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Content and Dynamics of Polyphenols in *Betula* spp. Leaves Naturally Growing in Estonia

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Abstract: The seasonal variation in the chemical composition and chemosystematics of the leaves of *Betula pendula* Roth., *B. pubescens* Ehrh., *B. humilis* Schrank and *B. nana* L. (*Betulaceae*), growing naturally in Estonia, and in *B. pendula* buds was studied. Polyphenols were analyzed by HPLC and HPLC-MS/MS. Hyperoside (423-3724 µg/g), myricetin glucuronide (106-1696 µg/g), quercetin glucuronide (206-1435 µg/g), myricetin glucoside (89-1197 µg/g), quercitrin (53-578 µg/g), and kaempferol glucuronide (77-342 µg/g) were found to be the main flavonoids in the birch leaves studied. The content of flavonoids in buds was lower than in leaves. The moderate correlations between the contents of the main polyphenols in the compared birch species were determined: *B. pendula* showed correlations with *B. pubescens* and with *B. humilis*. The seasonal variation of polyphenols was specific for each birch species, and no general tendency was observed. The presence of coumaric acid *O*-hexoside is not typical to *B. nana* and the content of some polyphenols can indicate the collecting time of plant material.

Keywords: *Betula pendula; Betula pubescens; Betula humilis; Betula nana;* polyphenols.

1. Introduction

Leaves and other parts of different birch species (*Betula* spp., *Betulaceae*), and products of birch such as buds, bark, essential oil, juice, wood, tar, etc. are used mainly for treating urinary tract disorders, severe infections and inflammations [1,2]. Birch leaves are especially popular as a remedy for a progressive diuresis [1]. In the former Soviet Union and Russia, buds of birch are, according to the U.S.S.R. 11th State Pharmacopoeia [3], widely used as a diuretic. According to the European Medicines Agency, birch leaf is a traditional herbal medicinal product that increases the amount of urine and helps flushing the urinary tract by acting as an adjuvant in minor urinary complaints [1].

The chemical composition of flavonoids as the main polyphenolic constituents of birch leaves has been investigated quite extensively [1], also within last ten years [4-11]. For example, Hänsel and Sticher [12] have mentioned the following flavonoids in the leaves of birch: quercetin-3-*O*-galactoside (=hyperoside), quercetin-3-*O*-glucuronide, myricetin-3-*O*-galactoside, quercetin-3-*O*-rhamnoside (=quercitrin), as well as other quercetin glycosides. Quercetin and hyperoside are reported as the principal flavonoids in birch leaves by Evans [13]. According to the 7th European Pharmacopoeia [14], the whole or fragmented dried leaves of *B. pendula* and/or *B. pubescens* as well as the leaves of

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hybrids of both of the species can be efficiently used for medical purposes. The leaves should contain not less than 1.5% of flavonoids, calculated as hyperoside, with reference to the dried drug.

Birch has been considered the most complex genus of all circumpolar genera because of its frequent hybridisation and high morphological variability. For example, some individual trees of *B. pendula* and *B. pubescens* show morphological characteristics that are intermediate between these two species. These morphological problems could be solved by studying the chemical composition of plant material. The latter chemotaxonomic studies have been based mainly on the composition of terpenoids and phenolics in birch leaves and other organs [6,9].

More than 65 species of *Betula* are known in Europe [1]. In Estonia there are four naturally growing birch species: silver birch (*Betula pendula* Roth, syn. *B. verrucosa* Ehrh.), white birch (*B. pubescens* Ehrh.), arctic dwarf birch (*B. humilis* Schrank, syn. *B. fruticosa* Pall.) and dwarf birch (*B. nana* L.) [15]. The content and composition of essential oil in the leaves of mentioned birch species was studied in our earlier publication [16].

This study was designed to assess the reliability of phenolic compounds as chemical indicators of birch species, and to evaluate their potential as chemosystematic markers. In our study, the seasonal dynamics of polyphenols content in the leaves of these four birch species was analysed for the first time.

2. Materials and Methods

2.1. Plant Material

Buds of *B. pendula* were collected in April in Tallinn, Harju County, Estonia, in 2009. All leaves samples were collected in the middle of June, August and October (Table 1). The plant organs were dried in a dark room at room temperature (20 ± 2 ºC) for ten days. Each dried sample was labeled, packaged in a paper-bag, and stored in the dark at room temperature until assayed. Voucher specimens (No Betulaceae/Bet1-4) have been deposited at the Institute of Pharmacy, University of Tartu, Estonia.

