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. Theses

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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 relevation 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 adequat 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 taditional 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.

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 exracts. We aimed to reveal the correlation between phenolic composition and radical scavenging activity of *S. tectorum* extracts.

- Ojective 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.

- Penolics 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.

MATERIALS AND METHODS

Plant material

Sempervivum tectorum was cultivated at the Research Station of the Corvinus University of Budapest, in Soroksár, leaf samples were collected in June 2007. Leaves of *Corylus avellana* were collected in Nógrád, Pest County, Hungary (June 2010).

Sample preparation

10-10 g lyophilized *S. tectorum* leaf samples were extracted with 500 mL chloroform, aceton, ethanol, 70% (v/v) ethanol, methanol and 80% (v/v) methanol in a Soxhlet apparatus. Additionally, the extract containig 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. A parallel of houseleek extract prepared with 80% (v/v) methanol was fractionated with Silicagel column chromatography. The sample was

eluted with 100–100 mL chloroform-methanol-water eluent mixtures of incresing polarity. For the preparation of houseleek decoction, 5 g lyophilized *S. tectorum* leaf sample was extracted with 500 mL hot water and allowed to infuse for forty minutes. For preparation of houseleek leaf juice, the sap of the leaves was squeezed fresh after collection and dried by lyophilization. The dried sap was redissolved in 80% (v/v) methanol, the removal of precipitated polysaccharides was performed by centrifugation. Dried *C. avellana* leaves (10 g) were extracted with 250 mL of *n*-hexane and chloroform in a Soxhlet apparatus consecutively, following ethyl acetate and ultimately methanol extraction.

Prior to evaluation all samples were submitted to SPE purification (500 mg/3 mL, LC-18 SPE cartridges) and filtered through regenerated cellulose syringe filters.

Quantitative phytochemical analyses

Contents of the main constituents in *S. tectorum* samples: flavonoids, total polyphenols, tannins, hydroxycinnamic acid derivatives, proanthocyanidins and anthocyanins were determined according to the methods of the Ph. Hg. VIII.

Antioxidant activity assays

Antioxidant activity of *S. tectorum* samples was determined by spectrophotometry in two *in vitro* decolorization assays using $ABTS^{\bullet+}$ [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] and $DPPH^{\bullet}$ [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. *S. tectorum* extracts prepared with solvents of different polarity, 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.

Peroxynitrite scavenging activity was determined in pyrogallol red bleaching test, an *in vitro* colorimetric assay, detection was performed at 542 nm. *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. Antioxidant activity was characterized as IC_{50} value (μg/mL).

HPLC and LC-MS/MS experiments

Instrumentation

Chromatographic separation was carried out with an ABL&E-Jasco system. Chromatographic separation with on-line mass spectral analyses were performed with an Agilent 6410B triple quadrupole equipped with an electrospray ionization source (ESI) coupled to an Agilent 1100 HPLC system.

For unambiguous identification retention times, UV and mass spectral data of compunds were compared to literature data and to those of authentic standards, where available.

Chromatographic conditions

All extracts both in qualitative and quantitative analyses were separated on C18-bonded silica columns. Binary gradient elution systems consisting of acetate or formate buffer in water and methanol or acetonitrile as organic modifier were applied.

Quantitative analyses

Quantity of hydroxycinnamic acid derivatives in *S. tectorum* leaf juice was determined by UV chromatograms, detected at 320 nm, while that of kaempferol glycosides was achieved in selected reaction monitoring (SRM) mode. Quantity of the main flavonoid compound was determined also by UV chromatograms detected at 350 nm. Before quantitation both fragmentor voltage and collision energy were optimized by parameter ramping with the use of the sample exract.

Quantitations were performed with the external standard calibration method. Standard solutions for the calibration were prepared at five different concentrations by the use of authentic reference compounds (caffeic acid, chlorogenic acid and rosmarinic acid) and the three isolated flavonol glycoside compounds (kaempferol 3-*O*-rhamnosyl-glucoside-7-*O*-rhamnoside, kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside and kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside) as standards, for determination of hydroxycinnamic acids and flavonol glycosides, respectively. Quality control samples 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* leaf juice.

Method recovery was tested by using the fortified sample recovery test, lyophilized *S. tectorum* leaf juice was spiked with known quantities of the standard solutions, in three parallels. Recovery (R) was calculated as $R = 100 (C_{found} - C_{initial}) / C_{added}$, where C_{found} = measured concentration in the fortified sample, $C_{initial}$ = initial concentration in the sample, C_{added} = concentration in the standard solution used.

