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FLAVONOID VARIATION IN THE LIVERWORT *CONOCEPHALUM CONICUM*: EVIDENCE FOR GEOGRAPHIC RACES

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Abstract—The flavonoids of 2 samples of *Conocephalum conicum* gametophyte tissue have been studied, one from U.S.A. and the other from Germany. Common to both samples were vicenin-2, lucenin-2, the 7-*O*-glucuronides of apigenin, chrysoeriol and luteolin and the previously unknown 7-*O*-glucuronide 4'-*O*-rhamnosides of apigenin, chrysoeriol and luteolin. Additionally the German sample contained the 7,4'-di-*O*-glucuronides of apigenin and luteolin and a new compound, apigenin 7-*O*-diglucuronide 4'-*O*-glucuronide. The North American sample contained, additionally, luteolin 7,3'-di-*O*-glucuronide, luteolin 7-*O*-glucuronide 3',4'-di-*O*-rhamnoside (a new triglycoside) and 2 further derivatives of luteolin 7-*O*-glucuronide. Evidence is presented for the existence of geographic races of *C. conicum* and for the qualitative invariability of the flavonoid patterns with changing season or environment.

INTRODUCTION

The northern hemisphere Marchantialean liverwort family Conocephalaceae is represented by the single genus *Conocephalum* Werber. and contains only 2 species, the common *C. conicum* (L.) Underw., with wide distribution in Europe, North Africa, Asia and North America and *C. supradecompositum* (S.O. Lindberg) Steph. which is limited to China and Japan [1].

C. conicum has been the subject of a limited number of chemical studies which described its terpene, paraffin [2,3] and polysaccharide [4] content. Pryce detected lunularic acid in *C. conicum* and more recently isolated lunularic acid decarboxylase from this source [5].

An earlier brief communication [6] reported that a luteolin-*O*-glycoside occurred in *C. conicum*. The present work reports the isolation of a variety of apigenin, chrysoeriol and luteolin glycosides from samples of *C. conicum* collected in Washington State (USA) and the Saarland (W. Germany).

RESULTS

Figure 1 represents a composite 2D PC which includes all major flavone glycosides isolated from the American and European examples of *C. conicum*. A number of flavonoids are common to both samples, but there are also considerable differences.

Common to both samples are two di-*C*-glycosides, **10** and **11**, which were unaffected by acid treatment. These were shown to be the luteolin- and apigenin-6,8-di-*C*-glucosides, lucenin-2 and vicenin-2 respectively, by MS, TLC and PC comparisons with authentic material. Other flavonoids common to both samples are the 7-*O*-glucuronides of apigenin, **1**, chrysoeriol, **2**, and luteolin, **3**, plus a group of 3 glycosides, **7**, **8**, and **9** which were all 4'-substituted (for appearance on a PC plus R_f values see Table 1).

Prolonged acid hydrolysis (2 hr) of **7**, **8** and **9** liberated apigenin, chrysoeriol and luteolin respectively. Brief acid hydrolysis (10 min) liberated the 7-*O*-glucuronides of the flavones plus rhamnose. As expected, β-glucuronidase hydrolysis gave glucuronic acid plus the 4'-*O*-rhamnosides of the flavones (which appeared as dark spots, unaffected by NH_3, with R_f values as listed in Table 2). The UV spectral data for **7**, **8** and **9** (Table 1) were also consistent with that for flavone 7,4'-diglycosides [7,8]. It is therefore concluded that **7**, **8** and **9** are the 7-*O*-glucuronide-4'-*O*-rhamnosides of apigenin, chrysoeriol and luteolin respectively.

The chrysoeriol derivative is the first reported naturally-occurring 7,4'-diglycoside of this flavone. Seshadri and Vydeeswaran [8] have synthesized the 7,4'-diglucoside, and our UV spectra are consistent with their data.

The major flavonoids of the European sample of *C. conicum* were components **12** and **14**. Prolonged acid hydrolysis showed that they were both derivatives of apigenin, and β-glucuronidase hydrolysis liberated glucuronic acid and apigenin as the only products. Their UV spectra indicated that **12** and **14** were both 7,4'-disubstituted apigenins.