Table 1. Plant material of *Betula* spp. growing naturally in Estonia and currently studied

No	Plant material	Collecting time	Collecting spot
1	Leaves of *B. pendula*	June	Tallinn
2	Leaves of *B. pendula*	August	Tallinn
3	Leaves of *B. pendula*	October	Tallinn
4	Leaves of *B. pubescens*	June	Tallinn
5	Leaves of *B. pubescens*	August	Tallinn
6	Leaves of *B. pubescens*	October	Tallinn
7	Leaves of *B. humilis*	June	Risti bog, Lääne County
8	Leaves of *B. humilis*	August	Risti bog, Lääne County
9	Leaves of *B. humilis*	October	Risti bog, Lääne County
10	Leaves of *B. nana*	June	Viru bog, Harju County
11	Leaves of *B. nana*	August	Viru bog, Harju County
11a	Leaves of *B. nana*	October	Viru bog, Harju County
12	Buds of *B. pendula*	April	Tallinn

2.2. Extraction of polyphenols

The dried plant samples were powdered in a mortar, sieved through a 1 mm sieve and macerated with a 10-fold excess (v/w) of 20% ethanol-water (10 ml) during 24 h hours at room temperature with eventual shaking. After filtration with a cotton filter, the solution was further transferred to a flask and 20% ethanol was added at 10 ml mark. After centrifugation at a cooling centrifuge Eppendorf 510R equipped with a swinging bucket rotor during 15 min at 4000 rpm 978 x g), the supernatants were analyzed and then kept at -20 °C.

At first, water, 20%, 50% and 96% ethanol were examined as extraction solvents for the leaves of *B. pendula* collected in April. All the four extracts were analyzed by HPLC and the chromatograms were compared (see paragraph "HPLC analysis") on the basis of chromatogram quality and net areas under chromatographic curves (AUC). The AUC values of different extracts 32122 (water), 34370 (20% ethanol), 27410 (50% ethanol), and 7589 (96% ethanol) at 280 nm were compared. Since 20% ethanol gave the highest AUC values, as well as the biggest numbers of various separated compounds, this composition of the solvent was used for further sample preparation.

2.3. HPLC analysis

For the identification and quantification of individual polyphenols and calculation of the total polyphenols by chromatographic method (TP_{AUC}) [17], the Agilent 1100 Series HPLC instrument consisting of an autosampler, solvent membrane degasser, binary pump and column thermostat was hyphenated with a UV-Vis diode array and ion trap mass spectrometric (LC-ESI-MS/MS) detectors. The MS analyses were performed in the negative ionization mode on an 1100 Series LC/MSD Trap-XCT equipped with an electrospray interface (ESI) (Agilent Technologies, Palo Alto, CA, USA). The conditions of MS^2 detection were: *m/z* interval 50-1000; target mass, 400; number of fragmented ions, two; maximal accumulation time, 100 ms; compound stability, 100%; drying gas nitrogen from generator; collision gas helium. The HPLC 2D ChemStation Software with a ChemStation Spectral SW module was used for the process guidance as well as processing of the results. Compounds were separated on a reversed-phase column Zorbax 300SB-C18 (150 x 2.1 mm i.d.; 5 μm particle size; Agilent Technologies) in the gradient of 0.1% formic acid in water and acetonitrile. The column temperature was 35 °C, eluent rate 0.3 ml/min, injection volume 5 μl.

Polyphenols were identified by comparing their MS/MS fragmentation spectra either with the fragmentation spectra of the respective commercial standards or with literature data [18] and quantitated using chromatographic peak heights at the wavelengths 370 nm (quercetin and luteolin glycosides), 306 nm (ferulic acid glycoside) and 330 nm (apigenin glycosides, chlorogenic and dicaffeoylquinic acids). The commercial standards of chlorogenic acid, quercetin, quercetin glucoside, quercitrin, (+)(-)-catechin, kaempferol, myricetin, epigallocatechin (all from Sigma-Aldrich), and procyanidine B1 (from Fluka) were used to build the respective calibration curves.

