Flavonoids as Chemotaxonomic Markers for *Erythroxylum australe*

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Methanolic leaf extracts of *Erythroxylum australe* F. Muell. produced eight *O*-conjugated flavonoids. Six of the flavonoid aglycones were dihydroisoflavones (all dihydro-orobol derivatives), one a flavanone, eriodictyol, and one a flavonol, quercetin. The major glycosides of the flavonoids included mono-glucosyl-rhamnosyls and dirhamnosyl-glucosides with either 3,5,7 or 3′,4′ linkage or a combination thereof. The array of flavonoids present in *E. australe* suggests kinship to *E. ulei* and linkage to the four cultivated alkaloid-bearing *Erythroxylum*, especially the ancestral *E. coca* var. *coca*. Because of the uniqueness of the flavonoids present in leaf tissue of *E. australe* they are unambiguously useful as chemotaxonomic markers for the taxon.

Key words: Erythroxylum australe, Flavanone, Isoflavone

Introduction

Our research on the presence of flavonoids in the *Erythroxylum* genus in 1999 gave an overview of past accomplishments of flavonoid research for the taxon (Johnson and Schmidt, 1999). This work included the summary detailed by Hegnauer (1981), inclusive also were the works by Bohm *et al.* (1981, 1982, 1988), Evans (1981) as well as other investigators. Commencing in 1997, Johnson *et al.* characterized the uniqueness of leaf flavonoids for several varieties of the genus *Erythroxylum* and how their leaf flavonoid(s) could be used as chemotaxonomic markers for the taxon (Johnson *et al.*, 1998, 2002, 2003; Johnson and Schmidt, 1999). As a continuation of our chemotaxonomic research on the *Erythroxylum* taxon, we: (*i*) report the presence of flavonoids present in the polar (methanolic) leaf extract from leaf tissue of *Erythroxylum australe* F. Muell.; (*ii*) describe their similarity and/or difference from those present in previously investigated *Erythroxylum* taxa, and (*iii*) determine their usefulness as chemotaxonomic markers for the taxon as well as their relationship to other varieties of the taxon. The current research is our first descriptive account of flavonoids present in leaf tissue of *Erythroxylum* endemic to the continent of Australia.

Materials and Methods

Plant material

Erythroxylum australe F. Muell. (*E. australe*) was collected and authenticated by Dr. Bruce Taylor, on the continent of Australia, December 1993. Dr. Taylor assigned the current *E. australe* sample as accession number 371. His description of the field collection site for *E. australe* follows: Location Killarney, SE Queensland 6 km SW of Killarney on road starting as Willow Street at the Condamine Bridge, 0.5 m past the entrance to 'Melrose'. Bush 1.0 m tall on the north side of road at the base of the twin forked "gum tree". Global Position Satellite (GPS) coordinates 28° 21′ 6″ S Lat × 152° 147′ E Long. Date collected, December 20, 1993.

Leaves and stems were harvested from the bush, air-dried, stored over Drierite desiccant bags (30 g/bag; W. A. Hammond Drierite Co., Xenia, OH, USA) and shipped to the Weed Science Laboratory at Beltsville Agricultural Research Center, Beltsville, MD, USA. Upon receipt, the plant material was examined by Drs. Lee Darlington and E. L. Johnson, and analyzed for alkaloid and leaf flavonoid constituents. For the current research, 0.030 kg/batches of *E. australe* leaf material were extracted to determine the flavonoid profile as described below.

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Isolation of leaf flavonoids

