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Polyphenol composition and antioxidant capacity of *Epilobium* species

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ABSTRACT

Epilobium species (*Onagraceae*) are commonly used herbal remedies in traditional, adjuvant therapy of benignus prostate hyperplasia (BPH), however the pharmacological and clinical standardization of commercially available *Epilobii herba* (willow-herb) remains difficult. Willow-herb products usually consist of mixtures from various species, with different phenoloid content, often only partially identified. The present study reports comprehensive LC–MS/MS investigation on the polyphenol composition of the most common *Epilobium* species, emphasizing the pharmaceutical importance of a uniform standardization protocol in case of their products. The antioxidant capacity of species was evaluated by a simple spectrophotometric method, using $ABTS^{\bullet+}$ (2,2′azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)).

High ratio of macrocyclic tannins, mainly oenothein B was identified in all *Epilobium* species examined. Flavonoid composition of *Epilobium* extracts showed several differences, especially comparing *E. angustifolium* to other species. Myricetin, quercetin, kaempferol and their various glycosides were dominant in samples, but their combination and ratio were distinctive in all cases. *Epilobium* extracts showed high radical-scavenger activity, comparable to that of well-known antioxidants, Trolox and ascorbic acid. Among species examined, extract of *Epilobium parviflorum* possessed the highest antioxidant capacity (EC_{50} = 1.71 ± 0.05 μg/ml).

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1. Introduction

Benign prostatic hypertrophy (BPH) is the most frequently occurring neoplasm in men. Its prevalence over 60 years of age is between 40% and 70%, while nearly 80% of men will develop BPH during their lifetime [1,2]. BPH is developed by a complex pathological process. Hormone imbalance, inflammation, oxidative stress as well as decreased apoptosis all play role in its evolvement [3–12]. Fifteen years ago the only treatment for BPH has been surgical. Since then, several promising medicines became available and the WHO has defined standards for medical BPH-trials. α_1-Adrenoceptor antagonists (e.g. prazosin, doxazosin, bunazosin, alfuzosin, tamsulosin, indoramin) and 5α-reductase inhibitors (e.g. finasteride, dutasteride) fulfil the WHO standards and they are proved to be clinically efficient in the therapy of BPH. However, when applied in a long-term therapy, they may cause several adverse effects, such as low blood pressure, ejaculatory disfunction or impotence [13–17]. Henceforth, due to complications associated with surgical procedure and the side effects of the afore-mentioned medicines, application of phytotherapy has been increasing significantly in the prophylaxis and treatment of BPH. The most commonly used products in Europe are composed of red stinkwood (*Pygeum africanum* (Hook.)), saw palmetto (*Serenoa repens* (Batr.)), pumpkin seed (*Cucurbita pepo* (L.)), stinging nettle (*Urtica dioica* (L.)) and willow-herb (*Epilobium parviflorum* (Schreb.)). These phytomedicines usually show multidirectional effect, thus yielding excellent results in the reduction of symptoms linked to prostate hypertrophy [18,19]. All of the major BPH-guidelines (of EAU, AUA or the German Urologists) acknowledge that plant extracts can play important role in the prophylaxis and in the treatment as supplements, emphasizing their most important advantage, the absence of adverse effects. However, due to the lack of evidences of clinical efficacy proved in long-term, placebo controlled, multicenter, clinical trials, none of these guidelines recommend phytotherapy alone in evolved stages of BPH [13].

Among the afore-mentioned herbal remedies *Epilobium* extracts are not the best known, but increasingly applied in the complementary therapy of BPH. According to our non-representative research via world wide web, at least three different *Epilobium* products (mostly as therapeutical teas or dietary supplements) are commercially available in each of EU member states. In United States and Australia the variety of *Epilobium*-based supplements is even wider.