For quantification of caffeoylquinic acids, luteolin and apigenin glycosides calibration curves of chlorogenic acid, luteolin and apigenin were used, respectively, using the ratio of molecular weights of the cognate standard substances.

Total phenol contents were estimated by the method of the net areas under chromatographic curves (TP_{AUC}) [16]. Birch extracts prepared as in the preceding paragraph were analyzed by UV-Vis chromatograms at 280 nm between 10 and 58 minutes and calculated using HPLC 2D ChemStation Software. AUC values of chlorogenic acid solutions with different concentrations at 280 nm were used for calibration.

2.4. Statistics

To elucidate the possible correlation of content of different polyphenols in birch ethanol extracts the obtained results were subjected to Pearson correlation (r) analysis followed by the two-tailed P value calculation by SPSS Data Editor.

3. Results and Discussion

3.1. Content and dynamics of polyphenols

Up to 32 of polyphenols and their glycosides were identified in the birch leaves and buds (Table 2). A total of 16 phenolic compounds were found in all the plant samples investigated, whereas glycosides of myricetin, quercetin and kaempferol were present as the main flavonoids. Kaempferol acetylglucoside was typical only for the *B. pendula* leaves collected in June and in August, quercetin 3-*O*-glycopyranosyl-1-6-galactopyranoside was determined in *B. nana* leaves only. Quercetin and

quercetin galloylglucoside were not found in the leaves of *B. pendula*. Also, several phenolic acids were observed in all the birch samples.

A number of the major phenolic acids, their glycosides and flavonoids were quantified in the birch leaves and buds (Table 3, Figure 1). Their content in the analyzed plant material varied over a wide range. So, there was even a 207-fold difference between the minimum and maximum content of procyanidin B1, 33-fold for (+)(-)-catechin, 23-fold for myricetin glucoside, 16-fold both for myricetin glucuronide and glucoside, 15-fold for quercitrin, and relatively smaller (4-9-fold) for other constituents. The total concentration of related major polyphenols varied 3 times. The content of hyperoside as the principal flavonoid of birch leaves was highest in the *B. pubescens* leaves collected in spring (3724 µg/g), whilst the leaves of *B. pendula* contained 5 times less hyperoside (735 µg/g). The leaves of *B. pubescens* collected in August or October (768-1255 µg/g) were slightly richer in polyphenols than the leaves of *B. pendula* from the same period (452-872 µg/g).

The buds of *B. pendula* contained considerably more procyanidin (1449 µg/g), catechins (1222 µg/g) and epicallocatechin (1839 µg/g) than the leaves (1-285, 18-586 and 57-416 µg/g, respectively) of the same and other species. However, the content of flavonoids in buds was clearly lower if compared with their amounts in leaves.

The moderate correlations between the contents of the main polyphenols in the compared birch species were determined. *B. pendula* showed correlations with *B. pubescens* (r=0.600, p=0.000) and with *B. humilis* (r=0.578, p=0.001). At the same time, correlations between *B. nana* and *B. pubescens* (r=0.558, p=0.011), as well as between *B. nana* and *B. humilis* (r=0.529, p=0.016) were found. The dynamics of the main polyphenols and total polyphenols (areas under chromatographic curves (TP_{AUC}) at 280 nm) was specific for all the birch species studied and no general tendency was observed (Table 2, Figure 1).

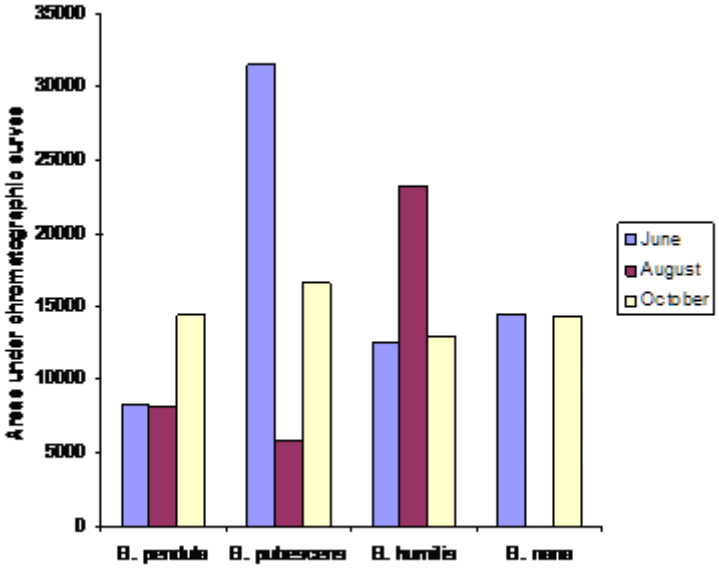

Figure 1. Dynamics of total polyphenols in 20% ethanolic extracts from the leaves of *Betula* spp. naturally growing in Estonia. Areas under chromatographic curves (AUC) at 280 nm. The leaves of *B. nana* collected in August were not analyzed.

