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# UNIFORMITY AND DISTINCTNESS OF *PHYLLOCLADUS* AS EVIDENCED BY FLAVONOID ACCUMULATION

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**Key Word Index**—*Phyllocladus*; Podocarpaceae; conifers; flavonoids; luteolin 3′-*O*-rhamnoside; luteolin 7-*O*-rhamnoside; chemotaxonomy.

**Abstract**—The conifer genus *Phyllocladus* is shown by comparative flavonoid chemistry to be remarkably homogeneous and quite distinct from other studied genera in the Podocarpaceae. It is characterized by the accumulation (in the foliage) of a predominance of flavone *O*-glycosides, and in particular, luteolin 7- and 3′-*O*-glycosides. Lower levels of flavonol *O*-glycosides are also evident. Two flavone glycosides are reported for the first time, luteolin 3′-*O*-α-L-rhamnopyranoside and luteolin 7-*O*-α-L-rhamnoside.

## INTRODUCTION

The 'celery-leaved' or 'celery-top' pines, *Phyllocladus* L.C. et A. Rich, are unusual conifers in that the functions of the leaves are carried out by flattened, fused branch systems called phylloclades [1]. Traditionally *Phyllocladus* has been treated as a genus in the Podocarpaceae [2] and five species have been recognized. Three are endemic to New Zealand, *P. alpinus*, *P. trichomanoides* ('tanekaha') and *P. glaucus* ('toatoa') [3]; one endemic to Tasmania, *P. aspleniifolius* [4] and one ranging from the Philippines to New Guinea, *P. hypophyllus* [2]. More recently it has been treated as a monotypic family the Phyllocladaceae [5, 6], but in the present paper the traditional treatment is followed.

Apart from the identification of cyanidin 3-*O*-glucoside in *P. aspleniifolius* [7] and a number of tentatively identified biflavones in *P. glaucus* and *P. trichomanoides* [8], there appears to have been no investigation of the flavonoids. As part of our continuing chemotaxonomic study of the New Zealand conifers, we now report on the flavonoid glycoside chemistry of *Phyllocladus*. Investigations of the flavonoids accumulated by other genera of the Podocarpaceae (*Dacrycarpus*, *Prumnopitys* and *Podocarpus*) have recently been published [9–11].

## RESULTS

In *Phyllocladus alpinus*, flavonols were generally found to be minor constituents and included quercetin 3-*O*-glucoside (as the only one obvious in the 2D-PC pattern), and the infrequently encountered quercetin 3′-*O*-glucoside together with traces of quercetin 3-*O*-rhamnoside and kaempferol 3-*O*-glucoside. The major flavonoids were flavones, with luteolin, luteolin 7-*O*-β-D-glucoside and luteolin 3′-*O*-rhamnoside being predominant. This is the first reported isolation of luteolin 3′-*O*-rhamnoside. It was identified primarily from its absorption spectra (which were equivalent to those of luteolin 3′-*O*-glucoside), and by hydrolysis and product analysis. $^1$H NMR spectroscopy defined the rhamnose: aglycone ratio as 1:1, and an α-glycosidic linkage was indicated both by the H-1″/H-2″ coupling constant of 1.5 Hz [12] and by the negative specific rotation [13]. $^{13}$C NMR spectroscopy confirmed the proposed structure and defined the rhamnose as being in the α-pyranose form [14].

Minor flavone glycosides isolated were luteolin 3′-*O*-glucoside, apigenin 7-*O*-glucoside and the as yet unreported [15] luteolin 7-*O*-rhamnoside. The latter was identified via hydrolysis and product analysis, its absorption spectra (which were equivalent to those of the 7-*O*-glucoside) and its $^1$H NMR spectrum which confirmed the presence of only one α-linked rhamnose.

A chromatographic survey of the other four species in this genus (using a refined technique to produce a pattern relatively free from fluorescent and tannin-related contaminants) revealed a similar array of flavonoids. Some variation within species was evident but, as with the differences between most species, these were qualitatively minor. Thus the major components luteolin 7-glucoside and luteolin 3′-rhamnoside were dominant in all five species except for *P. aspleniifolius* which appeared to lack the latter. Quercetin 3-glucoside (occasionally accompanied by kaempferol 3-glucoside and/or quercetin 3-rhamnoside) also occurred in all species. Of the minor components, apigenin 7-glucoside and luteolin were found in some samples of all species, the former being accompanied by apigenin 4′-glucoside in most *P. hypophyllus* samples. Luteolin 3′-glucoside had a more restricted distribution being regularly present only in *P. alpinus*. No clear distribution pattern emerged for luteolin 7-rhamnoside and quercetin 3′-glucoside which occurred sporadically in all species but *P. aspleniifolius* and *P. hypophyllus*. Distinguishing between most of these species by 2D-PC of their flavonoids was thus difficult, and generally only possible by a careful study of the minor flavonoids. Many of these minor flavonoids were only just discernible on a 2D-PC at 'standard' loading.

