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Flavonoids and Condensed Tannins from Leaves of Hawaiian *Vaccinium reticulatum* and *V. calycinum* (Ericaceae)[1]

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ABSTRACT: The flavonoids and condensed tannins of Hawaiian *Vaccinium reticulatum* Smith and *V. calycinum* Smith have been isolated and their structures determined. Flavonoids present in both species were quercetin, quercetin-3-O-glucoside, quercetin-3-O-galactoside, quercetin-3-O-methyl ether, isorhamnetin, and (-)-epicatechin. The condensed tannin contained procyanidin units with *cis* stereochemistry only. Extension and terminal units, and number-average molecular weight of the polymer were determined. A large quantity of neochlorogenic acid (a caffeic acid derivative) was also detected. The phenolic compounds of *V. reticulatum* from a population on Mauna Kea and two populations near Kīlauea, both on the island of Hawai'i, and from one population of *V. calycinum* on Kaua'i were qualitatively identical. The high degree of similarity supports the view that these species are closely related. It is suggested that the phenolic chemistry of the species may have been fixed in the progenitor of the Hawaiian *Vaccinium*.

IN THE RECENTLY PUBLISHED flora of the Hawaiian Islands, Vander Kloet (1990) suggested that the variation seen in *Vaccinium* on the Islands can best be accommodated by recognizing three species, *V. calycinum* Smith, *V. dentatum* Smith, and *V. reticulatum* Smith. Vander Kloet (1990) emphasized calyx lobe development, leaf persistence, and leaf blade size in his treatment and placed less emphasis on leaf margin, blade color, and indumentum, characters that had been used in earlier species delimitations (Skottsberg 1927, 1937, 1944, Degener 1940). In a more recent paper, Vander Kloet (1993) described seed and seedling characteristics, juvenile leaf features, and some aspects of the breeding biology of all three species. Homogeneous juvenile leaf features along with limited variation in seed size, shape, and sculpture led to the suggestion that the group is "... rather homogeneous and of recent origin" (Vander Kloet 1993).

Vander Kloet (1990) stressed the need for detailed studies of the group so that the significance of variation patterns may be assessed. As part of our ongoing study of polyphenolic compounds as systematic markers of Island taxa, we undertook an examination of the flavonoid chemistry of two species of Hawaiian *Vaccinium*. The high degree of chemical uniformity observed in the material studied to date is entirely in line with the suggestion that this group of species represents a closely related phylogenetic unit. The results of that study are presented in this paper.

MATERIALS AND METHODS

Source of Plant Material

Leaf samples of *Vaccinium reticulatum* amounting to about 10 g dry weight were removed from individual plants from three sites on the island of Hawai'i: (1) ca. 100 m east of the Desolation Trail trailhead; (2) west side of the road near the Volcano Observation Center; and (3) east flank of Mauna Kea at ca. 3000 m elevation. Eleven plants

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were sampled at site 1, 10 at site 2, and four at site 3. Efforts were made to avoid collecting nearest neighbors. Two specimens of *V. calycinum* Smith were collected on the north shore of Kaua'i by K. Marr. Vouchers have been deposited in UBC.

Isolation and Identification of Flavonoids

Air-dried leaf samples were extracted repeatedly with 80% aqueous methanol at room temperature. The combined extract of each sample was then evaporated to dryness and subjected to a combination of column and preparative thin-layer chromatography (TLC) according to described procedures (Wilkins and Bohm 1976, Gornall and Bohm 1980). Flavonoid structures were established using standard ultraviolet (UV) (Mabry et al. 1970) and mass spectral (MS) methods (Mabry and Markham 1975, Markham 1982). Sugar analysis was carried out using methods described by Ceska and Styles (1984) and Kartnig and Wegschaider (1971). It should be noted that detailed listings of UV spectral maxima, MS peaks, and ^{1}H and ^{13}C proton magnetic resonances (PMR) for the compounds described have been omitted to save space. In all cases, observed values agreed with published data.

Isolation, Purification, and Analysis of Condensed Tannins

Dried leaves were blended with 75% aqueous acetone containing 0.1% ascorbic acid, and the mixture was filtered. Acetone was removed by evaporation under reduced pressure. The aqueous residue was then subjected to column chromatography using Sephadex LH-20 according to the procedure described by Koupai-Abyazani and coworkers (1992).

Characterization of the Polymer

The structure of the purified tannin was determined using a hydrolytic procedure that employed phloroglucinol as the nucleophilic scavenger of the freed monomeric units. Resultant adducts were analyzed by high pressure liquid chromatography (HPLC) using a Lichrospher 100 RP-18 column (250 × 4 mm i.d.) with monitoring at 280 nm. A linear gradient of 1% aqueous acetic acid and methanol was used for elution (Koupai-Abyazani et al. 1993*a*,*b*).