Flavonoids were extracted from dried leaves of *E. australe* as described by Johnson *et al.* (1997). Two batches (0.030 kg/batch) of *E. australe* leaves were separately homogenized (anhydrously) in a Waring Blender for 30 s and placed in two glass beakers containing *ca.* 80 ml of 72% aqueous MeOH. The beakers were capped and stored overnight (in darkness) at 21 °C for flavonoid extraction. On the following day the crude extract was filtered through four layers of cheese cloth and extracted a second and third time each with 45 ml of 72% MeOH (*ca.* 30 min). The extracted fractions were combined, reduced *in vacuo* (45 °C) to *ca.* 5 ml, and 25 ml of HPLC grade water was added. The flask was gently agitated for 2 min, the concentrate (hue, greenish gray) was decanted, centrifuged at 20,000 × *g* for 30 min (4 °C) and the supernatant collected. To the pellet was added 5 ml of HPLC grade water and after re-suspending (*via* vortices), the centrifugation step was repeated two additional times, adding 5 ml HPLC grade water on each repeat. The resultant supernatants after centrifuging were combined, decanted into labeled round bottom flasks and dried *in vacuo* as above. This yielded 0.5 g of residue containing the *E. australe* flavonoid fraction, which was dissolved in 20 ml of 1% acetic acid (aq). This extract was then loaded, in 5 ml fractions, onto a Supelclean ENVI-18, 1 g, SPE Tube (Supelco, Inc., Bellefonte, PA) and subjected to clean-up procedures as follows. After successive washes with 1% HOAc (6 ml), 5% MeOH (4 ml) and 20% MeOH (4 ml) the flavonoids were eluted with 70% MeOH (6 ml). This fraction was then reduced *in vacuo* (45 °C) to dryness, redissolved in 10 ml 1% HOAc (aq), filtered through a 0.2 μm PTFE Whatman filter affixed to a 10 ml syringe (Whatman Laboratory Division, Clinton, NJ, USA), placed into a 15 ml screw cap vial and stored at 4 °C.

HPLC resolution

1 ml of the stored flavonoid fraction (above) was transferred to a 1.5 ml amber HPLC auto-sample vial. The vial was sealed and placed into the auto-sample carrier of a Hewlett-Packard (H-P) 1090M liquid chromatograph equipped with a ChemStation, diode array detector, Chem-Library (Hewlett-Packard, Avondale, PA, USA) and with a Gilson FC 204 fraction collector (Gilson Inc., Middleton, WI, USA) attached to the outlet port of the HPLC. A 100 μl sample was injected onto a Phenomenex Lunar C-18, 250 cm × 10 mm (i.d.), 5 μ silica/spherisorb semi-preparative/analytical column (Phenomenex, Torrance, CA, USA) for flavonoid separation. The HPLC conditions were: program: linear stepwise gradient mobile phase; solvent A: 100% HPLC grade H_2O; solvent B: MeOH/HOAc/H_2O (90:5:5); flow rate: 2 ml/min; detection: DAD UV at λ_{MeOH} 230 nm–470 nm; run time: 105 min (0.01 min, 25% B; 85.00 min, 42% B; 95.00 min, 65% B; 95.01 min, 25% B; 105.00 min, 25% B). After equilibration, the HPLC chromatogram was analyzed spectrally, and the primary flavonoids identified and collected by peak elution times (Fig. 1; Table I). Repetitive flavonoid sample injections and peak separations afforded *ca.* 8 mg of each flavonoid. The flavonoid fractions (primary peaks) were dried *in vacuo* (45 °C) and aliquots (*ca.* 2 mg) stored as above for ^{1}H NMR spectroscopy while the remainder was used for spectral analyses (UV and LC-MS). The classical shift reagents (Mabry *et al.*, 1970; Markham, 1982) were used with compounds (flavonoid peak fractions) #1 through #8 for *E. australe* (Fig. 2).

NMR spectrometry

Each stored flavonoid fraction was decanted into a flask, dried *in vacuo* as above, dissolved in 700 μl of MeOD-d_3 + 99.5% D and placed in NMR tubes. The tubes were sealed to prevent sample evaporation and/or oxidation during NMR data acquisition and storage. ^{1}H NMR spectra were acquired at 25 °C on a Bruker QE 300 MHz NMR spectrometer. A Mac NMR v.5 program on Power Macintosh 9500/120 was used for data collecting and processing. The proton spectra were determined at 300.6 MHz with a spectral width of 3100 Hz and 128 scans. Pre-saturation for 1.2 s at 4.8 ppm virtually eliminated the signal from water in the spectra which otherwise would interfere with the sugar proton peaks. COSY experiments were used to assign and/or confirm intra-molecular coupling. Subtraction of spectra from adjacent compounds (HPLC peaks) was used to compare the structural differences and similarities among structural analogues with different HPLC retention times. ^{1}H NMR data are presented in Table II.

