Flavonoids in the leaves of Polish species of the genus *Betula* L. IV. The flavonoids of *Betula pubescens* Ehrh., *B. carpatica* Waldst., *B. tortuosa* Ledeb., and *B. nana* L. leaves

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Abstract

Besides the flavonoids detected earlier, the leaves of purebred *B. pubescens* specimens were found to contain quercetin 3-arabinoxide, isorhamnetin 3-galactoside, luteolin 4'-glucoside, isoquercitrin, acacetin 7-glucoside, and perhaps scutellarein 7-glycoside, whereas *B. nana* exhibited a low content of myricetin 3-galactoside and quercetin 3-arabinoxide, and also possibly quercetin 7-rhamnoside. The qualitative composition of the flavonoids of desiccated *B. tortuosa* leaves seems to point to the validity of the assumption that this birch is of hybrid origin. The set of flavonoids in *B. carpatica* was almost identical with that in *B. tortuosa*.

INTRODUCTION

Betula pubescens Ehrh. (= *B. alba* L. in the part pertaining to *B. odorata*) occurs in the whole of Europe and in part of Asia (Browicz 1979). It grows on wet ground all over Poland except the Tatra Mountains, where it is replaced by *B. carpatica* of very similar morphology (Gos-tyńska-Jakuszczyńska 1980). Jentys-Szaferowa (1950) and also Bia-łobrzeczka and Truchanowicz (1960) have found *B. carpatica* to be an intermediate form between *B. pubescens* and *B. tortuosa*, this last, in turn, occurring in the mountains of Scandinavia and Scotland and in the Ural (Polyakov 1969, Kenworthy et al. 1972, Vaarama and Valanne 1963). Jentys-Szaferowa (1950) and Vaarama and Valanne (1970, 1973) hold *B. tortuosa* to have arisen from the crossing of *B. pubescens* with the fruticose, boreal *B. nana*. The last-mentioned species is only a post-glacial relic in Poland (Środoń 1979). For all these reasons the present investigations were carried out on specimens representative of all

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mentioned taxa of the genus *Betula* L. Moreover, a comparative analysis comprised two interesting trees which attracted botanists' attention by their morphological characters intermediate between *B. pubescens* and other birch species, i.e. *B. pendula* or *B. humilis*.

MATERIAL

Analysis was conducted on 10 ± 1 g of fresh leaves from each of the specimens listed in Table 1. The material was collected into ice in the period of full vegetation, i.e. when the qualitative flavonoid composition did not undergo any changes (Pawłowska 1980 a). As regards the specimens growing abroad and one Polish, morphological interesting specimen (denoted

Table 1
A list of specimens under study

Symbol of specimen	Locality	Notes
<i>B. pubescens</i>		locality occupied by this species only
Pub-1	Isle of Wolin	
Pub-2		
Pub-3		specimens 2,3 and 4 grow close to one another
Pub-4	Border of the Niepołomice Forest near Szarów	
Pub-5		
Pub-6		specimens 6 and 7 grow close to each other
Pub-7		
Pub-8	Botanic Garden of the Jagiellonian University	transplanted from Polesie Lubelskie
Pub-9		
Pub-10	Miechucino (Wysoczyzna Staniszevska)	
Pub-11		
int-1	Botanic Garden of the Maria Skłodowska-Curie University	desiccated material, presumably a hybrid of <i>B. humilis</i> $\times$ <i>B. pubescens</i>
int-2	Osadowo near Poznań	presumably a hybrid of <i>B. pendula</i> $\times$ <i>B. pubescens</i>
<i>B. carpatica</i>		
Car-1		
Car-2	Botanic Garden of the Jagiellonian University	
Car-3		
Car-4		
Car-5	Wielki and Mały Spękany Szczyt (Szczeliniec)	desiccated material
<i>B. tortuosa</i>		
Tor-1	Batvik (Skelleftea, Sweden)	
Tor-2		desiccated material
<i>B. nana</i>		
Nan-1	Botanic Garden of the Jagiellonian University	
Nan-2		

"int-2), only desiccated material of 2 g from each specimen was available and used for examination.

METHODS

EXTRACTION

The leaves were homogenized with methanol, left to stand for about 24 hours, and, following centrifugation, the extract was decanted, the remainder perfused with methanol. Extraction with methanol was repeated until the extract became colourless. Extracts from each specimen were mixed and next evaporated to the volume of 50 ml, and mixed with an equal amount of chloroform, thoroughly shaken and after about 20 minutes separated. The upper, aqueous methanol phase was condensed to about 10 ml.

