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PHENOLIC COMPOSITION AND ITS SEASONAL VARIATION IN *CALLUNA VULGARIS*

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Key Word Index—*Calluna vulgaris*; Ericaceae; phenolic acids, flavonoids; new dihydroflavonol glycoside; callunin; seasonal variation.

Abstract—Major phenolic compounds in the shoots of *Calluna vulgaris* were chlorogenic acid, the 3-*O*-glucoside, 3-*O*-galactoside and 3-*O*-arabinoside of quercetin, (+)-catechin, procyanidin D_1 and a newly identified phenolic glycoside, 3, 5, 7, 8, 4′-pentahydroxyflavanone 8-*O*-glucoside (callunin). Roots contained only (+)-catechin and procyanidin D_1 in high quantities. Smaller amounts of other phenolic compounds were present in both shoot and root. The number and the quantity of these substances were much higher in the summer months than in winter.

INTRODUCTION

Calluna vulgaris L. Hull occurs as the dominant plant over thousands of hectares of nutrient-poor heathland and moorland in northern and western Europe. Its success in these circumstances may be partly attributable to its capacity to tolerate soils of low mineral nutrient status. In addition, preliminary analysis has revealed that *Calluna* tissues have a high total phenolic content [1] and that some of these compounds are water leachable. It is therefore possible that allelopathic interactions with potential competitors together with effective anti-herbivore defences might contribute to the success of the plant. Allelopathic interaction involving phenolic compounds have been demonstrated previously in the ericaceous plants *Arctostaphylos glandulosa* [2] and *Erica australis* [3, 4].

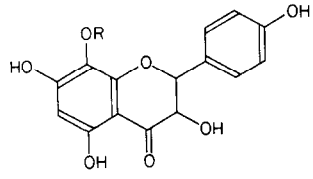
As part of an analysis of the basis of interaction between *Calluna vulgaris* and its associated species a detailed qualitative and quantitative determination of the phenolic composition of its shoots and roots has been carried out. The seasonal variation of phenolic contents has also been followed.

RESULTS AND DISCUSSION

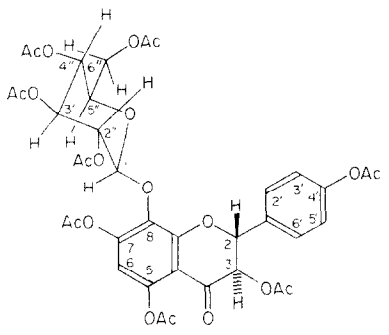
Phenolic composition

Fresh plant material (800 g) was extracted in MeOH–H_2O (4:1) and the EtOAc part of this extract (23 g) was separated by passing repeatedly through columns of Sephadex LH20 using EtOH, H_2O, EtOH–H_2O (1:1) and EtOH–EtOAc (1:1) for different mixtures of the compounds. The following major phenolic constituents were obtained: (1) chlorogenic acid (3.2 g), (2) quercetin 3-*O*-glucoside and 3-*O*-galactoside (2.4 g), (3) quercetin 3-*O*-arabinoside (1.3 g), (4) (+)-catechin (1.5 g), (5) procyanidin D_1 (1.2 g) and (6) a previously unknown compound, named callunin (1.1 g).