The genus *Epilobium* (*Onagraceae*) consists of over 200 species, 27 of them native to Europe, 12 native to Hungary. Most of them are characterized by very similar external habit. However, none of

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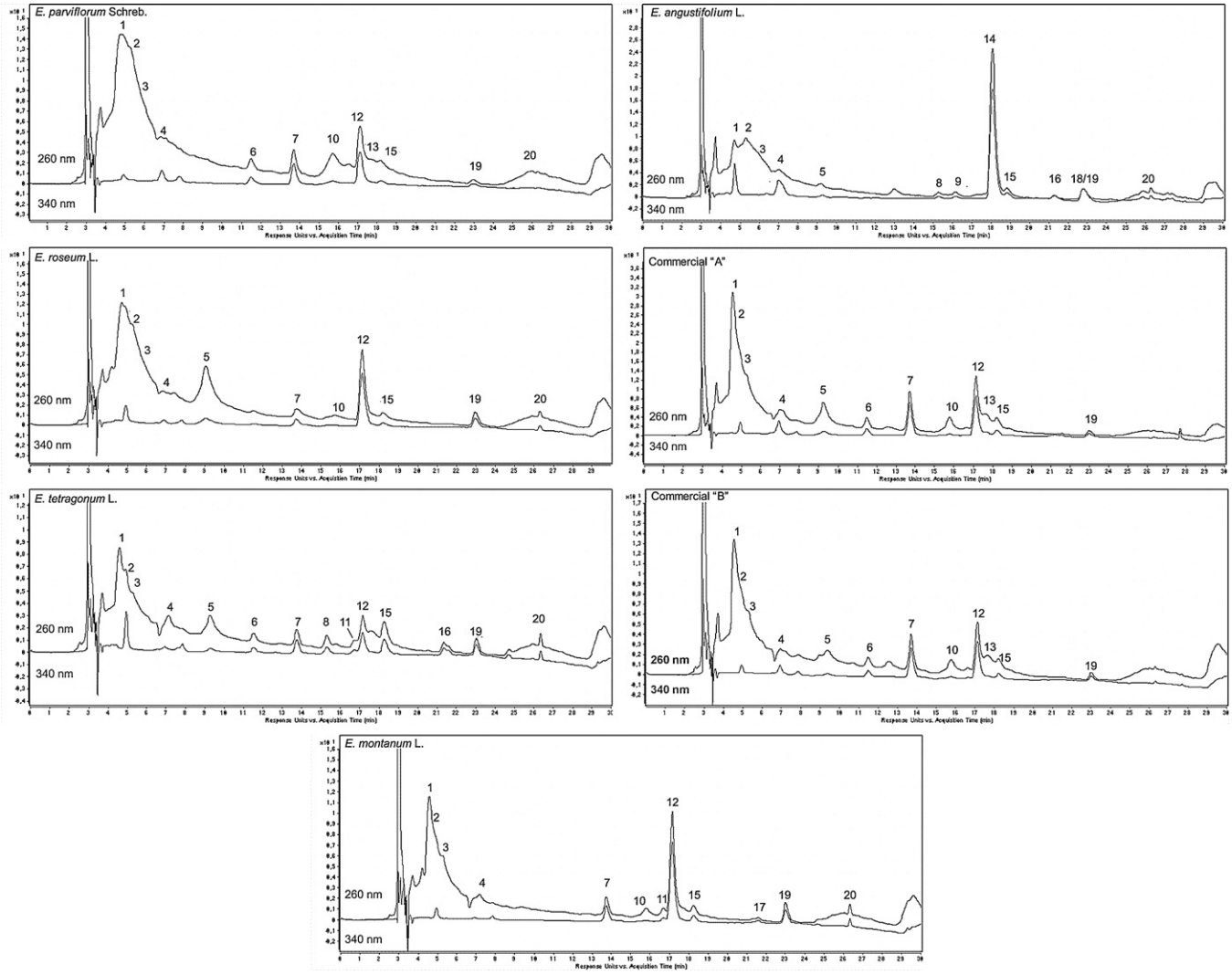


Fig. 1. UV chromatograms of *Epilobium* samples. Top line: recorded at 260 nm; bottom line: recorded at 340 nm.

the *Epilobium* species has ESCOP monography and none of them is registered in European Pharmacopoea, the most comprehensive scientific investigations have been made so far on two species: *Epilobium angustifolium* (L.) and *E. parviflorum* (Schreb.) [20–29]. Due to the notoriety of these two species, they are most often indicated as main components of purchasable willow-herb products, however Hungarian experiences contradict this. Because of the botanical similarity of diverse *Epilobium* species and their limited and variable presence in nature, willow-herb products available in Hungarian market often contain mixtures of even three or four species. Owing to the differences in composition of *Epilobium* varieties, biological equivalency of these commercial samples is debatable [28,30,31]. Therefore, the need of a uniform, evidence-based standardization of these supplements is increasing.

