Leaf Flavonoids as Chemotaxonomic Markers for Two *Erythroxylum* Taxa

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Erythroxylum coca var. *coca*, *Erythroxylum novogranatense* var. *novogranatense*, Eriodictyol, Flavonoids, Kaempferol, Luteolin

Leaf extracts of *Erythroxylum coca* var. *coca* Lam. (*E. c.* var. *coca*) yielded six *O*-conjugates of **Eriodictyol** flavonoids, while the equivalent extracts from *Erythroxylum novogranatense* var. *novogranatense* (Morris) Hieron (*E. n.* var. *novogranatense*) contained five flavonoids, two of which were *O*-conjugates of **Luteolin** and three were *O*-conjugates of **Kaempferol**. All six of *E. c.* var. *coca* methanolic extracted peaks (resolved by HPLC) were found to have a 2, 3 single bond, which in *E. n.* var. *novogranatense* is replaced by a 2-hydroxy allene. The other primary difference in the predominant flavonoids between these taxa is the chemical composition of the sugar and/or acyl *O*-conjugation and site(s) at which this conjugation occurred. The results suggest that the most abundant *O*-conjugated flavonoids of *E. c.* var. *coca* and *E. n.* var. *novogranatense* may be used as chemotaxonomic markers for the two taxa. Therefore, the *O*-conjugated peaks of **Eriodictyol** , are distinct chemotaxonomic markers for *E. c.* var. *coca* and the *O*-conjugated peaks **Luteolin** and **Kaempferol** for *E. n.* var. *novogranatense*. These taxa are two of the four cultivated *Erythroxylum* taxa that contain commercial quantities of the cocaine alkaloid in their leaves, this entity also sets apart the taxa from other members of *Erythroxylum*. We suggest that the biochemistry of flavonoids of other *Erythroxylum* taxa may also be species selective.

Introduction

Erythroxylum coca var. *coca* Lam (*E. c.* var. *coca*) *Erythroxylum novogranatense* var. *novogranatense* (Morris) Hieron (*E. n.* var. *novogranatense*) and *Erythroxylum novogranatense* var. *truxillense* (Rusby) Plowman, (*E. n.* var. *truxillense*) are three of four cultivated South American species of *Erythroxylum,* the other being *Erythroxylum coca* var. *ipadu* Plowman, leaves of which are used by the indigenous population medicinally, as a stimulant, and for nutritional properties (Gutirrez-Noriega, 1948; Schultes, 1981; Holmstedt *et al.*, 1977; Plowman, 1984). The leaves of each taxa contain terpenes, flavonoids, vitamins, and several ecgonine derivatives or hydroxytropane alkaloids (Hegnauer, 1960; 1981; Evans, 1981; Leete, 1979; 1982; 1990) of which the major, benzoylmethylecgonine (cocaine), is used medicinally as a local anesthetic. According to Bohm *et al.* (1982) the above *Erythroxylum* taxa have been taxonomically defined as from one, two, or to three. Morphologically, *E. n.* var. *truxillense* was also interpreted as an intermediate hybrid between *E. c.* var. *coca* and *E. n.* var. *novogranatense* (Bohm *et al.*, 1982). However, experimental evidence by artificial breeding and leaf flavonoid chemistry suggested that *E. n.* var. *truxillense* is not of hybridoginic origin. Thus, the three taxa are said to represent a linear evolutionary series, with *E. c.* var. *coca* as the ancestral taxon and *E. n.* var. *novogranatense* derived from *E. n.* var. *truxillense* (Bohm *et al.*, 1982). Therefore, to taxonomically cogitate their relationship is to regard *E. n.* var. *novogranatense* and *E. n.* var. *truxillense* as varieties discrete from *E. c.* var. *coca* (Bohm *et al.*, 1982).

An earlier account of the leaf flavonoid chemistry of *E. c.* var. *coca* was reported by Bate-Smith (1961). However, a more detailed study of the flavonoid chemistry (i.e., extraction, separation and identification) of the above *Erythroxylum* taxa was reported by Bohm *et al.* (1982).