Callunin (**1**), which crystallized from H_2O in long colourless needles, mp 187–188° yielded a phenolic aglycone (**2**), and glucose, after acid hydrolysis. The UV spectrum of callunin in MeOH (λ_{max} 293 and 328 nm) suggested that it was a glucoside of either a flavanone or a dihydroflavonol with three hydroxyl groups in the A-ring. A 35-nm bathochromic shift in NaOAc indicated that it contained a free 7-OH group [5]. The presence of an unsubstituted 5-OH group was shown by an HCl-stable bathochromic shift in $AlCl_3$ for each of the bands (24 nm in Band II). The proton NMR spectrum of callunin in d_6-acetone clearly showed that the A-ring contained only one aromatic proton (6-H, δ 6.00 ppm, singlet) and the B-ring, had four aromatic protons split into 2 doublets (3′-H + 5′-H, δ 6.87 ppm and 2′-H + 6′-H, δ 7.48 ppm; $J = 8.8$ Hz). The C-ring contained two diaxial protons giving two doublets ($J = 11.7$ Hz) at δ 5.15 ppm (2-H) and 4.58 ppm (3-H). A clear picture of both the phenol and the sugar part of the molecule emerged from the NMR spectrum of its octaacetate (**3**). The acetylation shifted the aromatic proton signals further downfield (6-H, δ 6.60 ppm, *s*; 3′-H and 5′-H, 7.20 ppm, *d*; 2′-H and 6′-H, 7.52 ppm, *d*, $J = 9$ Hz), spread the C-ring and sugar proton signals and confirmed that β-D-configuration of the glycosyl residue (axial anomeric proton). Decoupling of the proton signals showed that 2-H and 3-H in the C-ring were 1, 2 diaxial (2-H δ



1 R = Glc
2 R = H



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5.83 ppm, *d*; 3-H 5.52 ppm, *d*, $J = 12.5$ Hz), the β-D-glucopyranosyl ring protons were axial (1″-H, 5.00 ppm, *d*, $J_{1''2''} = 7.5$ Hz; 2″-H, 3″-H and 4″-H, 5.13 ppm, *m*; 5″-H, 3.46 ppm, *m*, $J_{4''5''} = 9.5$ Hz) and the C-6″ protons of glucose were gauche to C-5″ proton (6″-H, 4.39 ppm, *q*, $J_{5''6''} = 4.5$ Hz, $J_{6''6'''} = 12.5$ Hz; 6‴-H, 4.28 ppm, *q*, $J_{5''6'''} = 2.5$ Hz). The NMR of the octa-acetate also showed the acetate proton signals in distinct groups corresponding to their attachment to aromatic (δ 2.29, 2.31 and 2.36 ppm), C-ring (δ 1.72 ppm) and glucose (δ 1.97, 1.99, 2.02 and 2.03 ppm) part of the molecule.

The aglycone (**2**) obtained after acid hydrolysis, and purified by passing through Sephadex LH20 crystallized from EtOH–H_2O in pale yellow needles (mp 230–231°). The mass spectrum of this compound was typical of a dihydroflavonol [6] and showed that the A-ring contained three OH groups. The fragment ions containing the B-ring had the other two hydroxyl groups. Its UV spectrum in MeOH was similar to callunin (λ_{max} 296 and 328 nm), but the spectra deteriorated rapidly on addition of NaOMe and slowly in presence of NaOAc. This was interpreted to indicate that the glucose residue was attached to either the C-6 or C-8 hydroxyl position in callunin. According to Mabry [7], the decomposition of spectra in the presence of NaOMe and NaOAc is indicative of 5, 6, 7-trihydroxyl pattern in the A-ring of flavanones and dihydroflavonols. But, as the flavanones having free *ortho*-dihydroxyl groups and the flavones containing a 5, 7, 8-trihydroxyl system in the A-ring also deteriorated under similar conditions [5] and the C-6 and C-8 protons often gave an NMR signal in the same region of the spectrum, it was difficult to assign the position of the third hydroxyl group in the A-ring. However, failure of callunin to produce a peak in the region of 500–700 nm after treatment with 2, 6-dichloroquinone-4-chloroimide in pyridine and borate buffer at pH 9.2 (Gibbs reaction modified by King *et al.* [8]) clearly demonstrated that the C-8 position was not free, but substituted by the third hydroxyl group and glucose. The 7,8-*ortho*-dihydroxyl system in the A-ring of **2** was responsible for a 10-nm bathochromic shift in Band II in $AlCl_3$ over and above that observed with $AlCl_3$–HCl (5). This evidence confirmed the 3, 5, 7, 8, 4′-pentahydroxyflavanone 8-*O*-β-D-glucopyranoside structure (**1**) for callunin. This structure is also based on the analogy with previously reported compounds from *Calluna* flower and shoot, i.e. 3, 5, 7, 8, 4′-pentahydroxyflavone (**4**) [9] and 5,7,8,4′-tetrahydroxyflavone (**5**) [10].