Isolation of kaempferol glycosides was carried out by separation of 80% (v/v) methanolic *S. tectorum* extract with consecutive column chromatographic methods. Stationary phases applied (Silicagel 60, Sephadex LH20, MN polyamide SC-9) were chosen and combined subserviently, according to each analytes. In order to separate the analytes from polysaccharides, water soluble organic acids and other matrix constituents, the fractions were purified with phenyl SPE cartridges (500 mg/3 mL). Identity of the main flavonol compound was assayed by NMR spectroscopy. NMR experiments were carried out on a 600 MHz Varian DDR NMR spectrometer.

RESULTS

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, 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 of *S. tectorum* extracts prepared with solvents of different polarity were determined, results are expressed as pyrogallol.

Antioxidant activity assays

Antioxidant activity of *S. tectorum* extracts prepared with solvents of different polarity was higher in the assay containing $ABTS^{\bullet+}$ radical as compared to that containing $DPPH^{\bullet}$. Antioxidant activity data were plotted as IC_{50}^{-1} (mL/μg) against total polyphenol contents

(g/100 g) 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 $DPPH^{\bullet}$, as well as $ABTS^{\bullet+}$ radicals. 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.

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: $IC_{50} > 5000$ μg/mL, 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.

Peroxynitrite scavenging activity of houseleek leaf juice and that of extracts prepared with solvents of better selectivity for phenolics (70%, v/v ethanol and 80%, v/v methanol) was notably lower as compared to the standard solutions. *S. tectorum* samples were characterized by domination of kaempferol constituents glycosylated at the 3-*O* position, consequently neither structural requirements for peroxynitrite scavenging capacity – catechol group in B-ring or unsubstituted OH group at the 3-*O* position — were present. As the only exception, the scavenging activity of the 80% (v/v) methanolic extract was of the same order as that of rutin.

Qualitative HPLC and LC-MS/MS analyses

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, 301 and 317, generated by the loss of sugar units, furthermore the following fragment ions were observed: $[Y^3_0–H]^{\bullet-}$ 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. 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. According to the literature, relative abundance of the radical aglycone anion $[Y_0–H]^{\bullet-}$, deriving from homolytic cleavage of a sugar moiety, compared to that of the aglycone anion $[Y_0]^-$, deriving from a heterolytic cleavage, is in correlation with the position of glycosylation. Presence of the abundant $[Y_0\text{-}H]^{\bullet-}$ fragment ions in the mass spectra of flavonol glycosides

suggests loss of sugar moieties from the 3-*O* position The formation of the $[Y_0\text{-}2H]^-$ ion is explained by the elimination of two glycosyl radicals at the 3-*O* and 7-*O* positions successively. Substitution position of sugar moieties were tentatively characterized, according to fragmentation pattern of the flavonol glycosides.

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

According to the results of scavenger capacity assays, our further objective was high-performance liquid chromatographic evaluation of *S. tectorum* 80% (v/v) metanolic extract. Absorption maxima of distinctive UV spectra of flavonol compounds were characteristic of kaempferol and quercetin *O*-glycosides.

ESI-MS full scan mode analyses were performed, in order to identify the deprotonated molecular ions $[M\text{-}H]^-$, 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. In the 80% (v/v) methanolic extract of *S. tectorum* rutin and six kaempferol glycosides of different sugar composition (kaempferol 3-*O*-desoxyhexosyl-hexoside-7-*O*-desoxyhexoside, kaempferol 3-*O*-hexosyl-7-*O*-desoxyhexoside, kaempferol 3-*O*-desoxyhexosyl-pentoside-7-*O*-desoxyhexosyl-hexoside, kaempferol 3-*O*-desoxyhexosyl-pentoside-7-*O*-desoxyhexoside, kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside and kaempferol 3-*O*-hexoside) were described or tentatively characterized by LC–MS/MS.

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) metanolic extract was fractionated by Silicagel column chromatography. Fractions 4–9 of houseleek 80% (v/v) metanolic extract were evaluated by HPLC. According to UV spectral data, in fractions eluted later from Silicagel column were several flavonoid compounds detected, while fractions eluted foremost contained hydroxybenzoic and hydroxycinnamic acid dervatives.

In fractions of 80% (v/v) methanolic extract of *S. tectorum* sixteen flavonol glycosides and five phenolic acid derivatives were detected and tentatively characterized by LC–MS/MS. Besides the compounds described for the whole extract, five additional quercetin mono-, di- and triglycosides and four kaempferol di- and triglycosides were observed. While flavonols

were present in almost all fractions, the five caffeic, coumaric and gallic acid derivatives were detected in fractions eluted foremost from Silicagel column. Enrichment of constituents by Silicagel column chromatography enabled detection of compounds in the fractions which were not observed for the crude extract.