Determination of the biologically active compounds of *Epilobium* extracts is still ongoing, but several studies highlight the therapeutic importance of polyphenols and phytosterols [18,32,33]. Phenolic compounds are proved to have significant antioxidant capacity, while phytosterols inhibit Na+/K+-ATPase activity of prostatic membranes, suppressing the metabolism of the prostate in pathological cases [34–40]. One of the main tannin components of *Epilobium*, oenothein B is reported to inhibit prostatic 5α-reductase and aromatase enzymes, thus indirectly moderating the hormone imbalance, which is the main promoter of BPH [22,41].

The aim of this study was the more detailed investigation on the polyphenol composition of some *Epilobium* species, in order to find the possibly occurring differences among them, and to aid the establishment of a uniform, evidence-based standardization of their products. Since substances with antioxidant activity may be beneficial in the treatment of BPH [11,12,42], data on the antioxidant capacity of different *Epilobium* samples are also reported.

2. Experimental

2.1. Plant material

Five *Epilobium* species native to Hungary: *E. parviflorum* (Schreb.) (cultivated plant material; voucher No.: EPP0607/S), *E. angustifolium* (L.), *Epilobium montanum* (L.), *Epilobium tetragonum* (L.), and *Epilobium roseum* (L.) (collected plants: Budai-hegység, Mátra, Hungary; voucher Nos.: EPA0707, EPM0607, EPT0607, EPR0607) and two commercially available *E. parviflorum* samples (hereinafter marked with A and B) were studied. Plant material was identified macroscopically and microscopically in the Department of Pharmacognosy, Semmelweis University, Budapest, where the samples and herbarium specimen are deposited.

Table 1
Retention time and MS/MS data of the components observed in *Epilobium* extracts.

	Component	Rt (min)	$[M-H]^-$	Product ions (*m*/*z*)
1	Oenothein B	3.0–7.3	1567/783 $[M-2H]^{2-}$	915, 765, 450, 301
2	Different polyphenols	3.0–7.6	1473, 1208, 1065, 923	–
3	Caffeic acid–pentose ester	5.9	311	179, 149, 135
4	Chlorogenic acid	7.2	353	191, 135
5	Not identified	9.2	381	300, 283,229,185
6	Myricetin-3-O-hexose-gallate	11.8	631	479, 316, 151
7	Myricetin-3-O-hexoside	14.05	479	316, 287, 271, 179, 151
8	Not identified	15.5	615	300, 169
9	Quercetin-3-O-hexose-gallate	16.4	615	463, 300, 151
10	Ellagic acid–pentoside	16.5	433	300, 271, 228
11	Myricetin-3-O-pentoside	16.9	449	316, 287, 151
12	Myricetin-3-O-rhamnoside	17.3	463	316, 271, 179, 151
13	Not identified	18.0	301	284, 245, 200
14	Quercetin-7-O-glucuronide	18.2	477	301, 255, 179,151
15	Ellagic acid–hexoside	18.7	463	300, 271, 255
16	Quercetin-3-O-pentoside	21.5	433	300, 271, 255, 151
17	Kaempferol-3-O-hexoside	22.0	447	255, 227, 151
18	Kaempferol-7-O-glucuronide	22.9	461	285, 257, 229, 211, 169, 151
19	Quercetin-3-O-rhamnoside	23.2	447	300, 271, 255, 151
20	Kaempferol-3-O-rhamnoside	26.3	431	284, 255, 227, 151

2.2. Chemicals

Standard compounds: caffeic acid, chlorogenic acid, ellagic acid, gallic acid monohydrate, myricetin, quercetin, kaempferol, quercetin-3-O-ß-D-galactoside (hyperoside), and myricetin-3-O-ß-D-rhamnoside (myricitrin) were purchased from Sigma–Aldrich (Budapest, Hungary). Acetone applied for the extraction was of analytical reagent quality (minimum 99.5%), purchased from Molar Chemicals, Budapest, Hungary. HPLC grade water was prepared with a Millipore (Billerica, MA, USA) Direct Q5 equipment. Solvents employed for the sample preparation and for LC–MS analysis were of HPLC super gradient grade quality (Sigma–Aldrich, Budapest, Hungary). Solvents prepared for LC–MS analysis (acetic acid 2.5%, v/v) were additionally filtered through 0.45 μm (Millipore) membrane. The chemical reagent used in the antioxidant study, ABTS (2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) was purchased from Fluka–Biochemika, while potassium-persulfate, Trolox (5,7,8-tetramethylchroman-2-carboxyl acid) and ascorbic acid were purchased from Sigma–Aldrich (Budapest, Hungary).