The *Calluna* shoot contained a number of other phenolic compounds (Table 1); important among these were the phenolic glycosides orcinol-β-D-glucoside and arbutin, the 3-*O*-glucoside, 3-*O*-galactoside and 3-*O*-arabinoside of kaempferol, the flavanol aglycones quercetin and kaempferol, (−)-epicatechin and a number of procyanidins. Structures of these procyanidins have been published before [11]. A few minor compounds could not be identified completely because of their insufficient quantity; one such compound is a simple phenol thought to be of the phloroglucinol type (FIBI). This compound appeared in the newly formed *Calluna* shoot in the month of March and was later replaced by its glucoside (FIB) in the early summer months.

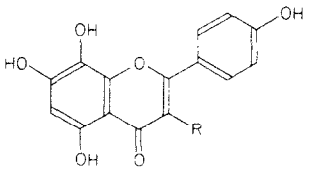
The presence together of 3-*O*-galactosides and 3-*O*-arabinosides of flavonols is known to be a regular feature of many ericaceous plants, e.g. *Rhododendron* and *Vaccinium* sp. [12]. *Calluna* also seems to follow this pattern. Quercetin-3-*O*-galactoside was already reported from *Calluna* flowers [9]. However, in the present case, this compound was found to be present with quercetin-3-*O*-glucoside in an equal mixture and it was only possible to separate them by repeated chromatography through a Sephadex LH20 column. The identities of their sugar residues were confirmed by GC after hydrolysis.

Calluna roots produced fewer phenols than the shoot. Other than (+)-catechin and procyanidin D-1, (−)-epicatechin, procyanidins A-2, B-1 and C-1, ferulic acid, vanillic acid and arbutin were present in the roots in small amounts.

Seasonal variation

The composition and the quantity of phenolic compounds in the *Calluna* plant varied considerably with season (Table 1). The smallest number of phenols was present in the months of January and February when most of the phenolic material in the shoot was in the form of chlorogenic acid and quercetin 3-*O*-glycosides. In March and April, these substances decreased in amount and a number of simpler phenolic compounds appeared in small quantities. These were gradually replaced by (+)-catechin, procyanidin D-1, chlorogenic acid, callunin, orcinol-β-D-glucoside, quercetin and its glycosides in the month of May and June. This pattern did not change significantly until December except for the occasional disappearance of callunin (in July and November).

Interpretation of the phenolic constitution of ericaceous roots is complicated by the presence of mycorrhizal fungi within many of the cortical cells [13]. These fungi may utilize or transform phenolic



4 R = OH

5 R = H

compounds formed in the roots. Roots generally contain only a few phenols in small quantities during the months of January to April. With the formation of new roots in May, greater numbers of phenolic compounds, some in large quantities, were produced. While chlorogenic acid and callunin were normally absent in roots they were detected in June samples. At this time of year, assimilation and root production was rapid and may have led to the production of an excess of compounds normally utilized by the mycorrhizal fungi. The presence of these compounds, specially callunin, in uninfected adventitious roots produced in the above ground part of the *Calluna* plant during August, favoured the view that these compounds did not co-exist with the mycorrhizal fungus. A closely related phenolic compound, poriolin (5, 7, 4'-trihydroxy-6-methylflavanone 7-*O*-glucoside) was found by Hillis and Ishikura [14] in

Table 1. Seasonal variation in the production of phenolic compounds of *Calluna* shoot and root