The genus *Phyllocladus* thus appears to be remarkably homogeneous with respect to flavonoid type (and pattern)

and is characterized by the predominance of flavone (in particular, luteolin) *O*-glycosides. This contrasts with the flavonoid profiles of other Podocarpaceae genera recently studied [9–11]. *Podocarpus sensu stricto* was shown to be characterized by the predominance of flavone *C*-glycosides, *Dacrycarpus* by the predominance of 3-methoxyflavones and the presence of B-ring trihydroxylation, and *Prumnopitys* by the predominance of flavonol 3-*O*-glycosides. Earlier studies of the leaf *n*-alkanes and the heartwood extractives [16] also indicate that *Phyllocladus* is uniform and distinct. This is supported also by a recent study of the flavanols in *Phyllocladus* which revealed that the three major flavanols are ubiquitous. In all species catechin and epicatechin (2*R*:3*R*) are accompanied by a unique 3-*O*-acylated *ent*-epicatechin (2*S*:3*S*), 'phyllo-flavan', the structure of which is the subject of a separate study [17].

## EXPERIMENTAL

*Plant material.* Two bulk samples of *P. alpinus* were collected and voucher specimens (CHR 388234, CHR 388235) were placed in the Botany Division, DSIR, herbarium. Smaller samples of *P. alpinus* (CHR 388243 and 13 others), *P. aspleniifolius* (CHR 388248, and two others), *P. trichomanoides* (CHR 388244 and 7 others), *P. hypophyllus* (CHR 203511 and three others) and *P. glaucus* (CHR 388271 and five others) were also collected.

*Sample extraction and workup*, P. alpinus. Dry foliage (50 g) was ground and extracted at room temp with 2 × 500 ml of EtOH–$H_2O$ (1:1). Column chromatography of the extract on microcrystalline cellulose (Merck) using the solvents (in order), 2% increasing to 10% HOAc, $H_2O$, EtOH, MeOH gave a series of fractions which were analysed by cellulose TLC (15% HOAc) and 2D-PC (TBA, 15% HOAc). Fractions were grouped according to their constituents and each combined group was separately rechromatographed on a small column of polyamide (Macherey and Nagel SC-6) in $H_2O$, the eluent being gradually changed to MeOH. Further purification by 1D- or 2D-PC was usually necessary as was a final clean-up on Sephadex LH-20 (in MeOH).

*Techniques of flavonoid identification.* Procedures used are as described in detail by Markham [18]. The known glycosides, apigenin 7-*O*-glucoside, luteolin 7- and 3′-*O*-glucoside, kaempferol 3-*O*-glucoside, and quercetin 3-*O*-glucoside and -rhamnoside were routinely identified by absorption spectroscopy, acid (HCl) hydrolysis followed by sugar analysis (PC) and aglycone analysis (UV spectroscopy, TLC cochromatography). Additionally, the glucosides were treated briefly with β-glucosidase in $H_2O$ [18], and each glycoside was cochromatographed with an authentic sample using at least three of the following: cellulose TLC (TBA, 50% HOAc, 15% HOAc); $SiO_2$ TLC ($C_6H_6$–HOAc–$H_2O$, 125:72:3, multiple runs); polyamide TLC (EtOAc–MeCOEt–HCOOH–$H_2O$, 5:3:3:1). Data for other glycosides are detailed below.

*Quercetin* 3′-*O*-β-D-*glucoside.* PC $R_f$s 0.53 (TBA), 0.07 (15% HOAc), 0.4 (50% HOAc); $\lambda_{max}^{MeOH}$ nm: 254, 270, 300, 365; (NaOMe) 278, 325, 432 (dec); ($AlCl_3$ and $AlCl_3$–HCl) 264, 270, 304, 356, 418; (NaOAc) 276, 324, 390; (NaOAc–$H_3BO_3$) 258 sh, 272, 310–330, 366.