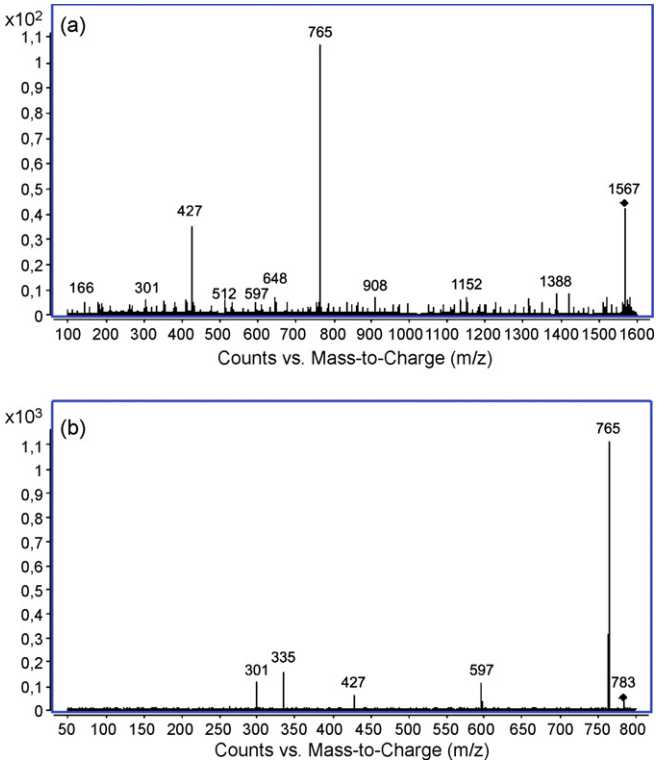


Fig. 2. CID spectra of oenothein B. (a) Single charged form ($[M-H]^-$) at (*m*/*z*) 1567; (b) double charged form ($[M-2H]^{2-}$) at (*m*/*z*) 783.

2.3. Sample preparation

Powdered plant material (5 g) was extracted three times in order, with 20 ml, diluted acetone (80, v/v%) in a sonicator (2 h). The extracts were evaporated to dryness at 50 °C, under reduced pressure. The residues were redissolved in 50 ml of HPLC grade methanol (extract solution).

In case of *Epilobium* samples, similarly to most of herbal extracts, purification by solid phase extraction (SPE) is desirable in order to discard the interfering matrix components and to eliminate chlorophyll that can adsorb on reversed-phase (RP) LC columns. SPE procedure was performed on a 12 port vacuum manifold processor (Licrolut extraction unit, Merck) with an octadecyl SPE microcolumn (LC-18, 500 mg, 3 ml, Supelco). 1.5 ml of extract solution was diluted with 3.5 ml 2.5% (v/v) acetic acid and homogenised by sonication. Afterwards the solution was loaded onto the SPE microcolumn previously activated with 3 ml methanol and 3 ml 2.5% (v/v) acetic acid. Cartridges were washed with 1.5 ml mixture of methanol and 2.5% acetic acid (70:30, v/v), then with 1.5 ml methanol. The loading solvent and the first washing fraction were collected into the same vial, and filtered on an Acrodisc Nylon, 0.20 μm membrane, Sartorius syringe filter (Sigma–Aldrich). This purified sample was used for analysis. The second washing fraction was used only to ensure that after the first washing no polyphenol residues remained on the cartridge.

3. LC-DAD-MS/MS analysis

Experiments were accomplished on an Agilent 1100 HPLC system (binary gradient pump, degasser, autosampler, diode array detector) coupled with Agilent 6410 Triple Quadrupole.