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Flavonoids are cited as being involved in plant environment interactions, and their apparition frequently suggested as an adaptive plant response in plants to high levels of solar radiation and elevated temperatures (Hoffmann *et al.*, 1983; Robbercht and Cadwell, 1986). Evidence also exists that indicate that flavonoids: (*i*) are ecologically important to plants (Rothschild, 1972; Palo and Robbins, 1991, Harborne, 1993); (*ii*) serve as defense mechanisms against herbivorous attack (Karban and Myers, 1989; Harborne, 1991; 1993); (*iii*) are natural antibiotics (Torrenegra *et al.*, 1989; Cuadra *et al.*, 1994); and (*iv*) enhancers of fertilization, i. e., pollen tube growth (Stanley and Linskens, 1974; Sedgley, 1975; Ylstra *et al.*, 1994).

The requirements for chemical constituents within plants to play a role as taxonomic markers have been detailed and numerous plant genera have been described using flavonoids as chemotaxonomic markers (Heywood, 1966; Ribéreau-Gayon, 1972). For a review of plant chemosystematics and a current review of flavonoid chemistry, readers should refer to Harborne and Turner (1984) and Harborne (1994). For the current research we consider the four cultivated *Erythroxylum* taxa distinctly set apart from other *Erythroxylum* taxa. This is because of the abundance of the cocaine-alkaloid (i. e., commercial quantities) in their leaves (Willaman *et al.*, 1961; Aynilian *et al.*, 1974; Holmstedt *et al.*, 1977; Evans, 1981; Plowman and Rivier, 1983; Johnson and Emche, 1994) and their cultivation by the Andean society over millennia for medicines (Schultes, 1981; Plowman, 1984). In addition, current ongoing investigations of Neo-tropical and Old World Species show that only the cultivated *Erythroxylum* taxa (see above), contains commercial quantities of the principle alkaloid benzoylmethylecgonine (E. L. Johnson, unpublished data).

In the current research, HPLC was used to separate, and both NMR and GC-MS were used to identify and confirm the flavonoid profile within methanolic leaf extracts of two of the four cultivated *Erythroxylum* taxa (*E. c.* var. *coca* and *E. n.* var. *novogranatense*) in order to investigate whether flavonoids can be used as intact chemotaxonomic chemical markers. This technique will be invaluable for identifying the two *Erythroxylum* taxa where living collections are not always accessible and flowers and/or fruits do not accompany leaf material shipped to investigators. Moreover, it establishes a chemotaxonomic precedence for *Erythroxylum* whereby four of the tropical (South American) species (*E. cataractarum* Spruce., *E. garcilipes* Peyr., *E. hondense* H. B. K., and *E. ulei* O. E. Schulz) which are morphologically similar to the cultivated taxa but do not contain the cocaine alkaloid, may be decorously distinguished by differences in their flavonoid chemistry. In addition, the current methodology provides a concise procedure for separating and identifying flavonoid conjugates within leaves of two of the four cultivated *Erythroxylum* taxa that produce commercial quantities of the cocaine-alkaloid so that the source taxon and confiscated (illicit) leaf material may be unambiguously identified. The current methodology should facilitate the separation of leaf flavonoids of the taxa so that their role(s) during herbivorous feeding, taxa fertilization (selfed and crossed) and usefulness as bioactive compounds characterized.

Materials and Methods

Plant material

Erythroxylum coca var. *coca* Lam., leaves were harvested from fields of Bolivia and Peru, 1994 and 1995, by the corresponding author, Dr. L. Darlington, Mr. M. Phelan and D. Augenstene. Leaves were oven dried in a circulating air oven (40 °C) placed in labeled plastic bags containing four Drierite desiccant bags (30 g/bag; W. A. Hammond Drierite Co., Xenia, OH., USA), shipped to the laboratory at Beltsville Agricultural Research Center (BARC) Beltsville, MD., and used for flavonoid analyses. A voucher specimen was deposited in the Weed Science Laboratory at BARC, Beltsville, MD. In addition, leaves of *Erythroxylum novogranatense* var. *novogranatense* (Morris) Hieron were harvested from the living collection (Johnson, 1996) at BARC, Beltsville, MD, and from Hawaii, 1995, (experimental field site). Leaves were oven dried as above for flavonoid analyses.

