Iridoid Glucosides from Four Balkan Endemics of the *Galium incurvum* Group (Rubiaceae)

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The Balkan endemics *G. mirum*, *Galium macedonicum*, *G. rhodopeum* and *G. aegeum*, from the *G. incurvum* group, were screened for iridoid glucosides. Eleven known iridoid glucosides were isolated, identified and analysed for the first time in the investigated plants. The main components appeared to be asperuloside and/or the non-acetylated iridoid acids – deacetylasperulosidic acid, scandoside, monotropein. Phylogenetic relationships are discussed.

Introduction

The narrow-leaved species of the *Galium incurvum* group (Ehrendorfer and Krendl, 1976) form a polymorphic polyploid complex, intriguing with its evolution and taxonomy. Ehrendorfer (1971, 1975, 1980) analyzes the interspecies relationships and the phytogeographical differentiation of this group in the Eastern Mediterranean area, accentuating on the role of the Balkan Peninsula as a centre of differentiation of the perennial *Galium* species, especially of the narrow-leaved morphological type. Later, as a result of systematic and cytogenetic studies on *Galium* from the Balkan Peninsula, new taxa were described (Ehrendorfer and Krendl, 1974; Krendl, 1987; Anchev, 1975, 1989) and new evidence about the distribution and systematics of the Eastern Mediterranean members of the *Galium incurvum* group were published (Ehrendorfer and Schönbeck-Temesy, 1982). All these studies elucidated some basic patterns of the evolution of this group and clarified taxonomic problems. At the same time new ones aroused, among them the phylogenetic relationships in the group and its members in Sect. Lejogalium (Ehrendorfer and Krendl, 1976).

In the Balkan Peninsula are distributed most of the eighteen European species from the *G. incurvum* group (Krendl, 1987). Seven of them occur in Bulgaria (Table I). *G. mirum* Rech. fil., *G. macedonicum* Krendl, *G. asparagifolium* Boiss. & Heldr., *G. rhodopeum* Velen. (diploid populations) and *G. velenovskyi* Ancev occur in the mountains of South-west Bulgaria up to 900 m on open slopes or in dry oak-eastern hornbeam communities. *G. rigidifolium* Krendl (*G. flavescens* auct. non Borb.) frequents open mountainous slopes up to about 1200 m, predominantly in the south-western part of the country. *G. aegeum* (Stoj. & Kitan.) Ancev is found in pine communities in Slavjanka and south Pirin Mts. between 1200 and 2000 m and *G. rhodopeum* (tetraploid populations) in the Central Rhodopes up to 1400 m.

Phytochemical evidence should clarify the classification problems in *Galium* and between closely related genera (Borisov and Zoz, 1975; Corrigan *et al.*, 1978). Iridoids and other compounds were used for this purpose. In the taxa of Rubioideae the iridoids asperuloside and/or deacetylasperulosidic acid were observed to be uniformly distributed (Inouye *et al.*, 1988). Because of their instability a limited taxonomic usefulness of the asperoloside type iridoids was suggested (Corrigan *et al.*, 1978).

In this paper, first results of our investigations are reported, on the correlation between the iridoid pattern with the morphological differentiation and ploidy levels of four Balkan endemics *G. mirum*, *G. macedonicum*, *G. rhodopeum* and *G. aegeum*.

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Table I. Morphological characteristics and ploidy levels of the species of the *Galium incurvum* group, distributed in Bulgaria.

Species	Plants caespitose (c), not caespitose (nc)	Inflorescence	Ploidy level
G.mirum	nc	broadly pyramidal	2×
G.macedonicum	nc	pyramidal	2×
G.rigidifloium	nc	broadly pyramidal	4×
G.asparagifolium	c	cylindrical	2×, 4×
G.rhodopeum	c	cylindrical	2×, 4×
G.velenovskyi	c	cylindrical	4×
G.aegeum	c	cylindrical	4×

Results and Discussion

The MeOH extracts of the four examined *Galium* species showed differences in the iridoid composition and the total iridoid content according to TLC (three mobile phases) and HPLC fingerprint chromatograms. The concentrated extracts were partitioned between chloroform and water and the water soluble parts were subjected to treatment with charcoal, droplet counter current chromatography (DCCC) separation, column chromatography (CC) on silica gel and low pressure liquid chromatography (LPLC) with various solvent systems.