SEPARATION, PURIFICATION AND IDENTIFICATION

The extract from the specimen Pub-8 was separated on Whatman No. 3 paper, and purified according to the methods earlier described (Pawłowska 1980 a). The fractions obtained were identified by thin-layer and one-dimensional paper chromatography, specific colour reactions, reduction, acid hydrolysis, and spectroscopy in visible and UV light (Pawłowska 1980 a).

ANALYSIS OF FLAVONOID COMPOSITION

Aqueous methanol extract (0.2 ml) from each specimens was analysed by two-dimensional thin-layer chromatography. Besides two versions of solvent systems (Pawłowska 1980 a), in the present examinations next versions were applied to plates coated with a 1-mm layer of MN 300 cellulose:

1. H_2O in the first dimension, BAW in the other (n-butanol: acetic acid: water 12:3:5 v/v);
2. 15% AcOH (acetic acid: water 3:17 v/v) in the first, and FAWiA (formic acid: amyl alcohol: water: isoamyl alcohol 1:1:1:1 v/v) in the other dimension;
- 3 a and 3 b. 0.4 and 0.6 ml of extract from a specimen were developed in 15% AcOH and FAWiA;
4. Reduction products—in the first dimension 10% FOH (formic acid: water 1:9 v/v) and BAW in the other.

The formerly applied co-chromatography with single standard glycosides was extended in the present study by combinations of various standard substances, e.g. hyperoside with quercitrin, hyperside with isoquercitrin and quercitrin, the examined fraction with hyperoside and quercitrin, the examined fraction with hyperoside, isoquercitrin and quercitrin, etc.

Table 2
Identifying analysis of fractions 23, 24, 25 and 27

Fraction	Rf values in systems				Colour in light		Effects of test reactions				Spectral maxima in methanol, nm
	BAW	FAWiA	15% AcOH	H ₂ O			ZrOCl ₂	ZrOCl ₂ + C ₆ H ₈ O ₇	AlCl ₃	diazoben- zidine	
					visible	UV					
23	0.50	0.53	0.22	0.	y	fl. y	fl. y	fl. y	y	p-ru	244; 268; 347
24	0.52	0.54	0.35	0.25		y	y	—	po. y	p-ru	
25	0.70	0.73	0.45	0.30	po.y	y-o	y-o	d-y	y	ru	
27	0.42	0.46	0.32			y	b-o	—	y-o		
	Products										
	of reduction				of hydrolysis						
Standards: Kaempferol Quercetin Myricetin Luteolin Glucose Galactose Rhamnose	colour	Rf values in systems			Rf values in systems						
		BAW	10% FOH	BAW ^a	A ^b	BAW ^c	B ^d				
	23				tr. 0.71	tr. 0.26	tr. 0.25				
	24	p-o	0.57	0.60	po. 0.86	po. 0.48	tr. 0.09	tr. 0.15			
	25	o	0.47	0.65	0.76	0.42	0.10	0.12			
	27	v-r	0.18	0.20	tr. 0.50	tr. 0.14	tr. 0.12				
					0.87	0.43					
					0.73	0.30					
					0.50	0.12					
					0.77	0.40					
							0.08	0.15			
							0.10	0.14			
						0.27	0.50				

Solvent systems: A—acetic acid; hydrochloric acid; water (5:1:5 v/v); B—benzene; n-butanol; pyridine; water (1:5:3:3: v/v).

^a—developed with ZrOCl₂; ^b—developed with diazobenzidine; ^c—developed with aniline phthalane in butanol; ^d—developed with ammonia solution AgNO₃; b—bright, d—dark, fl.—fluorescent, o—orange, p—pink, po—poor amounts, r—red, ru—russet, tr—trace amounts, y—yellow.