LC-MS procedures

Peaks #1 through #8 of HPLC flavonoid eluants were identified with LC-MS. A Hewlett-Packard 1100 HPLC with an autosampler attached to a Finnigan Mat (FM) 7000 MS was used for flavonoid analyses. The HPLC parameters were: injection volume: 5 μl; column: Phenomenex Luna 5 μ C18, 250 × 4.6 mm (Phenomenex, Torrance, CA, USA); flow rate: 0.75 ml/min (stop time, 15 min; post time, 2.00 min); solvents: **A**: 100% MeOH; **B**: 80% H_2O; **C**: 2% HOAc; **D**: 100% CH_3CN; during run; time; (in min): 0.00–6.00: 80% **B**, 20% **C**; 7.00–13.00: 40% **B**, 20% **C**, 30% **D**; 14.00–18.00: 10% **B**, 20% **C**, 50% **D**; 18.00–20.00: 80% **B**, 20% **C**; diode array detector: 210–300 nm. The Finnigan Mat 7000 was a triple quadrupole used with a single stage mass analysis. The first quadrupole was scanned while the second and third were set to reference. Ionization was accomplished by an atmospheric pressure ionization (API) source with a corona on 5 μA, where the reagent gas was determined by the composition of the HPLC mobile phase. Vaporizer and capillary temperatures were optimized on the protonated molecular ion of lysine at 375 and 140 °C, respectively. Mobile phase during optimization was 30% (v/v) CH_3CN and 70% (v/v) H_2O. HOAc was added to the mobile phase during response optimization and sample runs so that total content was constant at 4%. Skimmer pump pressure was ≈ 1100 Pa. All scans were acquired within 3 s.

LC-MS data for *E. australe*-peak flavonoid(s) [LC-MS (probe) 70 eV; rel. int.]: peak #**1**: 305 [M-176] (12), 308 [M-l73] (55), 360 [M-121] (28), 370 [M-111] (20), 410 [M-71] (12), 454 [M-27] (29); peak #**2**: 290 [M-321] (16), 304 [M-307] (100), 330 [M-281] (19), 342 [M-269] (10), 360 [M-251] (25), 380 [M-231] (26), 408 [M-203] (14), 436 [M-175] (11), 450 [M-161] (8), 481 [M-130] (35), 529 [M-82] (10); peak #**3**: 342 [M-252] (10), 359 [M-235] (100), 397 [M-197] (12), 408 [M-186] (25), 424 [M-170] (11), 481 [M-113] (27), 511 [M-83] (6), 539 [M-55] (17), 576 [M-18] (6); peak #**4**: 319 [M-162] (48), 366 [M-115] (100), 391 [M-90] (72), 397 [M-84] (98), 399 [M-82] (62), 410 [M-71] (67), 425 [M-56] (62), 448 [M-33](59); peak #**5**: 298 [M-151] (30), 312 [M-137] (16), 342 [M-107] (26), 359 [M-90] (100), 393 [M-56] (78), 397 [M-52] (72), 408 [M-41] (56), 415 [M-34] (49), 426 [M-23] (46), 442 [M-7] (38); peak #**6**: 306 [M-307] (31), 312 [M-301] (22), 350 [M-263] (38), 360 [M-253] (62), 365 [M-248] (21), 394 [M-219] (44), 414 [M-199] (40), 438 [M-175] (32), 482 [M-131] (29), 526 [M-87] (18), 570 [M-43] (14); peak #**7**: 285 [M-328] (46), 294 [M-319] (38), 310 [M-303](40), 355 [M-258] (49), 360 [M-253] (100), 361 [M-252] (50), 392 [M-221] (30), 414 [M-199] (47), 438 [M-175] (28), 482 [M-131] (18), 526 [M-87] (15), 570 [M- 43] (14); peak #**8**: 292 [M-347] (22), 300 [M-339] (33), 359 [M-280] (38), 408 [M-231] (100), 422 [M-217] (40), 459 [M-180] (22), 494 [M-145] (28), 511 [M-128] (36), 529 [M-110] (17), 586 [M-53] (12), 630 [M-9] (14).