Eleven pure iridoid glucosides were isolated and identified by ^{1}H and ^{13}C NMR spectra (El-Naggar and Beal, 1980; Boros and Stermitz, 1990) and comparison with authentic samples as the known iridoids **1-11** (Table II, Fig.1). They were found for the first time in the investigated plants. Main components appeared to be asperuloside (**1**) and/or the non-acetylated iridoid acids, deacetylasperulosidic acid (**2**), scandoside (**3**) and monotropein (**4**).

A TLC method combined with scanning densitometry at 235 nm was used for the analysis of the iridoids in the presence of external standards. The data are summarized in Table III.

In *G. mirum* and *G. rhodopeum* prevailed the non-acetylated iridoid acids, especially monotropein. In *G. mirum* was found the highest content (4.1%) of the iridoid acids **2-4** along with asperuloside, asperulosidic acid (**5**), 6-O-acetylscando-

Table II. ^{13}C NMR data of compounds **1–11** in D_2O at 62.8 MHz.

C	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1	93.0	99.8	98.0	94.7	99.6	97.7	99.6	97.3	93.7	97.2	99.8
3	150.0	151.7	149.3	147.7	155.5	153.2	155.7	96.2	150.8	150.4	155.8
4	104.7	112.5	115.8	116.5	110.3	116.2	107.3	45.5	105.7	11.5	107.5
5	36.0	42.5	45.8	38.1	41.5	46.3	40.8	36.3	36.7	35.3	41.2
6	86.3	75.3	81.8	132.2	74.9	83.8	74.8	88.5	87.5	39.0	74.9
7	128.2	129.6	129.4	138.7	131.7	126.8	131.7	127.2	125.6	130.0	129.6
8	142.2	150.2	147.0	85.8	144.8	149.4	144.7	148.7	148.1	142.0	150.0
9	43.5	45.6	48.8	45.0	45.3	41.6	45.2	43.7	44.0	50.0	45.1
10	60.8	61.1	60.6	67.4	63.7	60.4	63.7	62.5	59.5	60.6	60.9
11	172.0	172.1	171.1	175.0	174.7	174.9	174.7	174.4	174.5	173.0	170.6
1′	98.6	101.1	99.6	98.9	100.8	99.7	101.0	98.6	99.4	99.5	101.4
2′	72.6	73.7	73.7	73.5	73.5	73.7	73.5	73.5	73.4	73.6	73.6
3′	75.5	76.6	76.6	76.4	76.4	76.6	76.4	76.4	76.3	76.5	76.5
4′	69.6	70.4	70.4	70.4	70.2	70.4	70.2	70.3	70.4	70.4	70.3
5′	76.4	77.0	77.1	77.1	76.8	77.2	76.9	76.7	77.2	77.5	77.0
6′	61.5	61.5	61.5	61.5	61.5	61.6	61.5	61.5	61.5	61.5	61.5
MeCO	20.3				21.0	21.7	21.0	21.0			
MeCO	170.3				172.0	172.1	170.4	172.0			
OMe							52.6				52.7

The shift for C-6′ was arbitrary set as δ 61.5.

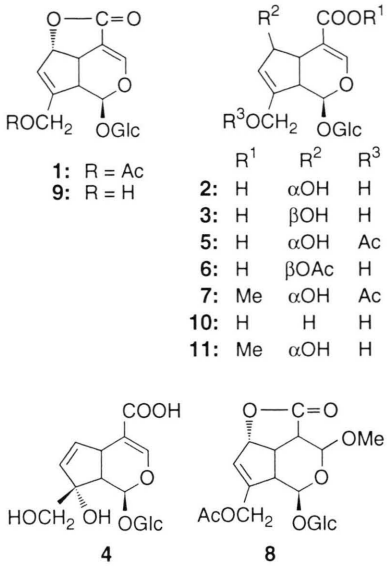

Fig. 1. Iridoid glucosides from the examined *Gallium* species.

side (**6**), as well as traces of daphylloside (**7**), iridoid V3 (**8**), deacetylasperuloside (**9**) and geniposidic acid (**10**). In *G. rhodopeum,* besides the acids **2-4,** were found asperulosidic acid and asperuloside. Compounds **7** and **8** might be formed as an artefacts during chromatography but their presence in these plants was proved by HPLC of the total extract.