Table 3

Flavonoids from the leaves of studied specimens of *B. turkiestanica*, *B. pubescens*, *B. carpatica*, *B. tortuosa*, *B. nana* and presumable hybrids of *B. pubescens*

No. of fraction	1	3	4	5	7	8	10	11	15	16	20	21	22	23	24	25	27
Name of compound	quercitrin	hyperoside	myricetin 3-diagalactoside	rutin	isorhamnetin 3-galactoside	isoquercitrin	kaempferol 3,4'-dimethyl ether	quercetin 3-gly- coside-7,4-di- methyl ether?	6-methoxy-kaem- pferide	scutellarein 7-glycoside?	quercetin 3-arabinoside	acacetin 7-glucoside	kaempferol	quercetin 7-rhamnoside?	kaempferol 3-glucosid	luteolin 4'-glucoside	myricetin 3-galactoside?
Specimen																	
Tur	+	+++	+	—	+	—	—	—	+	tr.	w.	tr.	—	—	tr.	+	—
Pub-1	+	+++	+	—	w.	—	+	—	+	—	+	—	+	—	w.	++	—
Pub-2	+	+++	+	—	w.	—	—	—	—	—	+	w.	—	—	w.	+	—
Pub-3	++	+++	+	—	+	+	w.	—	+	—	w.	—	tr.	—	+	++	—
Pub-4	+	+++	++	tr.	+	—	—	—	—	—	w.	w.	+	—	tr.	+	—
Pub-5	+	+++	+	—	+	—	—	—	—	—	+	w.	+	—	w.	+	—
Pub-6	++	+++	++	++	++	—	—	w.	—	—	tr.	++	+	w.	tr.	+	—
Pub-7	+	+++	++	++	+	w.	—	—	—	—	w.	+	+	w.	+	++	—
Pub-8	+	+++	—	++	+	++	+	—	+	w.	+	w.	tr.	+	+	+	tr.
Pub-9	+	+++	+	—	+	++	—	—	—	tr.	w.	+	+	—	w.	w.	—
Pub-10	w.	+++	+	—	++	+	w.	w.	+	+	++	++	+	—	+	+	—
Pub-11	+	+++	w.	—	w.	—	tr.	tr.	tr.	—	++	+	+	—	+	w.	tr.
Car-1	++	++++	+	w.	+	+	—	—	+	—	w.	+	++	w.	tr.	++	—
Car-2	+	++++	++	+	tr.	w.	tr.	—	+	—	tr.	tr.	w.	—	tr.	++	—
Car-3	++	+++	++	+	w.	tr.	w.	—	—	—	w.	tr.	w.	—	tr.	++	—
Car-4	++	+++	++	tr.	tr.	—	—	—	—	tr.	+	tr.	w.	—	tr.	++	—
Car-5	++	+++	+	w.	+	++	—	—	—	tr.	w.	w.	+	—	tr.	++	tr.
Tor-1	+	+++	+	++	w.	++	?	?	—	?	+	w.	++	w.	tr.	+	w.
Tor-2	+	+++	+	+	+	+++	?	?	+	?	w.	w.	+	w.	tr.	+	tr.
int-1	+	+++	+	++	+	w.	tr.	—	—	—	w.	+	+	+	tr.	+	tr.
int-2	+	+++	+	—	+	w.	w.	tr.	+	tr.	w.	w.	+	—	w.	+	—
Nan-1	+	+++	—	+	—	w.	tr.	—	—	—	tr.	—	—	+	tr.	—	—
Nan-2	++	+++	—	+	—	+	w.	—	—	tr.	w.	—	—	+	tr.	—	tr.

tr.—trace amounts, after spraying with $ZrOCl_2$, it is discernible in UV light only when 0.4 ml of extract has been mounted; w.—weak, visible in UV light only after 0.2 ml of extract has been mounted; "+"—discernible already in visible light; "++" to "++++"—large amounts of compound respectively

RESULTS AND DISCUSSION

IDENTIFICATION

The specific colour reactions (Table 2) and obtained spectra of methanol solutions indicate that compounds 23, 24, and 27 are flavonol glycosides, and 25 is a flavone glycoside.

FRACTION 23

The insignificant amount of the isolated compound 23 permitted only for hydrolysis which gave trace amounts of quercetin and of rhamnose (Table 2). The localization of compound 23 on two-dimensional chromatograms in version 3a and 3b would point to its being flavonol 7-glycoside. The R_f value of the analysed compound in 15% AcOH is identical with that given by Mabry et al. (1970) for quercetin 7-rhamnoside.

Fraction 24

Hydrolysis of compound 24 produced kaempferol and most probably glucose (Table 2). R_f values and localization on two-dimensional chromatograms show compound 24 to be probably kaempferol 3-glucoside.

Fraction 27

The hydrolyzate of compound 27 (of which a very small amount was isolated) was found to contain myricetin and probably galactose (Table 2). R_f values and localization on two-dimensional chromatograms of glycoside 27 and its reduction product indicate compound 27 to be in all likelihood myricetin 3-monoglactoside.