Results and Discussion

Leaf flavonoid chemistry

Methanolic extracts from leaves of *E. australe* separated by semi-preparative HPLC (see Materials and Methods) afforded eight major peaks that were well resolved (Fig. 1; Table I). Although peaks #2, #3, #5 and #6 were not baseline separated (*i.e.*, valley to valley), fractions were collected above the junction where the two components were well resolved. After collecting sufficient peak fractions from the methanolic leaf extract (*ca.* 8 mg of each flavonoid) the collected fractions were again subjected to HPLC separation (as above) to reevaluate their wavelengths (λ_{min} and λ_{max}; see Materials and Methods) and to ensure that the eluted peak(s) were flavonoids. After peak separation, acquired peak UV absorption spectra data for the flavonoids were compared with spectra data obtained from authentic flavonoids and the published reports of Mabry *et al.* (1970) and Markham (1982). Peaks #1–#8 were identified as flavonoids (Fig. 1) and yielded sufficient quantities for UV, EI-MS, LC-MS and ^{1}H NMR spectroscopic evaluation (*ca.* 2 mg/sample peak for ^{1}H NMR). Unlabeled peaks in the chromatograph were not flavonoids.

Peak retention times and elution order for the methanolic extracted flavonoids of *E. australe* during semi-preparative HPLC separation appear in Fig. 1 and Table I. Leaf flavonoids were identified with UV, EI-MS, LC-MS and ^{1}H NMR (see Materials and Methods for EI-MS, LC-MS; Table II for ^{1}H NMR data). Of the eight flavonoid glycosides extracted from *E. australe* leaf tissue, the aglycone moieties were; 2-methyl-3′-*O*-rhamnosyl-dihydro-orobol (peak #1), 3-*O*-rhamnosyl-7-*O*-glucosyl-quercetin (peak #2), 4′-*O*-dirhamnosyl-dihydro-orobol (peak #3), 5-*O*-rhamnosyl-7-methoxy-dihydro-orobol (peak #4), 7-*O*-rhamnosyl-5,3′,4′-

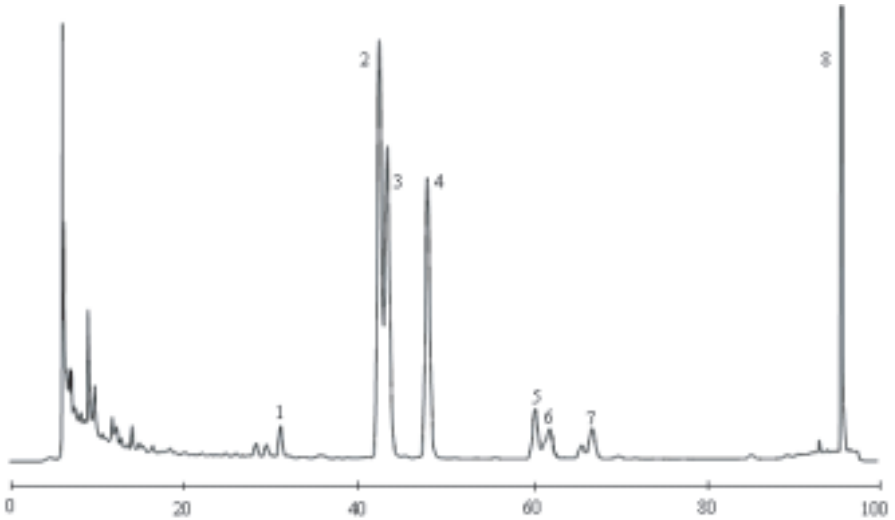

Fig. 1. HPLC chromatogram of the methanolic leaf extract of *E. australe*. Peaks #1–8 represent 1000 μg (s.e. ± 200 μg)/g dry weight leaf material.

Peak Number	Rt [min]	Compound
1	33.8	2-Methyl-3′-*O*-rhamnosyl-dihydro-orobol
2	45.0	3-*O*-Rhamnosyl-7-*O*-glucosyl-quercetin
3	46.7	4′-*O*-Dirhamnosyl-dihydro-orobol
4	51.3	5-*O*-Rhamnosyl-7-methoxy-dihydro-orobol
5	64.2	7-*O*-Rhamnosyl-5,3′,4′-OH-eriodictyol
6	65.8	5-Dehydroxy-7,3′-*O*-glucosyl-dihydro-orobol
7	70.9	3′-*O*-Rhamnosyl-7-*O*-glucosyl-dihydro-orobol
8	101.1	5-OH-6-Methoxy-dihydro-pseudobaptisin

Table I. HPLC retention times of the eight flavonoids found in *E. australe*.

Table II. ^{1}H NMR data for leaf flavonoids of *Erythroxylum australe* peaks #1–8 in MeOD-d_3 + 99.5% D.