Month	J	F	M	A	M	J	J	A	S	O	N	D
Shoot phenols												
Caffeic acid	—	1	2	2	1	—	—	—	—	—	—	—
Orcinol	—	—	2	1	—	—	—	—	—	—	—	—
Orcinol-β-D-glucoside	—	—	—	—	2	2	3	1	2	2	3	2
Arbutin	1	—	—	—	1	—	1	—	—	1	1	2
Chlorogenic acid	2	3	1	2	4	3	3	3	3	3	2	4
Isochlorogenic acid	—	—	1	2	—	—	—	—	—	—	1	—
F1B	—	—	—	—	1	2	2	—	—	1	2	—
F1B1	—	—	2	2	2	2	1	—	—	—	1	—
Callunin	—	—	—	2	4	3	—	4	2	2	—	2
Kaempferol	—	—	1	1	1	1	1	—	1	—	1	1
Quercetin	1	1	1	1	2	3	3	1	4	1	4	2
Kaempferol 3-*O*-glucoside	—	—	—	1	1	1	1	1	1	1	1	—
Kaempferol 3-*O*-galacoside	—	—	—	1	1	1	1	1	1	1	1	—
Kaempferol 3-*O*-arabinoside	—	—	—	1	1	1	1	1	—	—	1	—
Quercetin 3-*O*-glucoside	4	1	1	2	3	4	3	4	4	3	3	3
Quercetin 3-*O*-galacoside	4	1	1	2	3	4	3	4	4	3	3	3
Quercetin 3-*O*-arabinoside	1	1	1	1	2	2	1	1	2	2	2	1
(+)-Catechin	1	1	—	1	2	3	3	3	2	3	4	3
(−)-Epicatechin	1	1	—	1	1	1	1	1	1	1	2	1
Procyanidin D1	1	1	2	2	2	3	2	—	1	3	2	2
Procyanidin B1	—	1	1	1	1	1	1	—	—	1	1	1
Procyanidin B2	—	—	—	—	1	—	1	1	1	1	1	1
Procyanidin B3	—	—	—	1	1	1	1	—	1	—	—	—
Procyanidin B4	—	—	—	—	—	1	1	—	—	—	—	—
Procyanidin B5	—	—	—	—	—	1	1	—	1	—	—	—
Procyanidin C1	—	1	1	—	1	1	1	1	1	1	1	1
Root phenols												
(+)-Catechin	2	1	1	—	3	3	3	3	3	3	3	3
(−)-Epicatechin	—	—	—	—	1	1	2	2	2	1	1	1
Chlorogenic acid	—	—	—	—	—	3	1	—	—	—	—	—
Callunin	—	—	—	—	—	2	—	—	—	—	—	1
Arbutin	1	1	1	1	1	2	1	2	2	1	2	1
Orcinol-β-D-glucoside	—	—	—	—	1	1	—	—	—	—	1	2
Ferulic acid	1	1	1	1	1	—	—	1	—	1	1	—
Vanillic acid	—	—	—	—	1	1	1	1	1	1	1	—
Procyanidin D1	1	1	1	—	3	3	3	3	3	3	3	1
Procyanidin A2	—	—	—	—	1	1	1	1	2	1	1	1
Procyanidin B1	—	—	—	—	1	1	1	1	1	1	—	—
Procyanidin B2	—	—	—	—	—	1	1	—	—	—	—	—
Procyanidin B3	—	—	—	—	1	1	1	—	—	—	—	—
Procyanidin C1	—	—	—	—	1	1	1	1	1	1	1	—
Total phenol content in shoot (% dry wt)	16.0	15.6	15.3	16.2	23.4	21.7	26.5	19.4	28.4	23.0	21.9	19.8
Total phenol content in root (% dry wt)	2.5	2.2	2.8	4.8	6.8	7.8	9.5	5.7	9.5	8.7	6.1	4.6

Numbers represent relative intensity of the spot on paper chromatogram after spraying with 2,6-dibromoquinone 4-chloroimide, 1% solution in EtOH, followed by 10% $NaHCO_3$; (−) absent or present in trace quantity. Total phenol measurement was done with (+)-catechin as standard.

large quantities uninfected roots of *Pseudotsuga menziesii*, while the compound was completely absent in its mycorrhizal roots.

The total phenol content (Table 1) was in close agreement with the compositional change of phenolic compounds in the shoot and root of *Calluna* plant.