Fraction 25

Luteolin and glucose (Table 2) were products of hydrolysis of compound 25. The R_f values of this compound in BAW and 15% AcOH systems were most approximate, as compared with many different luteolin glycosides, to R_f of luteolin 4'-glucoside in these systems prepared by Harborne and Williams (1975).

Identification of the remaining flavonoid elements has been reported by Pawłowska (1980a, b).

QUALITATIVE FLAVONOID COMPOSITION

B. pubescens

The present investigations on specimens of purebred *B. pubescens*, growing in natural localities occupied by this species only (Table 1), cor-

roborated the findings of Hänsel and Hörhammer (1954) and Hörhammer et al. (1957), pointing to, the constant presence in their leaves of flavonol glycosides such as hyperoside (the highest amount) as well as quercitrin and myricetin 3-digalactoside, besides hesperidin and polyphenol acids. Moreover, the presence of acacetin was also borne out, but as 7-glucoside and only in some specimens (Table 3).

The occurrence of 6-methoxykaempferide and kaempferol-3,4'-dimethyl ether in leaf buds of *B. pubescens*, demonstrated by Wollenweber in 1975, was also observed in mature leaves but not in all specimens studied. Isorhamnetin, reported by the above-mentioned investigator, was found here already as 3-galactoside (in all specimens), and scutellarein probably as 7-glycoside in very few samples only. This indicated that the processes of glycosidation in this species, similarly as in *B. pendula* and *B. oycoviensis*, occur during maturation of leaves (Pawłowska 1980a, c). Besides the flavonoids mentioned above, kaempferol 3-glucoside (24), and luteolin 4'-glucoside (25) were detected in all, and isoquercitrin in some *B. pubescens* trees studied (Table 3).

B. nana

In the leaves of this species the presence of quercitrin, hyperoside, rutin, kaempferol 3,4'-dimethyl ether, hesperidin, and polyphenol acids (Hänsel and Hörhammer 1954, Wollenweber 1975) was confirmed. Furthermore, quercetin 7-rhamnoside and negligible amounts of myricetin 3-monogalactoside were encountered therein (Table 3).

B. tortuosa and *B. carpatica*

Jentys-Szaferowa (1950) maintains that *B. tortuosa* resembles *B. nana*. This resemblance is manifested by some morphological characters of leaves, i.e. the position of their broadest part, the angle of the second pair of lateral veins, the angle of the base, and the ratio of the leaf length to the distance of its first denticle from the base. Likewise Vaarama and Valanne (1970, 1973), after a biometric analysis and physiological experiments, concluded that *B. tortuosa* was a hybrid of *B. nana* and *B. pubescens*. According to Jentys-Szaferowa (1950) the crossing between *B. nana* and *B. pubescens* occurred probably in the late glacial period, when *B. nana* still occupied vast expanses of Central Europe and at the same time *B. pubescens* began to appear. This is the more likely because sediments from that period in Poland were found to contain remains of a birch very similar to *B. tortuosa* or *B. carpatica* (Wasilikowa 1964, Ralska-Jasiewiczowa 1966). Today such hybrids are frequent in Finnish Lapland and in Scotland (Elkington 1968, Kallio and Mäkinen 1978). Kenworthy et al. (1972) obtained a high percentage of viable progeny by

experimental crossing. Rehder (1974) mentions the hybrid of *B. nana* × *B. pubescens* as *B. intermedia* Thomas.

In the present study the desiccated *B. tortuosa* material was found to contain two flavonol diglycosides: myricetin 3-digalactoside, present in specimens of purebred *B. pubescens* (1, 2, 3, 5, 9, 10 and 11), and rutin—in *B. nana* leaves. Besides, *B. tortuosa* contained isorhamnetin 3-galactoside and luteolin 4'-glucoside, which were present in *B. pubescens* leaves. Quercetin 7-rhamnoside, in turn, was detected in both *B. nana* and *B. tortuosa* (Table 3). Thus, the biochemical characters studied bear out a hybrid origin of *B. tortuosa*.