Table 2. Polyphenolic compounds detected in the 20% ethanol extracts from the leaves and buds of *Betula* spp. naturally growing in Estonia

No	Rt (min)	$[M-H]^-$	Main fragments	Compound	Consisting in sample No
1	1.0	179	161	Caffeic acid	11, 12
2	1.3	191		Quinic acid	1-12
3	1.3-4.1	331	169, 271, 211	Galloyl glucose	2, 3, 7, 8, 9, 10, 12
4	4.2-21.5	483	169, 331	Gallic acid-4-*O*-(6'-*O*-galloyl)glucoside	10-12
5	7.2	305	179, 125, 261	Epigallocatechin	1-12
6	8.8	593	425, 407, 289, 467, 303, 177	Prodelphinidin B3 isomers	2-11
			425, 407, 289, 467, 303, 177	[20]	
7	9.8	593	423; 441; 305; 425; 467; 575		
			423; 441; 305; 467; 575		
8	10.5	593			
9	11.2	593			
10	11.8	353	191, 179, 135, 173	Cryptochlorogenic acid	1-12
11	19.6	353	191, 179	Chlorogenic acid	1-12
12	23.8	353	191, 179, 161	Neochlorogenic acid	1-12
13	14.4-24.9	337	163, 191, 119	Coumaryl quinic acid	1-12
14	15.9-17.5	577	425, 407, 451, 289	Procyanidin B1	1-12
15	17.0	289	245; 205; 179	(+)(-)-Catechin	1-12
16	18.4	325	163, 119	Coumaric acid *O*-hexoside	1-9
17	19.9	355	193	Ferulic acid *O*-hexoside	4
18	22.0	341	179	Caffeic acid *O*-hexoside	3, 10-12
19	27.6	327		Betuloside	3, 6, 7-12
20	28.0	367	191	5-*O*-Feruloyl quinic acid	4, 7-9
21	32.8	493	317	Myricetin glucuronide	1-12
22	33.5	479	317	Myricetin hexoside	1-12
23	35.7	615	463, 301	Quercetin galloylglucoside	4-6, 10-12
24	37.5		301, 300, 179	Quercetin-3-galactoside (hyperoside)	1-12
25	38.7		300; 301	Quercetin-3-glucoside (isoquercitrin)	
25	37.9	477	301, 227, 151	Quercetin glucuronide	1-12
26	41.1	433	301	Quercetin pentoside	1-12
24	41.4	447	285, 245, 217	Kaempferol hexoside	1-12
25	41.9	505	301; 300; 463	Quercetin hexoside-malonate	1-3, 7-12
26	42.4	461	285	Kaempferol glucuronide	1-12
27	44.8	447	301; 285; 255	Quercetin rhamnoside (Quercitrin)	1-12
28	47.7	533	285	Kaempferol hexoside-malonate	7
29	49.7	625	301, 463	Quercetin 3-*O*-glucopyranosyl-1-6-galactopyranoside	10, 11
30	49.9	489	285	Kaempferol acetylglucoside	1,2
31	52.2	301	179; 151; 273; 257; 107	Quercetin	4-12
32	54.9	503	285	Kaempferol acetylglucuronide	1-3, 7-9

For sample numbers, see Table 1

Table 3. Content (µg/g) of main phenolic components (>15 µg/g) in the 20% ethanolic extracts from the leaves and buds of *Betula* spp. naturally growing in Estonia