EXPERIMENTAL

Extraction of phenols. Plant material (800 g) was extracted ×4 with MeOH–H_2O (4:1). The combined extracts (2 l.) were evapd to remove MeOH, the residue diluted with H_2O (400 ml) and extracted with EtOAc (4 × 400 ml). The EtOAc extract was dried and evapd *in vacuo*, giving a powdery residue (23 g). This was dissolved in hot EtOH (50 ml), cooled, filtered and then passed through a column of Sephadex LH20 (60 cm × 5 cm) using EtOH as eluting solvent. Each of the 11 fractions obtained (containing mixtures of different compounds) were rechromatographed repeatedly in a smaller column (40 cm × 2.5 cm) using EtOH, H_2O, EtOH–H_2O (1:1) and EtOH–EtOAc (1:1) as solvents until individual compounds were obtained in pure form. The monthly sampling was carried out in the Cropton forest near Rosedale in North Yorkshire and the plant materials were extracted in MeOH–H_2O (4:1). The total phenol content was measured in this extract according to the method of Forrest and Bendall [15]. The MeOH–H_2O extract was evaporated *in vacuo* to remove MeOH and the aqueous residue was extracted ×4 into EtOAc. This extract was concentrated and a known quantity of it was separated on Whatman No. 1 paper (25 × 25 cm^2) in 6% HOAc (solvent A) in the first direction and *i*-BuOH–HOAc–H_2O 14:1:5 (solvent B) in the other direction.

All phenolic glycosides were hydrolysed individually in 2 N HCl at 98° for 30 min. The aglycone was extracted in EtOAc, evaporated to dryness and purified by passing through a small column of Sephadex LH20 (15 cm × 2 cm). The aqueous fraction was treated with a mixture (1:1) of Amberlite IR-45 (OH) and IR-120 (H) ion exchange resins until neutralized and the sugars were identified by spraying with 1% *p*-anisidine HCl in BuOH (+ heating) after chromatography on cellulose layers in (1) *n*-PrOH–EtOAc–H_2O, 6:1:3 (developed twice), (2) EtOAc–HOAc–H_2O, 14:3:3, and (3) pyridine–EtOAc–H_2O, 2:8:1. Finally, they were confirmed by GC after derivatization, into their TMS ethers by dissolving in pyridine (1 cm^3) and BSTFA (1 cm^3). The column packing was 2% SE52 on Chromosorb WHP (100–120 mesh) and the N_2 carrier flow rate was 30 cm^3/min. Callunin octa-acetate was prepared according to [11] and purified on precoated Si gel layers developed in EtOAc–C_6H_6 (2:5). The common phenolic compounds and the glycosides were identified by their R_f value, response to UV light, their colour reactions with spray reagents and by UV spectroscopy [5]. Quercetin from all sources were confirmed by mass spectroscopy. ^{1}H NMR spectra were obtained with a 220-MHz spectrometer.