Elution was performed on a 5 μm Supelcosil C-18 column (250 mm × 4.6 mm; 25 °C, flow-rate: 1 ml/min). Separation was achieved with a gradient elution, which was an improved version of a method previously developed in our laboratory [30]. Initial ratio

Table 2
Presence of characteristic compounds in *Epilobium* extracts.

Component no.[a]	E.p	E.a.	E.r.	E.t.	E.m.	A	B
1	+++	++	+++	+++	+++	+++	+++
2	++	++	++	++	++	++	++
3	+	++	+	++	+	+	+
4	+	+	+	+	+	+	+
5	–	+	+	+	–	++	+
6	+	–	–	+	–	+	+
7	++	–	++	+	++	++	++
8	–	+	–	+	–	–	–
9	–	+	–	–	–	–	–
10	++	–	+	–	+	++	++
11	–	–	–	+	+	–	–
12	+++	–	+++	++	+++	+++	+++
13	+	–	–	–	–	+	+
14	–	+++	–	–	–	–	–
15	+	+	+	++	+	+	+
16	–	+		+		–	–
17	–	–	–	–	+	–	–
18	–	+	–	–	–	–	–
19	+	+	++	++	++	+	+
20	+	+	+	+	+	–	–

(+++) High ratio; (++) medium ratio; (+) low ratio; (–) minor or absent compound. E.p.: *Epilobium parviflorum* Schreb.; E.a.: *Epilobium angustifolium* L.; E.r.: *Epilobium roseum* L.; E.t.: *Epilobium tetragonum* L.; E.m.: *Epilobium montanum* L.; A: commercial sample A; B: commercial sample B.

[a] Numeration according to Table 1.

of eluents was: acetonitrile–acetic acid (2.5%, v/v; pH 2.47) 11:89 (v/v). In the first 20 min the ratio of eluents changed to 21:79 (v/v). This was followed by a linear gradient up to 41:59 (v/v) until the 25 min. In the last 3 min (up to the 28th) the gradient went up to 100:0 (v/v). Data were acquired in the range of 200–400 nm and integrated at 260 and 340 nm.

Measurements were executed with electrospray ionisation (ESI) in negative ion mode with the following settings: nitrogen gas temperature 350 °C, drying gas flow rate 9 l/min, and nebulizer pressure 45 psi. Capillary voltage was 4000 V, while fragmentor voltage was 135 V. Collision energy was altered between 10 and 60 eV depending on structure of the molecule examined. The full mass scan ranged between (m/z) 50 and 1600 Da. Reduction of flow rate was achieved by help of a splitter (40% to MS, 60% to waste).

Components were identified based in their UV and MS spectral data and if possible, they were compared to those of authentical standards.

4. Measurement of antioxidant capacity

ABTS assay described by Arts et al. [43] was employed with minor modifications to determine the antioxidant capacity of samples. The antioxidant capacity of samples was related to that of well-known antioxidant compounds, Trolox and ascorbic acid.

2.45 mM water solution of potassium persulfate ($K_2S_2O_8$) was used to dissolve 10 mg (one tablet) of ABTS to give a final concentration of 7 mM. During solution a free radical is formed (ABTS$^\bullet$). This stock solution of free radicals was stored in darkness for 12–16 h, diluted with methanol (approximately 50-fold dilution) just before the measurement to a final absorbance of 0.8 ± 0.02 at 734 nm.

Different concentrations (10, 20, 30, 40 and 50 μg/ml) of *Epilobium* extracts were prepared with methanol (70%, v/v). Reference substances, Trolox and ascorbic acid, were dissolved in methanol (70%, v/v) and examined in seven diverse concentrations (between 10 and 250 μM). Based on previous observations, 70% (v/v) methanol is appropriate for clear resolution of extracts and reference compounds and does not interfere with the measurement in the volume applied.

The absorbance of radical solution was read before every single measurement (A_0). Afterwards 100 μl of sample was added to 2400 μl of radical solution, homogenised and the reduction of absorbance was observed between the 30 s and the 10 min (A_{10}), until the reaction was complete and reached a plato rate. Each experiment was carried out in triplicate.

The percentage reduction of ABTS$^\bullet$ was calculated by help of the following equation:

$$\% = \frac{100(A_0 - A_{10})}{A_0}$$

The concentration of free radicals was calculated by means of absorbance–inhibition ratio.