Brief acid hydrolysis partly converted **12** to a mixture of apigenin 7-*O*-glucuronide and apigenin 4'-*O*-glucuronide [both with R_f values 0·55 (TBA), 0·28 (15% HOAc)]. The structure of **12** is therefore consistent with

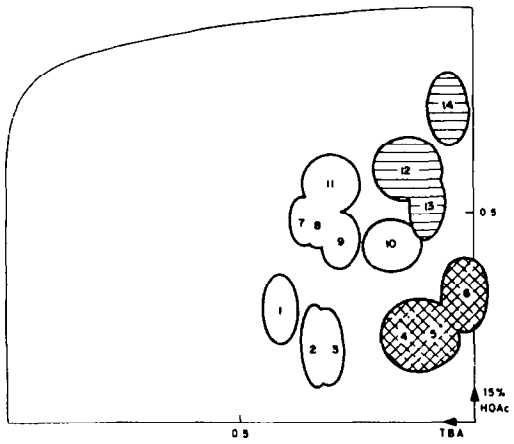

Fig. 1. PC pattern of the major flavonoids isolated from *Conocephalum conicum*. Horizontal hatching indicates spots unique to the European sample, crossed hatching indicates spots unique to the North American sample and unhatched spots are common to both samples.

Spot no. Compound structure

1 Apigenin 7-*O*-glucuronide.
2 Chrysoeriol 7-*O*-glucuronide.
3 Luteolin 7-*O*-glucuronide.
4 Luteolin 7,3'-di-*O*-glucuronide.
5 Luteolin 7-*O*-glucuronide derivative.
6 Luteolin 7-*O*-glucuronide-3',4'-di-*O*-rhamnoside + derivative.
7 Apigenin 7-*O*-glucuronide 4'-*O*-rhamnoside.
8 Chrysoeriol 7-*O*-glucuronide 4'-*O*-rhamnoside.
9 Luteolin 7-*O*-glucuronide 4'-*O*-rhamnoside.
10 Luteolin 6,8-di-*C*-glucoside.
11 Apigenin 6,8-di-*C*-glucoside.
12 Apigenin 7,4'-di-*O*-glucuronide.
13 Luteolin 7,4'-di-*O*-glucuronide.
14 Apigenin 7-*O*-diglucuronide-4'-*O*-glucuronide.

apigenin 7,4'-di-*O*-glucuronide. This was confirmed by co-chromatography with the authentic compound from *Marchantia polymorpha* [7].

The other apigenin glucuronide was studied similarly. Brief acid hydrolysis partly converted **14** to an intermediate glycoside with a free 4'-hydroxyl group [R_f 0·29 (TBA), 0·40 (15% HOAc)] as the major product plus a small amount of the 7- and 4'-*O*-glucuronides of apigenin. The major product was a 7-glycoside (UV data) and its R_f values on PC are close to those expected for an apigenin diglucuronide [cf. apigenin 7,4'-di-*O*-glucuronide, R_f 0·21 (TBA), 0·55 (15% HOAc)]. On this basis the intermediate product was considered to be apigenin 7-*O*-diglucuronide.

Longer hydrolysis (1 hr) converted most of **14** to a mixture of apigenin and the 7-*O*-diglucuronide. Minor products were the 7- and 4'-*O*-monoglucuronides, and a trace of the 7,4'-di-*O*-glucuronide. The hydrolysis data thus establishes the structure of **14** as apigenin 7-*O*-diglucuronide 4'-*O*-glucuronide. This is a new apigenin derivative. The only other known flavonoid glycoside which contains a disaccharide of glucuronic acid is acacetin 7-*O*-diglucuronide which was isolated from *Clerodendron trichotomum* [9]. The acid hydrolysis results give a guide to the relative ease of cleavage of the various glycosidic links in this triglycoside. For example, since apigenin 7-*O*-diglucuronide and apigenin are the major hydrolysis products, it follows that the 4'-glucuronide linkage is the most readily hydrolysed and that the interglycosidic linkage (within the disaccharide) is more stable than the 7-glucuronide linkage. The relative stabilities of the three linkages to acid are thus, interglycosidic > 7-glucuronide > 4'-glucuronide.

The remaining component in the European sample (spot 13), was shown to be luteolin 7,4'-di-*O*-glucuronide. This compound was previously isolated from *Marchantia polymorpha* [7] and *M. berteroana* [10], and the identity was established by enzymic hydrolysis, UV spectroscopy and 2D PC.