Compound	Leaves											Buds
	Betula pendula			*Betula pubescens*			*Betula humilis*			*Betula nana*		*Betula pendula*
	J	A	O	J	A	O	J	A	O	J	O	Apr
Procyanidin B1	28	114	36	41	1	94	1	192	80	285	207	1449
(+)(-)-Catechin	18	169	36	128	44	115	74	282	183	586	369	1222
Epigallocatechin	57	243	170	121	105	193	156	364	261	416	217	1839
Chlorogenic acids	477	478	124	852	150	246	1118	2010	950	1568	1572	154
Myricetin glucuronide	141	106	206	1194	118	110	316	842	485	1696	139	72
Myricetin hexoside	149	89	109	52	724	798	180	398	276	748	1197	52
Hyperoside or isoquercitrin	735	452	872	3724	768	1255	860	1164	702	1623	423	545
Quercetin glucuronide	566	431	320	890	206	572	691	1435	646	244	238	99
Kaempferol glucuronide	94	238	77	139	80	98	281	342	100	133	113	77
Quercitrin	222	193	578	548	64	209	382	481	282	39	53	30
Total	2487	2513	2528	7689	2260	3690	4059	6075	3965	7338	4528	5539

J – June, A – August, O – October, Apr - April

3.2. Discussion

Seasonal dynamics of polyphenols in *B. pendula* leaves in mosaic urban environment and in different weather conditions during their vegetation period was studied by Kavelenova et al [19]. The maximum phenol content was observed in the first and second decades of May with a transition to a lower level in the middle of July and rising again in late summer and autumn. We can see analogical results in the total content of polyphenols in *B. pubescens*, but not in the other birch species studied. The same tendency of dynamics of hyperoside as the main flavonoid was observed in the leaves of *B. pendula* and *B. pubescens*. The clear dynamics of quercetin and myricetin content was observed by Graglia et al [11] in *B. nana* leaves: the maximum amounts were found in June and minimum levels from July to August.

During leaf expansion and maturation, the content of flavonoid aglycones decreases in *B. pendula* and *B. pubescens* [6]. The same was concluded by Laitinen et al [10] with regard to the contents of hyperoside in mixed *B. pendula* and *B. pubescens*. This was not observed in our study probably because the leaf dimensions do not change from mid-June when the plant samples were collected. On the other hand, young leaves do not always contain higher concentrations of phenolics than mature leaves [10]. The content of hyperoside was maximal in the leaves of the mentioned species collected by us in October.

Different amounts of derivatives of kaempferol, myricetin and quercetin were found in *B. pendula* and *B. pubescens* leaves [7]. As that was determined by Valkama et al [9], the total concentration of flavonoids was lower in *B. nana* than in the two *B. pubescens* subspecies. In our study, the level of flavonoid concentrations in different birch species is rather similar.

Several factors can affect the content of flavonoids in birch species. The tendency of a decrease in the polyphenol content during drought years was observed [19]. The summer of 2009 was extremely warm and dry in Estonia that may explain the data we have obtained. The investigation conducted by Stark et al [4] showed that the temperature sum was correlated positively and soil P concentration was correlated negatively with the concentrations of foliar flavonoids in *B. pubescens*. However, we can

see higher amounts of flavonoids in the leaves of the same species collected in October than in August which was a lot warmer month. Moreover, latitudinal and regional variations in the composition and concentrations of flavonoids in *B. pubescens* leaves were found [4]: concentrations of quercetin derivatives were correlated positively with latitude. As mentioned by Keski-Saari et al [5], flavonol glycosides may have a UV-B protective role in birch species. We agree with Laitinen et al [8] that the secondary chemistry of birch shoots is under strong genetic control. But later that is affected by different environmental factors.

3.3. Conclusions

The glycosides of myricetin, quercetin and kaempferol were found to be the main flavonoids in the birch leaves studied. Hyperoside is the principal flavonoid of *B. pendula* and *B. pubescens* leaves. The buds of *B. pendula* contained clearly less of flavonoids and much more other polyphenols such as procyanidin, catechins and epicallocatechin than leaves. The content of quercetin 3-*O*-glycopyranosyl-1-6-galactopyranoside was typical only to *B. nana* but coumaric acid *O*-hexoside was not found in *B. nana*. Quercetin and quercetin galloylglucoside were not determined only in the leaves of *B. pendula*. The mentioned polyphenols may be used as chemosystematic markers for *B. nana* and *B. pendula*.

The seasonal variation of polyphenols was specific for each birch species. The presence of several polyphenols can determine the collecting time of plant material.

By the content of polyphenols, the leaves of *B. pendula, B. pubescens* and also *B. humilis* can serve as a good source for natural medicines with a traditional background.

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