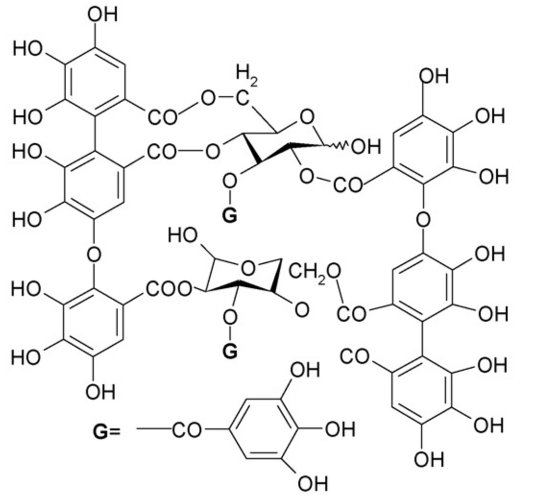
$$A = \varepsilon c L$$

ε is the molar absorptivity ($1.5 \times 10^4\ M^{-1}\ cm^{-1}$); c the concentration (M); L the path length (1 cm).

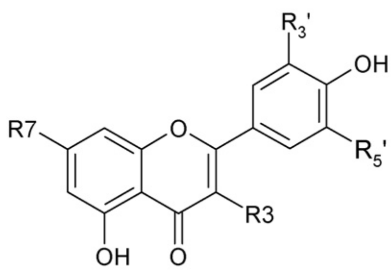
Antioxidant capacity was defined as the concentration (μg/ml) of samples necessary to decrease the initial radical concentration by 50% (EC_{50}).

5. Results and discussion

The LC–MS/MS examination of *Epilobium* species resulted in detection of 20 diverse and distinctive components; thus far 16 of them have been identified. *Epilobium* species contain these components in various combinations and ratios. UV chromatograms of samples are shown in Fig. 1. The method applied did not allow precise separation in case of peaks Nos. 1–3 detected at 260 nm, but partial identification of these components was managed to be completed based on their MS/MS data (Table 1). Compound No. **1** could be detected in the whole extension of the wide peak appearing between 3.7 and 7 min, at (m/z) 783 and 1567 Da. It is presumable, that both molecular ions assign to the same component (No. **1**). Upon examination of the total ion chromatograms, the main peak of (m/z) 783 was accompanied by a small peak of its isotopic (C^{13}) form with 0.5 spacing. This finding allows to assume that (m/z) 783 Da is a double charged ($[M-2H]^{2-}$), while (m/z) 1567 Da is a single charged molecular ion form ($[M-H]^-$) of compound No. **1**. After MS/MS fragmentation component No. **1** was identified as oenothein B. However, the literature relates to the presence of this compound in several *Epilobium* species



Oenothein B



Flavonoid component	R_3	R_7	R_3'	R_5'
Myricetin-3-O-hexose-gallate	hexose-gallate	OH	OH	OH
Myricetin-3-O-hexoside	hexose	OH	OH	OH
Quercetin-3-O-hexose-gallate	hexose-gallate	OH	OH	H
Myricetin-3-O-pentoside	pentose	OH	OH	OH
Myricetin-3-O-rhamnoside	rhamnose	OH	OH	OH
Quercetin-7-O-glucuronide	OH	glucuronide	OH	H
Quercetin-3-O-pentoside	pentose	OH	OH	H
Kaempferol-3-O-hexoside	hexose	OH	H	H
Kaempferol-7-O-glucuronide	OH	glucuronide	H	H
Quercetin-3-O-rhamnoside	rhamnose	OH	OH	H
Kaempferol-3-O-rhamnoside	rhamnose	OH	H	H

Fig. 3. Chemical structure of oenothein B and flavonoids identified in *Epilobium* species.