^{13}C-NMR spectra were recorded in water: d_6-acetone (1 : 1 vol/vol) on a Varian XL (300 MHz) spectrometer. ^{1}H-NMR analysis was carried out in d_4-methanol on a Bruker WH-400 (400 MHz) spectrometer. Optical rotations of polymers dissolved in methanol-water (1 : 1 vol/vol) were measured in a 10 mm path length cell at 20°C in a Jasco J710 spectrophotometer.

RESULTS

Flavonoids

Twenty-five individuals of *V. reticulatum*, representing three populations, and two of *V. calycinum* were subjected to 2D-TLC analysis. The chromatograms exhibited identical patterns of flavonoids with only slight variation in concentration (by visual inspection). Five flavonoids (UV absorbing) and a phenylpropanoid (blue fluorescense) were observed as the major spots on the chromatograms. These compounds were isolated by column chromatography and purified by means of preparative TLC. Initial comparisons of these compounds with standards using TLC indicated that two were flavonoid monoglycosides and the other three were flavonoid aglycones. UV analysis showed that the monoglycosides were 3-O-substituted flavonols (Markham 1982). The glycosidic components were shown to be glucose and galactose. The MS spectra of these compounds showed an ion with $m/z = 302$, which is characteristic of quercetin, and ions with $m/z = 153$ and $m/z = 137$, which represent the A-ring and B-ring fragments of quercetin, respectively (Mabry and Markham 1975). The two glycosides were, therefore, quercetin-3-O-glucoside (1) and quercetin-3-O-galactoside (2). The UV data of the third compound revealed that it was also a 3-O-substituted flavonol (Markham 1982), but acid hydrolysis resulted in unchanged com-

pound and no sugar. The color reaction of the compound with diphenylboric acid ethanolamine complex was the same as for 3-O-substituted quercetin derivatives. The compound ran faster than quercetin in nonpolar solvent systems. The MS spectrum of this compound showed an ion at $m/z = 316$, which is consistent with a flavone having four OH groups and one O-methyl group. Ions at $m/z = 301$, which arises by loss of the methyl group; $m/z = 153$, which arises from the A-ring; and $m/z = 137$, which arises from the B-ring, are consistent with the behavior of quercetin 3-O-methyl ether (3). The other two aglycones were identified as quercetin (4) and isorhamnetin (5) by comparison of their TLC, UV, and MS data with those of standards. No isorhamnetin glycosides were detected.

The ^{1}H-NMR spectrum of the isolated phenylpropanoid indicated that it was a caffeic acid derivative (Ibrahim and Barron 1989). Comparison of the MS data with those reported by Sakushima et al. (1985) suggested that the compound is neochlorogenic acid (6).

Condensed Tannins

An aqueous acetone extraction of *Vaccinium* leaves yielded the purified polymer after a combination of liquid partitioning and chromatography on Sephadex LH-20 column. The polymer had an $E^{1\%}$ value of 134 at its absorption maximum near 278 nm, which is typical of a procyanidin (PC) polymer, and an optical rotation of $[\alpha]^{20} + 153$, which corresponds to a molar proportion of subunits with 2,3-*cis* stereochemistry of 96% (Porter 1989). TLC analysis and the spectrophotometric measurement of the hydrolysis products (Czochanska et al. 1980) revealed that the polymer contained PC units. No prodelphinidin (PD) units were detected.

These observations were further confirmed by ^{13}C-NMR spectroscopy of the polymer, which indicated the presence of PC units (signal near 145 ppm) with *cis* stereochemistry (signal near 76 ppm) (Porter 1989). No signal was observed for PD or *trans* units in the spectrum. The number-average molecular weight (MW) (M_n) of the polymer was estimated to be in the range of ca. 1160 (assuming an average unit MW of 290) by integrating the signals near 73 ppm (C-3 of PC units) and 67 ppm (C-3 of terminal flavan-3-ol units). This was in total agreement with the results of HPLC analysis of the phloroglucinol adducts of the polymer (Koupai-Abyazani et al. 1993*a*,*b*). Acid hydrolysis of the polymer in the presence of phloroglucinol yielded the extension units as flavan-3-ol-phloroglucinol adducts and terminal units as flavan-3-ols. Analysis of the hydrolysis products by RP-HPLC indicated the presence of (-)-epicatechin-4-phloroglucinol (7) and (-)-epicatechin (8).