The qualitative flavonoid composition in the *B. carpatica* trees under study was almost identical with that in the desiccated *B. tortuosa* material (Table 3). This birch was found to lack only compound 13 which may be a substrate in hyperoside synthesis and, therefore, is of low taxonomic value. Besides, the possibility of a degradation of compound 13 during desiccation of *B. tortuosa* material cannot be ruled out. A more essential difference between the two taxa seems to concern the ratio of diglycoside content, in favour of rutin for *B. tortuosa*, though the material was desiccated, whereas *B. carpatica* contained more myricetin digalactoside. In order to define this difference more precisely, it would be necessary to analyse the two diglycosides quantitative in fresh leaves collected from a larger number of specimens. *B. tortuosa* contained more isoquercitrin which may be a product of partial decomposition of rutin in the desiccated sample. In the *B. carpatica* leaves the luteolin glucoside content was higher than in *B. tortuosa* (Table 3).

Biometric studies of the leaves (Jentys-Szaferowa 1950), showed *B. carpatica* to be an intermediate form between *B. tortuosa* and *B. pubescens* with respect to certain characters. A morphological analysis of hulls and druples, has confirmed the intermediate character of this taxon (Białobrzaska and Truchanowicz 1960). This would indicate that *B. carpatica* is a fixed hybrid.

If the composition of flavonol, glycosides and luteolin glucoside in fresh material is borne out for a larger number of *B. tortuosa* specimens, there seems to be no reasonable basis for differentiation of these birch taxa as separate species, as has been done by Regel (1868) and Natho (1976).

Presumable hybrids of *B. pubescens* from natural localities

Although, owing to their morphology, four of the specimens studied were also established as *B. pubescens*, they contained rutin (Table 3). Besides, two of them, growing close to each other (Pub-6 and Pub-7), additionally contained myricetin digalactoside, similarly as did *B. carpatica* and *B. tortuosa*. It should be emphasized that Pub-4, Pub-6, and Pub-7 grew on the border of the Niepołomice Forest, together with *B. humilis*.

Pub-6 and Pub-7 as well as Pub-8 also contained quercetin 7-rhamnoside, which had been encountered in *B. humilis* (Table 3). Identical biochemical characters were observed in an interesting specimen from the Botanic Garden in Lublin which, despite its resemblance in the shape of leaves to *B. pubescens*, raised some doubts as to its classification. The presence of rutin and quercetin 7-rhamnoside seems to show that Pub-4, Pub-6, Pub-7, Pub-8 and int-1 are natural hybrids of *B. humilis* × *B. pubescens*, though morphologically they are very similar to *B. pubescens*. This is the more likely because Boińska (1974) has also discovered such hybrids of *B. humilis* × *B. pubescens* in the localities occupied by both parental species.

Likewise interesting was a specimen int-2, which came from Osadowo near Poznań, where *B. pendula* and *B. pubescens* were growing side by side. The morphology of this specimen seemed to suggest an intermediate character between *B. pendula* and *B. pubescens* (Staszkievicz, unpublished). It has a qualitative composition identical with that of Pub-1 and Pub-5 (Table 3). This indicates, as we assume, that the qualitative analysis of flavonoids does not permit any differences to be found between a taxon of hybrid origin and those of introgressive character.

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Flawonoidy liści polskich gatunków z rodzaju Betula L. IV. Flawonoidy liści Betula pubescens Ehrh., B. carpatica Waldst., B. tortuosa Ledeb. i B. nana

Streszczenie

Przeprowadzona analiza flawonoidów z liści osobników czystej *B. pubescens* wykazała w nich obecność hyperozydu, kwercetryny, 3-dwugalaktozydu myrcetyny, 3-arabinozydu kwercetyny, 3-galaktozydu izoramnetyny, 4'-glukozydu luteoliny, izokwercetryny, 7-glukozydu akacetyny, betuletolu (6-metoksy-kempferyd), 3,4'-dwumetylokempferolu oraz prawdopodobnie 3-glikozydu 7,4'-dwumetylokwerceptyny i 7-glikozydu skutelarny. Wśród badanych osobników stwierdzono takie, których skład jakościowy flawonoidów wskazywał na ich charakter mieszańcowy: *B. pubescens* × *B. humilis*. U *B. nana* znaleziono: hyperozyd, kwercetrynę, rutynę, 3-arabinozyd kwercetyny oraz prawdopodobnie 7-ramnozyd kwercetyny i nieznaczne ilości 3-galaktozydu myrcetyny. Jednocześnie stwierdzono, że skład jakościowy flawonoidów *B. carpatica* i *B. tortuosa* był identyczny i potwierdzał ich mieszańcowy charakter (*B. nana* × *B. pubescens*).