The major flavonoids in the Washington sample of *C. conicum* were the di-*C*-glucosides vicenin-2 and lucenin-2 (spots **11** and **10**). These were accompanied by three other spots, **4**, **5** and **6**, which were absent in the European sample. Prolonged acid hydrolysis (1·5 hr) showed that all three were luteolin glycosides.

Compound **4** was hydrolysed completely with β-glucuronidase to luteolin and glucuronic acid and had UV spectra consistent with it being a 7,3'-disubstituted luteolin. Co-chromatography confirmed its structure to be luteolin 7,3'-di-O-glucuronide, a compound which has previously been isolated from *M. polymorpha* [7], *M. berteroana* [10] and *M. macropora* [11].

Table 1. R_f and spectral data of new flavone glycosides from *Conocephalum conicum*

Component no.		5	6 and 6B	7	8	9	14
Spot colour	(UV/NH_3)	yellow	dark	dark	dark	brown	dark
R_f	TBA	0·06	0·03	0·46	0·40	0·35	0·09
	15% HOAc	0·15	0·26	0·46	0·42	0·37	0·69
	30% HOAc	0·38	0·47	0·80	0·72	0·65	
Spectral maxima (nm)	MeOH	270,321	269,325	266,319†	265,332†	266,331†	268,325†
	NaOMe	279,300*sh*	287,319*sh*	282,360*†	280,308*sh*	267,291*sh*	286,360*sh**†
		404	377*		363*†	360*†	
	NaOAc	267,308*sh*	269,325		268,328		
		347*sh*,399					
	$NaOAc-H_3BO_3$	269,325	269,327				
	$AlCl_3$	260*sh*,275,	255,278,290*sh*,				
		291*sh*,344,380	338,378				
	$AlCl_3$-HCl	260*sh*,275,	255,277,289*sh*				
		219*sh*,343,379	337,376				

* Shift accompanied by decrease in intensity. † Solvent = H_2O.

Table 2. Comparative R_f data for the flavone rhamnosides

Flavonoid	R_f		
	TBA	15% HOAc	30% HOAc
Apigenin 4′-rhamnoside	0·83	0·27	0·55
Chrysoeriol 4′-rhamnoside	0·74	0·27	0·55
Luteolin 4′-rhamnoside	0·70	0·20	0·38
Luteolin 3′,4′-dirhamnoside	0·54	0·33	0·56

Compound **5** was separable from **4** only by prolonged PC in TBA. The amount isolated was insufficient for full structure analysis but it was shown to produce luteolin 7-*O*-glucuronide on acid hydrolysis and to be unaffected by hydrolysis with β-glucosidase, β-glucuronidase or α-rhamnosidase (present in our pectinase*).

Spot **6** was initially thought to be one compound but it was finally resolved (with difficulty) into two, **6A** (R_f 0·16) and **6B** (R_f 0·11) on polyamide TLC. Compound **6B** was separated from **6A** by treating **6** with β-glucuronidase. This process removed glucuronic acid from **6A** (PC evidence) and left **6B** unchanged and thus separable by PC (TBA).

On prolonged acid hydrolysis both **6A** and **6B** gave luteolin as the sole aglycone, together with a little luteolin 7-*O*-glucuronide. On mild acid hydrolysis they both gave luteolin 7-*O*-glucuronide as an end product. The UV spectral data for **6A** + **6B** and **6B** are the same (Table 1) and are consistent with both **6A** and **6B** being 7,3′,4′-tri-substituted luteolins (7,4′-disubstitution is excluded by the NaOMe-induced shift of band II [7]).

Treatment of compound **6** (**6A** + **6B**) with β-glucuronidase converted **6A** completely into a 3′,4′-disubstituted product which on brief acid treatment gave luteolin plus rhamnose. The chromatographic relationship of this product to luteolin 4′-*O*-rhamnoside (see Table 2) suggests that it is a dirhamnoside and its UV spectra are identical with those of the previously isolated luteolin 3′,4′-di-*O*-glucuronide [12]. As expected however, the mobility of the dirhamnoside is higher than the diglucuronide in TBA and lower in 15% HOAc. Thus the structure is considered to be luteolin 3′4′-di-*O*-rhamnoside and accordingly, that of compound **6A**, luteolin 7-*O*-glucuronide-3′,4′-di-*O*-rhamnoside.