The ethyl acetate–soluble fraction, obtained during the tannin extraction procedure, was examined by TLC on cellulose (6% aqueous acetic acid). Development of a deep red spot after spraying the chromatogram with vanillin/HCl reagent indicated the presence of flavan-3-ols. Further analysis of this fraction by 2D-TLC and HPLC showed compound 8, (-)-epicatechin, to be present. Identical results were obtained for all collections of *Vaccinium*.

DISCUSSION

The flavonoid profile of *Vaccinium calycinum* and *V. reticulatum* consists of quercetin, its 3-O-glucoside, 3-O-galactoside, 3-O-methyl ether, 3′-O-methyl ether (isorhamnetin), and (-)-epicatechin. Other than minor concentration differences among individuals, this profile appears to be invariant. Quercetin or its glycosides have been reported from several other species of *Vaccinium*: *V. vitis-idaea* L., *V. myrtillus* L., and *V. uliginosum* L. (Hattori 1962), *V. myrtillus* (Azar et al. 1987), *V. macrocarpon* Ait. (Kuznetsova et al. 1990), and *V. erythrocarpum* Michx. and *V. japonicum* Miq. (Vander Kloet and Bohm 1991). Kaempferol derivatives, not observed in our study, are reported less often from members of the genus. Kaempferol glycosides were reported from *V. japonicum* but not from *V. erythrocarpum* (Vander Kloet and Bohm 1991). Harborne and Williams

(1973) reported quercetin in all 13 *Vaccinium* species tested in their survey of the family, whereas they recorded kaempferol from only six. The presence of quercetin 3-O-methyl ether and 3′-O-methyl ether (isorhamnetin) represents unusual sightings in the genus. The presence of these two compounds may provide useful markers in the search for related species.

The condensed tannins identified from the two species studied here are also very similar to tannins obtained from other species of *Vaccinium*. Czochanska et al. (1980) showed that the condensed tannins from the fruit of *V. corymbosum* L. had 100% *cis* stereochemistry and 100% PC units. The polymers of ripe and unripe fruit from *V. corymbosum* and *V. oxycoccus* L. were reported to contain 100% PC units with 87–95% *cis* stereochemistry (Porter 1988).

Studies of flavonoids of other Hawaiian endemic species have yielded interesting and variable results. One of the most striking examples of flavonoid variation recorded for an Island system involves Hawaiian *Bidens* (Asteraceae) (Ganders et al. 1990). Those workers reported differences in flavonoid profiles between individuals within populations that were greater than differences observed between some species pairs. Gardiner (1976) reported "many ... differences" among populations of tetraploid species of Hawaiian *Lipochaeta* (Asteraceae) as opposed to populations of the diploid species, which, though chemically simpler, appeared to be more homogeneous. The seven species of *Scaevola* (Goodeniaceae) endemic to the Hawaiian Islands were shown to share a suite of flavone and flavonol glycosides with only minor differences noted (Patterson 1984). Examination of exudate flavonoids of individuals from three populations of *Wilkesia gymnoxiphium* A. Gray, a member of the silversword alliance, showed a very high level of homogeneity with only quantitative differences as judged from chromatographic spot sizes (Bohm and Fong 1990). In a study of natural hybridization between *Dubautia ciliolata* (DC.) D. Keck subsp. *glutinosa* G. Carr and *D. scabra* (DC.) D. Keck subsp. *scabra* it was shown that only minor quantitative variation of flavonoids existed among individuals of the two species (Crins et al. 1988). It might be noted that conservative flavonoid profiles have been reported for other Island systems. A case in point is the study on the Juan Fernández Islands in which Pacheco and coworkers (1985) observed moderately uniform flavonoid profiles for several species of the endemic genus *Robinsonia* (Asteraceae).

With the exception of *Bidens*, and the diploid species of *Lipochaeta*, the tendency seems to be for flavonoid profiles within Hawaiian genera to be conservative. This is also the case for the two species of *Vaccinium* described in our study. The conservative nature of flavonoid profiles in these taxa suggests that their flavonoid biosynthetic capabilities may already have been fixed at the time of arrival of the first propagules, and that the phenolic phenotype has not been challenged in the habitats currently occupied. Gardiner (1976) suggested that the somewhat depauperate flavonoid profile of *Lipochaeta* may have been the result of a release from predator and/or pathogen pressure when the organisms entered their new environments. This would be difficult to establish with any degree of certainty. What can be done, however, is a continued search for patterns of variation in a larger number of endemic species with comparisons, whenever possible, with likely ancestral taxa. In the case of Hawaiian *Vaccinium*, a wider sampling of all three taxa is planned along with examination of the other members of section *Macropelma*.

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