On the other hand, asperuloside was the main component in *G. macedonicum* and *G. aegeum*. *G. macedonicum* was characterized with the highest content of asperuloside (4.3%), followed by deacetylasperulosidic acid (1.5%). Seven additional iridoids were isolated and identified as the known iridoids **3-9** together with the methyl ester of the deacetylasperulosidic acid (**11**). In *G. aegeum* the content of asperuloside reached 2.3%. Iridoids **2-7** and **9-11** were also identified.

A high content of sucrose was found in all plants with the exception of *G. mirum.*

Our studies showed that asperuloside, a main component in many *Galium* plants, is much more stable then the minor components daphylloside, asperulosidic acid and 6–0-acetylscandoside during chromatography and duration, contrary to the suggestion (Corrigan *et al.*, 1978) for a limited usefullness of asperuloside in taxonomy, because of its instability.

The biosynthesis of the *Galium* iridoids proceeds via iridodial and deoxyloganic acid to deacetylasperulosidic acid and asperuloside (Uesato *et al.*, 1986; Inouye *et al.*, 1988). It seems that *G. rhodopeum* and *G. mirum* contain more primitive iridoids (non-acetylated iridoid acids) while in *G. macedonicum* and *G. aegeum* prevail the products of further biosynthetic transformations (asperuloside). The ratio of the non-acetylated iridoid acids (**2**+**3**+**4**) and asperuloside seems to be a characteristict feature. For *G. macedonicum* and *G. aegeum*, this ratio is less than 1, while in *G. mirum* and *G. rhodopeum*, more than 1. Thus, the iridoid chemi-

Table III. Content of iridoid glucosides in the examined *Galium* species.

Compound	Iridoid: %*			
	G. macedonicum	*G. mirum*	*G. rhodopeum*	*G. aegeum*
Asperuloside (**1**)	4.3	0.2	0.2	2.3
Non-acetylated acids (**2+3+4**), total:	2.3	4.1	1.7	1.0
Deacetylasperulosidic acid (**2**)	1.5	0.9	0.3	0.1
Scandoside (**3**)	0.7	0.5	0.3	0.4
Monotropein (**4**)	0.1	2.7	1.1	0.5
Asperulosidic acid (**5**)	0.7	0.3	0.2	0.3
6-O-Acetylscandoside (**6**)	0.7	0.4	–	0.7
Daphylloside (**7**)	0.1	tr	–	0.3
Deacetylasperuloside (**9**)	0.1	tr	–	–
Geniposidic acid (**10**)	–	tr	–	tr
Deacetylasperulosidic acid methyl ester (**11**)	0.1	–	–	0.2
Total iridoids	8.3	4.8	2.1	4.8
Ratio **2+3+4/1**	0.5	20.5	8.5	0.4

tr < 0.1%; * of plant dry weight.

cal profile and the morphological characters suppose the development of two evolutionary lines in the *G. incurvum* group, those of the not caespitose and caespitose plants, in this case represented by *G. mirum* and *G. rhodopeum.* Of course, this suggestion could only be proved after evaluation of the intra-specific variability.

To clarify further phylogenetic connections and ecogeographical differentiation in the *G. incurvum* group, the iridoid composition of the closely related species *G. rigidifolium* Krendl, *G. asparagifolium* Boiss & Heldr. and *G. velenovsky* Ancev are to be studied, in parallel with some of the wide-leafed *Galium* species in the flora of the Balkan Peninsula.

Materials and Methods

The NMR spectra were measured on a Bruker 250 MHz spectrometer. Scanning was performed at 235 nm on a Shimadzu CS-930 densitometer in a zigzag reflection mode with a slit of 0.4x0.4 mm. Analytical HPLC was performed on an ISCO HPLC-system with a C18 Whatman ODS-3 column.