[23,44], the MS/MS-fragmentation pattern of oenothein B has not been reported before. CID (collision-induced dissociation) spectra of both presumptive oenothein B molecular ions ((*m*/*z*) 1567 and 783 Da) are shown in Fig. 2. Oenothein B appeared dominantly in front of the peak, later it was accompanied by several high molar-mass components (indicated with No. **2**: (*m*/*z*) 1473, 1208, 1065, and 923 Da), which are most probably various macrocyclic gallo- and ellagi-tannin derivatives. High ratio of tannins was characteristic of all *Epilobium* species without exception. Caffeic acid–pentose ester (No. **3**: Rt: 5.9 min; (*m*/*z*) 311 Da) was identified according to MS/MS data alone. In case of other polyphenols and flavonoids good resolution was achieved with the method applied. Chlorogenic acid (No. **4**, (*m*/*z*) 353 Da) was the first component clearly noticeable at both wavelengths. It was present in all samples examined. Flavonoids were represented mainly by a variety of myricetin-, quercetin- and kaempferol-glycosides, which occurred in different ratio in diverse *Epilobium* species. Ratio of components was evaluated by a rough estimation, based on the peak areas of UV and TIC chromatograms of samples (Table 2). The most probable glycosylation position was determined based on the method of Hvattum and Ekeberg [45] and

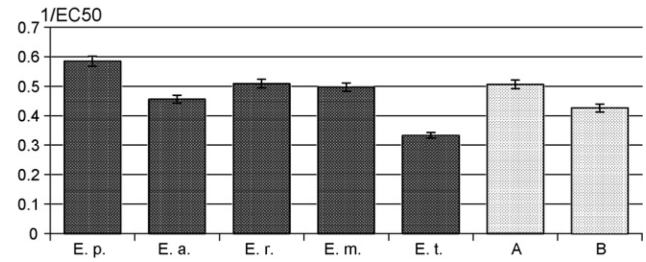


Fig. 4. Antioxidant capacity of samples, defined as their $1/EC_{50}$ value. E.p.: *Epilobium parviflorum* Schreb.; E.a.: *Epilobium angustifolium* L.; E.r.: *Epilobium roseum* L.; E.t.: *Epilobium tetragonum* L., E.m.: *Epilobium montanum* L.; A: commercial sample A; B: commercial sample B.

Ablajan et al. [46]. Fragmentation patterns of compound No. **6** and No. **9** suggested that in these flavonol-glycosides a gallic acid group is bounded to the molecule, most likely to the sugar component. Compounds of this type are quite sporadic in the flora. Component No. **6** was present in *E. parviflorum*, *E. tetragonum* and identified as myricetin-3-O-hexose-gallate. Component No. **9** was found to be quercetin-3-O-hexose-gallate and occurred in *E. angustifolium*. Chemical structure of oenothein B and flavonoids found in *Epilobium* extracts are shown in Fig. 3.

The most remarkable differences have been observed between *E. angustifolium* and other species. Exclusively *E. angustifolium* contained flavonol-glucuronide components (No. **14**: quercetin-glucuronide; No. **18**: kaempferol-glucuronide), which were not present in any other *Epilobium* species examined. Moreover quercetin-glucuronide seemed to be the main component of *Epilobium angustfolium*, while in other species, myricitrin (compound No. **12**: (*m*/*z*) 463 Da) was found to be the main flavonoid.

Epilobium species have been examined with respect to their possible antioxidant effect. Results obtained from the antioxidant assay presented that *Epilobium* extracts possess significantly high radical-scavenger activity (EC_{50} value was between 1.71 and 3.00 μg/ml). All samples examined were good antioxidants, comparable to Trolox or ascorbic acid (Trolox EC_{50}: 7.96 ± 0.238 μM; ascorbic acid EC_{50}: 14.29 ± 0.43 μM). Among *Epilobium* species and commercial samples, *E. parviflorum* showed the highest antioxidant capacity (1.71 ± 0.05 μg/ml) (Fig. 4).

6. Conclusion

This study presents detailed investigation of the polyphenol composition of various *Epilobium* species, emphasizing the pharmaceutical importance of a uniform standardization in case of their commercially available products. The LC–MS/MS method enables effective comparison of different species and exact identification of their chemical composition. Tandem mass spectrometry supplied new informations about oenothein B and the analysis confirmed its significant presence in *Epilobium* species examined. The LC–MS/MS method is considered reliable, but further NMR investigation is undoubtedly inevitable to confirm the obtained structural data.

Epilobium extracts are successfully applied in BPH. The measured strong antioxidant action, among others, most probably contributes to the beneficial effect. According to our result, flavonoid composition and antioxidant activity of *Epilobium* species shows several differences. However, influence of these differences on the pharmacological action requires further investigation; application of only one species is recommended.

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