Compound **6B** on the above evidence appears to be a derivative of 3′,4′-di-*O*-substituted luteolin 7-*O*-glucuronide. Mild acid hydrolysis of **6B** did yield some luteolin 7-glucuronide but the major product was a low mobility compound [R_f 0·14 (TBA), 0·06 (15% HOAc)] which was also produced without the accompanying luteolin 7-glucuronide by α-rhamnosidase (i.e. pectinase) treatment. This product possessed an *o*-dihydroxyl group in the B-ring and was converted to luteolin 7-glucuronide on acid hydrolysis. β-Glucuronidase had no effect on either **6B** or its low mobility hydrolysis product suggesting that the glucuronic acid moiety is derivatized.

* We have established that Koch-Light pectinase ex. *A. niger* has some α-rhamnosidase activity. For example it cleaved rhamnose completely from both rutin and quercetin 3-rhamnoside after 3 days in distilled water. Kaempferol 7-neohesperidoside and rhamnosyl luteolin-8-*C*-xyloside however were unaffected.

DISCUSSION

Conocephalum conicum contains a number of new and novel flavonoids. In common with other liverworts of the order Marchantiales, flavone *O*-glucuronides are a feature of its flavonoid chemistry. Although both populations studied contained a common set of flavone glycosides (see Fig. 1), the most interesting result is that these are accompanied by a set of 7,4′-glucuronides of apigenin and luteolin (**12**, **13**, **14**) in the European sample, which are not present in the American sample, and a complex set of glycosides of luteolin (**4**, **5**, **6**) which are present in the American but not in the European sample.

The possibility that these differences are due to a seasonal or climatic effect was eliminated by the finding that the flavonoid pattern in the European sample is the same in mid-winter (when the American sample was collected) as in mid-summer. Moreover, the flavonoid pattern of the German material remained unchanged after 2 yr culture in a greenhouse at constant temperature and humidity. These results confirm that flavonoids, at least for some liverworts, represent reliable taxonomic characters qualitatively independent of season and site of growth.

The present evidence thus suggests that *C. conicum* exists as two chemically distinguishable geographic races, as two chemically distinguishable geographic races and the marked, but less distinctive morphological differences between the 2 populations [13] tend to support this view. The full extent of flavonoid variation in *C. conicum* however must await a survey across its full Northern Hemisphere distribution. Of particular interest would be a study of Chinese or Japanese *C. conicum* in conjunction with the solely Asian species, *C. supradecompositum*.

EXPERIMENTAL

Plant material. The American sample of *Conocephalum conicum* (L.) Underw. was collected in December in Snohomish County, Washington (voucher specimen, B. G. Brehm and P. C. Comp in the Herbarium, Reed College). The European sample of *C. conicum* was collected in June, 1972, at Steinbachtal, Saarland. Further collections were made in January, 1975, from the above locality, from Stiftswald St. Arnual Saarland, and from material originally collected at Steinbachtal, and grown on garden soil in a greenhouse at the Universität des Saarlandes for over 2 yr (voucher specimens for all samples, collected by R. Mues, are held in the Herbarium of Fachrichtung Botanik, Universität des Saarlandes).

Isolation procedure. C. conicum gametophyte tissue was air-dried prior to extraction. Flavonoids were extracted as described previously [7]. Individual compounds were isolated by 2-D PC followed by purification by 1-D PC (often 'overrun' to achieve separation). Chromatographic, spectroscopic and hydrolytic procedures used to elucidate the flavonoid glycoside structures have been described previously [7,10]. Enzymic hydrolyses were all carried out in dist. H_2O, overnight.

The identities of known flavone glycosides isolated from this plant were in all cases checked by 2-D co-chromatography with authentic material on paper or TLC cellulose using TBA and HOAc (15 or 30%) as solvents. Solvent used with polyamide plates was MeOH–HOAc–H_2O, 8:1:1.

4′-Rhamnosides of luteolin. The 4′-rhamnoside and 3′-4′-dirhamnoside of luteolin were produced by β-glucuronidase hydrolysis of compounds **9** and **6** respectively and purified by 2-D PC. UV data for luteolin 4′-rhamnoside: λ_{max} (MeOH) 268, 285*sh*, 326; (+NaOAc) 273, 315*sh*, 350 nm and for luteolin 3′,4′-dirhamnoside: λ_{max} (MeOH) 268, 285*sh*, 321; (+NaOAc) 276, 311*sh*, 366*sh* (+NaOAc–H_3BO_3) 268, 285*sh*, 321 nm.

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