for *C. avellana* leaves, we detected flavonol 3-*O*-hexosides in the extracts.

According to our results, HPLC-ESI-MS/MS in negative ionization mode provided a selective and sensitive tool for qualitative, as well as for quantitative evaluation of phenolic compounds present in extracts of traditional herbal remedies.

9. ÖSSZEFOGLALÁS

Standardizált növényi készítmények fejlesztéséhez elengedhetetlen a minőségbiztosítás. Megnehezíti a feladatot, hogy a gyógynövények kivonatai – és különösen a fenoloid vegyületeket tartalmazók – számos, szerkezetileg nagyon különböző komponensből álló komplex elegyek.

A *Sempervivum tectorum* L. és *Corylus avellana* L. levelét a tradicionális gyógyászatban elsősorban gyulladáscsökkentő hatása miatt alkalmazzák. Farmakológiai hatásait flavonoid és egyéb fenol-sav komponenseikkel hozták összefüggésbe.

Munkánk célja volt a *S. tectorum* és *C. avellana* fenoloid összetételének átfogó vizsgálata, különös tekintettel flavonoid vegyületeikre. Munkánk során nagyhatékonyságú folyadékkromatográfiával kapcsolt és elektroporlasztásos ionforrással szerelt tandem tömegspektrometrás módszereket alkalmaztunk.

Összefüggést találtunk a vizsgált *S. tectorum* kivonatok összes polifenol tartalma és szabadgyökfogó aktivitása között, a továbbiakban a legnagyobb antioxidáns kapacitással rendelkező kivonatok fenoloid összetételét vizsgáltuk. Egyszerű és gyors HPLC módszereket fejlesztettünk *S. tectorum* 80% (v/v) metanollal és etanollal készült kivonatok, valamint présnedv flavonoid és fenolkarbonsav komponenseinek elválasztására. Elsőként alkalmaztunk LC–ESI–MS/MS módszert negatív ionizációval a kövirózsa flavonol-*O*-glikozid, egyszerű sav, hidroxibenzoe- és hidroxifahéjsav származékainak vizsgálatára. Szelektív HPLC-UV és HPLC-DAD-ESI-MS/MS módszereket fejlesztettünk és validáltunk a tradicionális gyógyászatban leggyakrabban alkalmazott *S. tectorum* présnedv legfontosabb kempferol-3-*O*-glikozidjainak és kávésav-származékainak mennyiségi meghatározására.

Kiegészítettük a *C. avellana* levél flavonol-glikozidjaira vonatkozó adatokat HPLC-DAD-ESI-MS/MS módszerek alkalmazásával. A már korábban is detektált flavonol-3-*O*-ramnozidok mellett flavonol-3-*O*-hexozidok jelenlétét igazoltuk.

Eredményeink megerősítették, hogy a negatív ionizációs HPLC-ESI-MS/MS módszerek szelektív és érzékeny eszközt biztosítanak a vizsgált tradicionális gyógynövények fenoloid vegyületeinek kvalitatív és kvantitatív vizsgálatához.

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LIST OF PUBLICATIONS**Papers of the author in the scope of the dissertation**

Riethmüller E, **Alberti Á**, Tóth G, Béni Sz, Ortolano F, Kéry Á. (2013) Characterization of diaryl-heptanoid- and flavonoid-type phenolics in *Corylus avellana* L. leaves and bark by HPLC/DAD-ESI/MS. *Phytochemical Analysis*, 24: 493-503.

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ACKNOWLEDGEMENTS

I wish to thank

Dr. Anna Blázovics, D.Sc. director of the Department of Pharmacognosy, who has provided me the opportunity to complete my doctoral studies in the department.

Dr. Éva Szőke, D.Sc. chairwoman of the Doctoral School of Pharmaceutical Sciences, former head of the department, who has always ensured the chance for me to carry on my research work in the Department of Pharmacognosy and has always supported the improvement of my scientific skills.

Dr. Ágnes Kéry, Ph.D. my tutor, for her guidance and kind support during my Ph.D. studies and research work. Besides her valuable professional and human advices, I am especially grateful to her for her confidence in me as she taught me self-reliance and let me accomplish my ideas.

Dr. László Kursinszki, Ph.D., who was always ready to help me concerning chromatographic questions.

Dr. György T. Balogh, Ph.D. and **Árpád Könczöl** (Compound Profiling Laboratory, Gedeon Richter Plc.), for their generous help and for the opportunity to work together.

Dr. Balázs Blazics, Ph.D. and **Dr. Szabolcs Szarka**, Ph.D. former colleagues, for the invaluable discussions and advices regarding chromatography and mass spectrometry.

Dr. Szabolcs Béni, Ph.D. (Department of Pharmaceutical Chemistry, Semmelweis University), who performed NMR analyses and helped readily in any questions.

Dr. Mahmoud Al-Khrasani, Ph.D. and **Erzsébet Lackó** (Department of Pharmacology and Pharmacotherapy, Semmelweis University), who carried out antinociceptive assays.

Dr. Eszter Riethmüller, **Dr. Péter Bányai** and **all the Ph.D. student fellows** for their help and the good times we had in- and outside the laboratory.

Anna Kriston who shared her know-how of laboratory work with me.

Rudolfné Mathuny for her indispensable help in lyophilization of the samples.

Lenke Tóth and **all colleagues**, who helped me during my Ph.D. studies.

Finally, I thank my **family** and **husband, Ádám** for their support, patience and encouragement.