Callunin (**1**), long colourless needles from H_2O, had mp 187–188° R_f: A. 0.67, B. 0.54; Colour: UV/± NH_3, invisible; 2, 6-dibromoquinone-4-chloroimide (DBC) followed by 10% $NaHCO_3$ solution: Prussian blue turning to blue-green. UV: λ_{max} (MeOH) 293 nm (ε 11,888), 328 nm (ε 6923); (NaOMe): 246 (ε 15,664), 328 (ε 21,119) ($AlCl_3$): 317 (ε 19,625), 396 (ε 3000); ($AlCl_3$ + HCl): 316 (ε 18,500), 396 (ε 3000); (NaOAc): 250 (ε 6524), 328 (ε 23,000); (NaOAc + H_3BO_3): 290 (ε 11,886), 330 (ε 8268); NMR [$(CD_3)_2CO$] δ 2.90–3.90 ppm (*m*, glucose protons), 4.58 ppm (1 H, *d*, *J* = 11.7 Hz, C-3), 4.80 ppm (1 H, M, C-1″ of glucose), 5.15 ppm (1 H, *d*, *J* = 11.7 Hz, C-2), 6.00 ppm (1 H, *s*, C-6), 6.87 ppm (2 H, *d*, *J* = 8.8 Hz, C-3′ and C-5′), 7.48 ppm (2 H, *d*, *J* = 8.8 Hz, C-2′ and 6′). NMR, callunin octa-acetate ($CDCl_3$): δ 1.72 ppm (3 H, *s*, C-ring acetate proton), 1.97, 1.99, 2.02, 2.03 ppm (4 × 3 H, *s*, glucose acetate protons), 2.29, 2.31, 2.36 ppm (3 × 3 H, *s*, aromatic acetate protons), 3.46 ppm (1 H, *q*, C-6″), 5.00 ppm (1 H, *d*, *J* = 7.5 Hz, C-1″), 5.13 ppm (3 H, complex *m*, C-2″, 3″ and 4″), 5.52 ppm (1 H, *d*, *J* = 12.5 Hz, C-3), 5.83 ppm (1 H, *d*, *J* = 12.5 Hz, C-2), 6.60 ppm (1 H, *s*, C-6), 7.20 ppm (2 H, *d*, *J* = 9 Hz, C-3′ and 5′), 7.52 ppm (2 H, *d*, *J* = 9 Hz, C-2′ and 6′). MS: major ions same as those of aglycone. Hydrolysis products: (**2**) and glucose.

Aglycone (**2**), pale yellow needles from EtOH–H_2O, had mp 230–231°. R_f: A. 0.41, B. 0.65; Colour: UV/± NH_3 invisible; 2, 6 DBC/$NaHCO_3$: grey turning to blue green. UV: λ_{max} (MeOH): 296 nm (ε 11,193), 328 (ε 7045) (NaOMe): 250, 310, 380 degenerate rapidly, ($AlCl_3$): 265 (ε 5784), 329 (ε 15,784), 341 (sh), 432 (ε 2598), ($AlCl_3$ + HCl): 319 (ε 16,078), 418 (ε 2549), (NaOAc): 245, 310, 383 degenerate slowly, (NaOAc + H_3BO_3): 301 (ε 13,369), 380 (sh). MS: *m/z* 304 (49), $M^{+\cdot}$, $C_{15}H_{12}O_7$; 302 (4), $[M-2]^{+\cdot}$ $C_{15}H_{10}O_7$; 182 (15), $C_8H_6O_5$; 169 (100) $[A_1+1]^+$ $C_7H_5O_5$; 168 (37), $A_1^{+\cdot}$, $C_7H_4O_5$; 167 (18), $[A_1-1]^+$, $C_7H_3O_5$; 140 (11), $[A_1-28]^{+\cdot}$, $C_6H_4O_4$; 136 (30), $B_3^{+\cdot}$, $C_8H_8O_2$; 135 (6), $[B_3-1]^+$, $C_8H_7O_2$; 134 (28),$[B_3-2]^{+\cdot}$ $C_8H_6O_2$; 107 (78), B_4^+ and $[B_1-1-28]^+$, C_7H_7O; 106 (10), $[B_4-1]^+$ C_7H_6O.

Unidentified compounds: F1B, colourless plates from MeOH, mp 185–186°. R_f A. 0.76, B. 0.64; colour: UV/±NH_3: invisible; 2, 6 DBC/$NaHCO_3$: deep blue turning to purple. UV: λ_{max} (MeOH): 282 nm, 325 nm; (NaOMe): 282, 328; ($AlCl_3$): 306, 366; ($AlCl_3$ + HCl): 303, 366; (NaOAc) and (NaOAc + H_3BO_3): 282, 325. Hydrolysis products: F1B1 and glucose.

F1B1, R_f A. 0.50, B. 0.90. Colour: UV/± NH_3: Invisible; 2, 6 DBC/$NaHCO_3$: purple; UV: λ_{max} (MeOH): 287 nm, (NaOMe): 245, 321; ($AlCl_3$): 308, 360; ($AlCl_3$ + HCl): 307, 360; (NaOAc): 320, (NaOAc + H_3BO_3), 289.

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