*Luteolin* 3′-*O*-α-L-*rhamnopyranoside.* PC $R_f$s 0.69 (TBA), 0.20 (15% HOAc), 0.51 (50% HOAc); $\lambda_{max}^{MeOH}$ nm: 244 sh, 271, 342; (NaOMe), 280, 330, 402; ($AlCl_3$ and $AlCl_3$–HCl) 258, 278, 297 sh, 350, 385; (NaOAc) 276, 316, 395; (NaOAc–$H_3BO_3$) 272, 345. Specific rotation $[\alpha]_D^{20} = -95°$ (MeOH). $^1$H NMR (i) (90 MHz, δ, TMS ether in $CHCl_3$–$CCl_4$): 7.64 (*m*, H-2′), 6.87 (*dd*, *J* = 8.7, *ca* 2 Hz, H-6′), 6.88 (*d*, *J* = 8.6 Hz, H-5′), 6.48 (*s*, H-3), 6.32 (*d*, *J* = 2.1 Hz, H-8), 6.20 (*d*, *J* = 2 Hz, H-6), 5.16 (*d*, *J* = *ca* 2 Hz, H-1″), 4.0–3.4 (*m*, H-2″, 3″, 4″, 5″), 1.23 (*d*, *J* = 3.5 Hz, H-6″); (ii) (300 MHz, δ, DMSO-$d_6$): 12.93 (5-OH), 7.71 (*d*, *J* = 2.2 Hz, H-2″), 7.62 (*dd*, *J* = 8.2, 2.2 Hz, H-6′), 6.97 (*d*, *J* = 8.2 Hz, H-5′), 6.77 (*s*, H-3), 6.43 (*d*, *J* = 1.7 Hz, H-8), 6.17 (*d*, *J* = 1.7 Hz, H-6), 5.43 (*d*, *J* = 1.5 Hz, H-1″), 3–3.75 (*m*, sugar protons), 1.14 (*d*, *J* = 6.5 Hz, Me), integration as expected for a mono-rhamnoside. $^{13}$C NMR (DMSO-$d_6$, δ, 30°): 181.6 (C-4), 164.9 (C-2), 163.4 (C-7), 161.5 (C-5), 157.3 (C-9), 152.3 (C-4′), 144.4 (C-3′), 122.1/122.4 (C-1′, 6′), 116.5/116.8 (C-2′, 5′), 103.1/103.4 (C-3, 10), 99.9 (C-1″, 99.1 (C-6), 94.0 (C-8), 71.9 (C-4″), 70.3/70.1/69.6 (C-2″, 3″, 5″), 17.9 (C-6″).

*Luteolin* 7-O-α-L-*rhamnoside.* PC $R_f$s 0.52 (TBA), 0.29 (15% HOAc), 0.6 (50% HOAc): $\lambda_{max}$ (MeOH) 258, 270 sh, 354; (NaOMe) 270, 300 sh, 402; (NaOAc) 258, 270, 356; (NaOAc–$H_3BO_3$) 262, 378; ($AlCl_3$/HCl) 272, 295 sh, 364, 388. $^1$H NMR (300 MHz, δ, DMSO-$d_6$): 7.55 (*d*, *J* = 2.5 Hz, H-2′), 7.35 (*dd*, *J* = 8, 2.5 Hz, H-6′), 6.84 (*d*, *J* = 8 Hz, H-5′), 6.74 (*d*, *J* = 2.5 Hz, H-8), 6.73 (*s*, H-3), 6.38 (*d*, *J* = 2.5 Hz, H-6), 5.4 (*br s*, H-1″), 1.13 (*d*, *J* = 6.5 Hz, Me).

*General method for PC survey of other species.* Dry foliage (1 g) was extracted with EtOH–$H_2O$ (1:1) and the extract chromatographed as a band on paper in 15% HOAc. The resultant PC was cut into two sections with $R_f$ ranges *ca* 0–0.5 (just below a fluorescent band) and 0.5–1.0, and each section was eluted separately with EtOH–$H_2O$. Aliquots of the eluents (≡ extract from 100 mg dry foliage) were first passed through a plug of polyamide powder in a Pasteur pipette to remove tannins. The treated aliquots were then analysed by 2D-PC, the low $R_f$ range in TBA–50% HOAc and the other in TBA–15% HOAc. The 0.5–1.0 range fraction contained most of the fluorescent components but no flavonoids. Chromatograms were routinely sprayed with Naturstoff-reagenz A (Roth) as an aid to flavonoid identification.

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