	Peak Number							
Proton	1	2	3	4	5	6	7	8
2′	7.0–7.4	7.608	7.325	7.709	7.305	7.0–7.4	7.0–7.4	7.709
3′		OH	OH	OH	OH			
4′	OH	OH		OH	OH	OH	OH	
5′	7.0–7.4	6.867	6.912	7.047	6.921	7.0–7.4	7.0–7.4	7.051
6′	7.0–7.4	7.647	7.362	7.759	7.349	7.0–7.4	7.0–7.4	7.751
2	3.53/5.66		5.455dd	5.427dd	5.359	5.642dd	5.792dd	5.489
C**H**$_3$	1.15							
3	3.632		3.601	3.601	2.564dd	3.652	3.652	3.825
5						7.791		
6	6.530	6.209	6.470	6.868	6.213	6.501	6.530	
8	6.865	6.401	6.731	6.909	6.383	6.865	6.865	6.614
Sugar H-1	3.797	5.125	4.062	3.841	4.608	5.196	4.131	5.232
		3.782						4.522
Sugar C**H**$_3$	1.182	1.510	0.947	1.082	0.951		1.184	1.092
OC**H**$_2$O								6.352
OC**H**$_3$				3.942				3.904

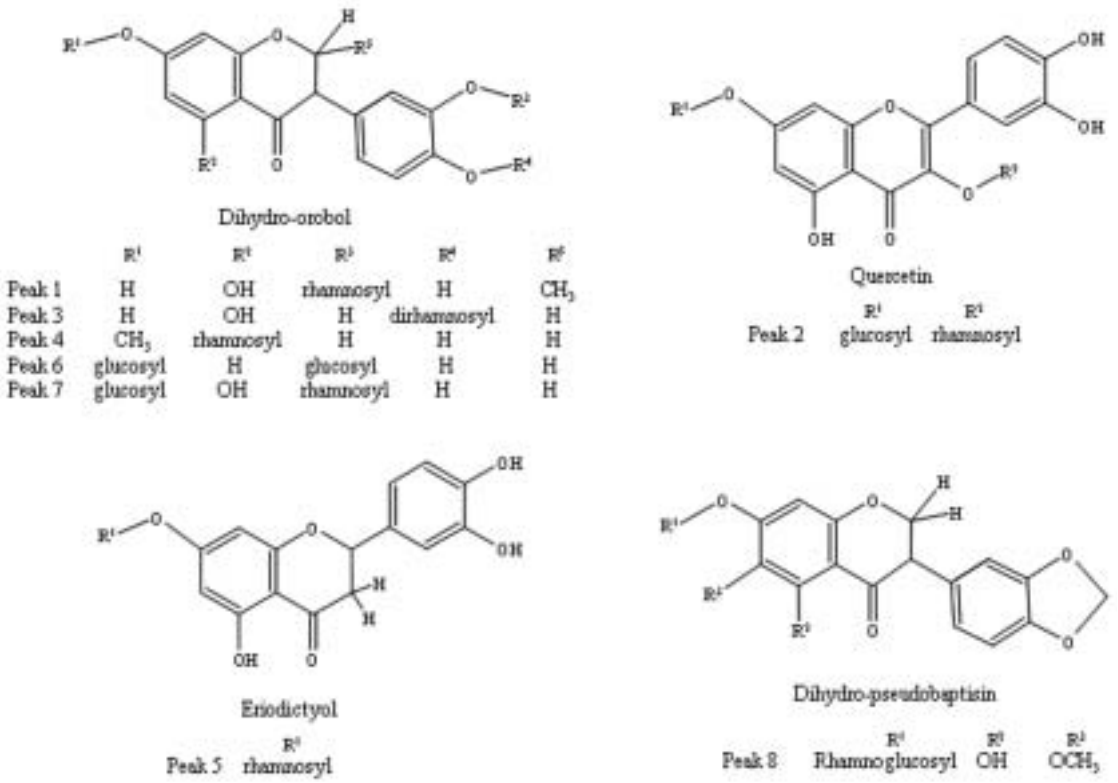

Fig. 2. Peaks #1–8 isolated from methanolic leaf extracts of *E. auistrale.*

OH-eriodictyol (peak #5), 5-dehydroxy-7,3′-*O*-glucosyl-dihydro-orobol (peak #6), 3′-*O*-rhamnosyl-7-*O*-glucosyl-dihydro-orobol (peak #7) and 5-OH-6-methoxy-dihydro-pseudobaptisin (peak #8: Fig. 2).