S. tectorum leaf juice

Ten flavonol glycosides and sixteen simple organic and phenolic acids were detected in *S. tectorum* leaf juice: in addition to flavonol glycosides described for the 80% (v/v) methanolic extrct and for its fractions, fumaric acid esters of kaempferol 3-*O*-desoxyhexosyl-pentoside-7-*O*-desoxyhexosyl-hexoside and kaempferol 3-*O*-desoxyhexosyl-pentoside-7-*O*-desoxyhexoside, acids revealing the crassulacean acid metabolism (isocitric acid, malic acid, etc.), glycosides and other derivatives of hydroxybenzoic (gallic acid) and hydroxycinnamic acids (coumaric, caffeic and ferulic acid).

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. UV spectra of compounds detected in the ethanolic extract of *S. tectorum* were characteristic of kaempferol and quercetin *O*-glycosides, as well as of hydroxybenzoic and hydroxycinnamic acid derivatives.
Twelve flavonol glycosides and ten organic and 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, simple phenolics were principally characterized as galloyl glycosides and esters of coumaric acid, while among flavonols kaempferol glycosides prevailed (with seven mono-, di-, tri- and tetraglycosides), quercetin glycosides were less frequent (with four mono-, di- and triglycosides) and myricetin 3-*O*-hexoside was the only myricetin constituent detected in our samples.

C. avellana ethyl acetate and methanolic extracts

Studies on the extracts of *C. avellana* leaves revealed that flavonoids were the major constituents of the samples. In the ethyl acetate extract five flavonoid glycosides (myricetin 3-*O*-desoxyhexoside, quercetin 3-*O*-hexoside, quercetin 3-*O*-desoxyhexoside, kaempferol di(desoxyhexoside), kaempferol 3-*O*-desoxyhexoside) and rosmarinic acid were detected. The

main compound of the extract was myricetin 3-*O*-rhamnoside (myricitrin). In the methanolic extract, additionally to rosmarinic acid and the flavonoids mentioned above, myricetin 3-*O*-hexoside and one caffeic acid derivative was detected. The main compound of this extract was also identified as myricitrin.

Quantitative analyses

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

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 and mass spectra of the reference compounds. The applied gradient elution ensured good resolution, thus proper selectivity for all hydroxycinnamic acid derivatives. The relative standard deviation for intra- and inter-day precision was < 15% for all compounds, while intra- and inter-day accuracy ranged from 73.0% to 125.4%. Regression was quadratic polynomial for all analytes. Caffeic acid and chlorogenic acid content of the leaf juice was 2.69 ± 0.06 mg/100 g dried juice (RSD%: 2.08) and 3.54 ± 0.27 mg/100 g dried juice (RSD%: 7.68), respectively. Quantity of rosmarinic acid was < LOD. Results for method recovery were 87.19% (RSD%: 1.06), 82.41% (RSD%: 1.11) and 88.26% (RSD%: 1.16), for caffeic acid, chlorogenic acid and rosmarinic acid, respectively.

Determinatin of kaempferol glycosides in S. *tectorum* leaf juice

Quantity of kaempferol 3-*O*-β-[α-rhamnopyranosyl-(1→2)-glucopyranoside]-7-*O*-α-rhamnopyranoside (structure was confirmed by NMR spectroscopy) 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 → *m/z* 593) was chosen as quantifier ion. The applied gradient elution ensured good resolution for the main flavonoid compound, thus the selectivity was proper also for the DAD method. Kaempferol 3-*O*-rhamnosyl-glucoside-7-*O*-rhamnoside content of the 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. Quantifier ions were indicated by evaluation of mass spectra of the compounds. Fragment ion *m/z* 285 (transition *m/z* 577 → *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 → *m/z* 285) was chosen for kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside. Kaempferol 3-*O*-desoxyhexoside-7-*O*-desoxyhexoside and kaempferol 3-*O*-hexoside-7-*O*-desoxyhexoside content of the 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.

Results for method recovery ranged from 71.61% to 110.43%. 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%.

CONLCUSIONS

- 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 peroxynitrite 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 cultivated *C. avellana* leaves, two flavonol 3-*O*-hexosides and a kaempferol disaccharide 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 intensitiy of diagnostic fragment ions deriving from collision-induced dissociation of compounds enabled tentative characterization of flavonol glycosides, as well as that of phenolic acids.

SUMMARY

Quality control is essential for innovation of standardized herb extracts. However, it can be difficult, since medicinal plant extracts – and particulary 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.

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