Plant material

Above ground parts of *G. mirum* (The foothills of the central Rhodopes, Besaparsky ridove, 350 m, 11.06.1992, A9234), *G. macedonicum* (Struma valley, north of Kresna, 250 m, 2.07.1992, A9275), *G. rhodopeum* (Besaparsky ridove, 300 m, 11.06.1992, A9232) and *G. aegeum* (Slavjanka Mt., 1400 m, 4.08.1994, A94116) were collected during flowering. Voucher specimens were determined by Dr. M. Anchev and deposited in the herbarium of the Institute of Botany, Bulgarian Academy of Sciences, Sofia (SOM).

Isolation

Dried ground aerial parts of *G. macedonicum* (318 g) were extracted twice with 3 l ethanol. After concentration the residues were dissolved in water and extracted twice with 200 ml $CHCl_3$. The water soluble parts were treated with charcoal (Merck, Cat. 2184) and eluted with 1 l H_2O and 0.5 l portions of 5% MeOH (residue 0.9 g), 30% MeOH (0.8 g), 50% MeOH (1.6 g), MeOH (0.9 g), MeOH-Me_2CO (1:1, v/v) (0.6 g) and MeOH-$Cl(CH_2)_2Cl$ (1:2, v/v) (0.7 g). The combined 30% MeOH, 50% MeOH, MeOH and MeOH-Me_2CO fractions were separated by ascending droplet counter current chromatography (DCCC) with $CHCl_3$-MeOH-H_2O (35:65:40), collecting 15 ml fractions. Fr. 19–22 (461 mg) afforded pure **1**, fr. 28–32 (258 mg) pure **7**, fr. 35–40 (82 mg) pure **8**. Fr. 1–5 (669 mg) were additionally separated on silica gel with $CHCl_3$-MeOH-H_2O-HCOOH (75:24:1:0.2) to give fr. 26–27 of pure **5** (43 mg) and fr. 33–35 – pure **6** (32 mg) and at the end with MeOH (298 mg) The last fraction was separated on silica gel with EtOAc-i-PrOH-H_2O (6:3:1) yielding **2** (42 mg), **3** (15 mg) and **4** (5 mg). Fr. 8–12 (380 mg) from the DCCC column were purified on a Lobar RP-18 column with 10% MeOH to pure **9** (fr.18; 9 mg) and **11** (fr.38–50; 60 mg).

The same way from *G. mirum* (121 g), *G. rhodopeum* (20 g) and *G. aegeum* (68 g) were isolated: from *G. mirum* - compounds **1-10;** *G. rhodopeum* – **1-5**, **10** and *G. aegeum* – **1-7**, **9-11** (Fig.1).

Analysis of iridoids

Sample preparation

Dried ground aerial parts (0.4 g) of *G. macedonicum, G. mirum, G. rhodopeum* and *G. aegeum,* respectively, were extracted with MeOH (2x6 ml). After concentration and addition of water (3 ml), extraction with $CHCl_3$ (3x2 ml) was carried out. The water layer was treated with neutral aluminium oxide (1 g). After filtration and washing with 3 ml H_2O and 3 ml H_2O-MeOH (1:1, v/v), the combined filtrates were concentrated and dissolved in 2 ml MeOH-H_2O (1:1, v/v).

TLC analysis

Aliquots (5.0 μl) of the sample solutions together with 5.0 μl of the standard solutions were applied to three silica gel plates (Merck Cat. 5554), developed with $CHCl_3$-MeOH-H_2O-HCOOH (75:24:1:0.2), EtOAc-i-PrOH-H_2O (6:3:1) and $CHCl_3$-MeOH-H_2O (60:22:4), respectively, and determined by densitometry at 235 nm.

HPLC analysis

10 µl of the sample solutions were injected. Gradient elution was used: pump A H_2O-MeOH (95:5) and H_3PO_4 (15 µl/100 ml mobile phase) and pump B MeOH. The substances were detected at 233 nm. The flow-rate was 0.8 ml/min.

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