Of note was the absence of kaempferol in leaf tissue of *E. australe.* In polar methanolic leaf tissue extracts from three of four alkaloid bearing taxa of *Erythroxylum* [*E. novogranatense* var. *novogranatense*, *E. novogranatense* var. *truxillense* and *E. coca* var. *ipadu* (from Colombia, South America); Johnson *et al.*, 1997, 1998, 2002, 2003, as well as the previous work of Bohm *et al.*, 1981] kaempferol was found. However, *E. ulei* and *E. australe*, with similar arrays of flavonoids, both lacked the presence of kaempferol (Johnson and Schmidt, 1999). We recognize that there are differences (quantitative) in flavonoid compositions of leaf tissue harvested from greenhouse, field and/or feral grown *Erythroxylum*, and that changes in their flavonoids will occur under different environmental conditions or when improperly stored. However, our analyses of methanolic extracts from leaf tissue of *E. australe* did not contain kaempferol. In light of the absence of kaempferol in *E. australe* and the presence of the flavanone eriodictyol, we summarize that there is the possibility of the relationship to the ancestral taxon *E. coca* var. *coca*, (Bohm *et al.*, 1982) since both contain the eriodictyol flavanone (Johnson *et al.*, 1997). We will discuss this finding later.

Dihydro-orobol, an isoflavone, was the predominate flavonoid present in *E. australe* (peaks #1, 3, 4, 6 and 7; Fig. 2). There was an array of positional structural differences on the A, B and C rings (Fig. 2). Of particular interest is the difference observed for *O*-conjugation found between peaks #1, #3, #4, and #7 of dihydro-orobol where conjugation of the *O*-rhamnosyl occurred at the 3′ position of ring B (peaks #1 and #7), as a 4′-*O*-dirhamnosyl on ring B (peak #3) and at the 5 position of ring A (peak #4; Fig. 1 and 2). The other isoflavone found (peak #6) contained two glucosyls, one at position 7 (ring A) and one at 3′ (ring

B). It is interesting to note that peak #6 is the only flavonoid found in *E. australe* to lack an OH at the 5 position (ring A). Two other flavonoids were also found in the methanolic extract from *E. australe* leaf tissue (peaks #2 and #5), the former, the flavonol quercetin (peak #2), which contained an *O*-rhamnosyl at position 3 of ring C and a 7-*O*-glucosyl (ring A; Figs. 1 and 2), the latter, the flavanone eriodictyol (peak #5), which contained 5, 3′, 4′-OH (rings A and B), and an *O*-rhamnosyl at position 7 (Figs. 1 and 2).

Chemistry

The HPLC pattern of flavonoid elution from extracted leaf tissue of *E. australe* is shown in Fig. 1. Peak retention times for the eight flavonoids, with exception of peaks #2 and #3, were well resolved with no overlapping or shouldering. Close peak retention time (*i.e.*, peaks #2 and #3) was due to structural conjugation and differences in aglycone substitution on the parent flavonoid, and was resolved by ^{1}H NMR spectroscopy as detailed by Johnson *et al.* (1997, 1998) (Figs. 1 and 2; Table II). Therefore, each peak-to-peak difference must be considered structurally significant. As in our previous studies, 'difference spectra' were used to demarcate unique structural features that would distinguish two compounds in adjacent peaks by subtracting out the chemical structures that are similar (Johnson *et al.*, 1997). For example, on peak #2, hydroxylation occurred at positions 5, 3′, 4′, with an *O*-rhamnosyl at position 3 and an *O*-glucosyl at position 7, whereas, for peak #3, hydroxylation occurred at positions 5, 7, 3′, with an *O*-dirhamnosyl at the 4′ position (Figs. 1 and 2; Tables I and II).

Cognate flavonoid chemistry

The flavonoids of *E. australe* (peaks #1–#8) differed from flavonoids in the four cultivated taxa of *Erythroxylum* where the prevalent flavonoid constituents were dihydro-isoflavones, whereas, those present in the four cultivated *Erythroxylum* taxa were mostly flavonols, flavanones and dihydroflavonols (see Johnson *et al.*, 1997, 1998, 2002; Johnson and Schmidt, 1999). However, we have reported the presence of isoflavones in a feral *Erythroxylum* taxon, *E. ulei*, which is indigenous to Peru (Johnson and Schmidt, 1999; von Schulz, 1907). It was interesting to note flavonoid extracts from the two feral taxon that we have examined (*i.e.*, *E. ulei* and *E. australe*) contained no cocaine alkaloids in their leaf tissue (Johnson and Schmidt, 1999; Johnson, unpublished data). Whether or not there is a relationship in *Erythroxylum* between the presence of isoflavones and the lack of cocaine alkaloids requires further investigation.

This is our second examination of flavonoids extracted from leaf tissue of a feral non-alkaloid-bearing taxon of *Erythroxylum* for use as chemotaxonomic markers. The first was *E. ulei*, which displayed an array of six distinct flavonoids which could be used unambiguously as chemotaxonomic markers for the taxon (Johnson and Schmidt, 1999). The primary flavonoids present in leaf tissue of *E. ulei* were isoflavones that included orobol, genistein, baptigenin and the flavonol quercetin (Johnson and Schmidt, 1999). We also found isoflavones in *E. australe*, as well as a flavanone and flavonol (*i.e.*, dihydro-orobol and pseudobaptisin, eriodictyol and quercetin). It is of interest that the two feral taxa did not contain kaempferols which were found in three of the four cultivated alkaloid-bearing taxa of *Erythroxylum* (Johnson and Schmidt, 1999; Johnson *et al.*, 2002). It should be noted that the absence of the flavonol kaempferol in *E. australe* leaf tissue does not make it biochemically equivalent to *E. coca* var. *coca* or *E. ulei* (Tables I and II; see Johnson *et al.*, 1997; Johnson and Schmidt, 1999).

Of the eight peaks found in *E. australe*, five were dihydro derivatives of orobol. This is the second variety of seven *Erythroxylum* taxa investigated to contain orobol and interestingly, they are both feral taxa. Orobol is a widely occurring isoflavone (Bohm, 1998) and McCormick *et al.* (1986) reported methyl ether and *C*-prenyl orobol derivatives are commonly found in Asteraceae. In our research on *E. australe* we found five orobol derivatives, one being pseudobaptisin, a methyl ether orobol derivative.

Quercetin was present in the methanolic leaf extract of *E. australe* and existed as 3-*O*-rhamnosyl-7-*O*-glucosyl with 5,3′,4′-hydroxylation. In our investigation of leaf flavonoids in *Erythroxylum* taxa, with exception of *E. coca* var. *coca* and *E. novogranatense* var. *novogranatense*, quercetin has been found in both cultivated and feral *Erythroxylum* taxa and is considered ubiquitous (Bohm *et al.*, 1981, 1982, 1988; Johnson *et al.*, 1997, 1998, 2002; Johnson and Schmidt, 1999). It may be noted that the quercetin found in *E. coca* var. *ipadu*, currently under cultivation in Colombia, South

America, glycosylation occurred at the 3 position as an *O*-rhamnosyl as in *E. australe.* However, at position 7 conjugation differed, wherewith in Colombian *E. coca* var. *ipadu* there was hydroxylation, whereas in *E. australe* glycolation (Johnson *et al.*, 2003). In addition to quercetin, the isoflavone dihydropseudobaptisin containing a 6-methoxy-7-*O*-rhamno-glucosyl was present in *E. australe* and differed from the isoflavones found in *E. ulei* (Johnson and Schmidt, 1999). This is our first report of the presence of pseudobaptisin in leaf tissue of *Erythroxylum*.

The presence of the flavanone eriodictyol in *E. australe* may suggest a relationship to the ancestral taxa *E. coca* var. *coca* (Johnson *et al.*, 1997). This is because six of the flavonoids found in *E. coca* var. *coca* leaf tissue were the flavanone eriodictyol, in addition, it was also found in Colombian *E. coca* var. *ipadu*, a cultigen of *E. coca* var. *coca* (Johnson *et al.*, 1997, 2003; Plowman, 1979). Furthermore, we have used Amplified Fragment Length Polymorphism (AFLP) to identify and determine the relationships of the four cultivated and the feral taxa *E. ulei* (Johnson *et al.*, unpublished data). Our dendrogram strongly suggests that *E. ulei* was more related to *E. coca* var. *coca* than to the *E. novogranatenses* (Johnson *et al.*, unpublished data). We will also use AFLP to determine if there is a relationship between *E. australe, E. ulei* and the parental *Erythroxylum* taxon, *E. coca* var. *coca*.

In our study of flavonoids in an *Erythroxylum* taxon indigenous to the continent of Australia, it was of interest to note that many of the flavonoids present in *E. australe* are similar to those encountered in the *Erythroxylum* taxa autochthonous to the Andean region of South America. We cannot at this time demarcate the direction of gene flow for the *Erythroxylum* taxa. Apparently, there is a trans-continental relationship that exists among and between the taxa in terms of the presence of leaf flavonoids which requires further study. In a previous study Johnson and Schmidt (1999) noted that preliminary observations indicated diminished flavonoid patterns for *Erythroxylum* taxa grown under controlled environments and greenhouses as compared to field grown plants. Therefore, it should be noted that these *E. australe* accessions were collected from feral plants and because of the uniqueness of the array of flavonoids present, they are unambiguously useful as chemotaxonomic markers for the taxon.

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Bohm B. A. (1998), Introduction to Flavonoids. Harwood Academic Publishers, Amsterdam, The Netherlands.

Bohm B. A., Phillips D. W., and Ganders F. R. (1981), Flavonoids of *Erythroxylum rufum* and *Erythroxylum ulei*. J. Nat. Prod. **44**, 676–679.

Bohm B. A., Ganders F. R., and Plowman T. (1982), Biosystematics and evolution of cultivated coca (Erythroxylaceae). Syst. Bot. **7**, 121–133.

Bohm B. A., Loo T., Nicholls K. W., and Plowman T. (1988), Flavonoid variation in *Erythroxylum*. Phytochemistry **27**, 833–837.

Evans W. C. (1981), The comparative phytochemistry of the genus *Erythroxylum*. J. Ethnopharmacol. **3**, 265–277.

Hegnauer R. (1981), Chemotaxonomy of Erythroxylaceae (including some ethnobotanical notes on old world species). J. Ethnopharmacol. **3**, 279–292.

Johnson E. L. and Schmidt W. F. (1999), Flavonoids as chemotaxonomic markers of *Erythroxylum ulei.* Z. Naturforsch. **54c**, 881–888.

Johnson E. L., Schmidt W. F., and Norman H. A. (1997), Leaf flavonoids as chemotaxonomic markers for two *Erythroxylum* taxa. Z. Naturforsch. **52c**, 577–585.

Johnson E. L., Schmidt W. F., and Norman H. A. (1998), Flavonoids as markers for *Erythroxylum* taxa: *E. coca* var. *ipadu* and *E. novogranatense* var. *truxillense*. Biochem. Syst. Ecol. **26**, 743–759.

Johnson E. L., Schmidt W. F., and Cooper D. (2002), Flavonoids as chemotaxonomic markers for cultivated Amazonian coca. Plant Physiol. Biochem. **40**, 89–95.

Johnson E. L., Schmidt W. F., Emche S. D., Mossoba M. M., and Musser S. M. (2003), Kaempferol (rhamnosyl) glucoside, a new flavonol from *Erythroxylum coca* var. *ipadu*. Biochem. Syst. Ecol. **31**, 59–67.

Mabry T. J., Markham K. R., and Thomas M. B. (1970), The Systematic Identification of Flavonoids. Springer-Verlag, New York.

Markham K. R. (1982), Techniques of Flavonoid Identification. Academic Press, London.

McCormick S., Robson K., and Bohm B. A. (1986), Flavonoids of *Wyethia angustifolia* and *W. helenioides*. Phytochemistry **25**, 1723–1726.

Plowman T. (1979), The identity of Amazonian and Trujillo coca. Bot. Mus. Leaf. Harv. **27**, 45–68.

Plowman T. (1984), The ethnobotany of coca (*Erythroxylum* spp., Erythroxylaceae). Adv. Econ. Bot. **1**, 62–111.

von Schulz O. E. (1907), Erythroxylaceae. In: Das Pflanzenreich, Vol. 4 (134) (Engler A., ed.). Verlag von Wilhelm Engelmann, Leipzig, pp. 1–164.