Isolation of leaf flavonoids

Dried leaves (0.02 kg) of *E. c.* var. *coca*, and *E. n.* var. *novogranatense* were separately homogenized in a Waring Blender for 30 sec. The homoge-

nized leaf samples were individually placed in labeled beakers, extracted overnight (21 °C) in capped beakers containing *ca* 80 ml of 72% MeOH. The crude extracts were filtered through four layers of cheese cloth and the leaf homogenates extracted a second and third time with 45 ml of 95% MeOH (*ca* 30 min). The extracted fractions were combined with the original, reduced *en vacuo* (55 °C) to *ca* 5 ml, and 25 ml of HPLC grade water added. The flasks were gently agitated for 2 min, the residues (hue, greenish gray) were decanted and centrifuged at 20,000×*g* for 30 min (4 °C). The resultant supernatants were decanted into labeled round bottom flasks and dried *en vacuo* as above. This yielded a 2.0 g residue for *E. c.* var. *coca* and 2.0 g for *E. n.* var. *novogranatense* with a golden brown hue, that contained the flavonoid fractions. The flavonoid fractions were dissolved in 10 ml of HPLC grade MeOH, filtered through a 0.2 μm PTFE Whatman filter affixed to a 10 ml syringe (Whatman Laboratory Division, Clinton, NJ., USA), eluted into 15 ml screw cap vials and stored at 4 °C.

HPLC chromatography

From each stored flavonoid fraction (above), 1 ml was extracted and individually placed into a 1.5 ml amber HPLC autosample vial and sealed. The vials were placed into the autosample carrier of a Hewlett-Packard (H-P) 1090M Liquid Chromatograph equipped with 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 100μl sample of each fraction was separately injected onto a Supelcosil LC-8-DB, 15 cm × 4.6 mm (i.d) 5μ octyldimethylsilyl deactivated base semi-prep analytical column (Supelco Inc., Bellefonte, PA, USA) for flavonoid separation. The HPLC conditions were: Program: Linear stepwise gradient: Mobile phase: Solvent A: 100% HPLC grade HOH: Solvent B: MeOH:-HOAc:HOH (90:5:5, v/v): Flow Rate 3 ml/min: Detection: DAD UV at λ_{min}^{MeOH} 230 nm – λ_{max}^{MeOH} 450 nm: Run time 45 min (0.01 min, 20% B; 14.50 min, 28% B; 15.01, 35% B; 42.00 min, 42%B; 45.00 min, 25% B). After equilibration, the HPLC chromatogram was divided into six regions, and the primary flavonoid fractions collected by peak elution time with the Gilson FC 204 fraction collector which afforded *ca* 200 mg of each flavonoid. The flavonoid (primary peak) fractions were dried *en vacuo* (40 °C) and aliquot (*ca* 2 mg) stored as above for ^{1}H NMR spectroscopy while the remainder was used for spectra analyses (UV and GC-MS). The classical shift reagents (Mabry *et al.*, 1970; Markham, 1982) were used with compounds (flavonoid peak fractions) **#1** through **#6** for *E. c.* var. *coca* and **#1** through **#5** for *E. n.* var. *novogranatense* (data not presented).

NMR spectrometry

The stored flavonoid fractions were decanted into labeled flasks, dried *in vacuo* as above and dissolved in 700 μl of MeOD-d_3 99.95 +% D. ^{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 32 scans. Pre-saturation for 1.2 sec 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 intermolecular coupling. Subtraction of spectra between adjacent peaks was used to compare the structural differences and similarities among structural analogues with differences in HPLC retention time.

GC-MS procedures

Peak extracts of *E. c.* var. *coca* (**#1–6**) and *E. n.* var. *novogranatense* (**#1–5**; *ca* 7 μg) were individually dissolved in 20 μl 1:1 BSTAF and pyridine, decanted into ampules (sealed), then heated at 60 °C for 1 hour (derivatization) and evaporated to dryness with N_2. Peak samples were individually dissolved in a 1%, 1:1 BSTFA: pyridine mixture. EI spectra were acquired on a Finnigan-MAT TSQ – 70B triple stage mass spectrometer. Acquisition conditions were: Ion Source temp 150 °C: Ionization energy 70 eV: Emission current 200 μA: Scan range *m/z* (rel. int) 100–1600 in 2 sec: Sample introduction *via* direct probe (*ca* 1 to 2μl): Program: From 50 °C to 800 °C at 4 °C/sec.

MS data (fully derivatized product) E. c. var. *coca peak* **#1** flavonoid(s): (EIMS {probe}70 eV; rel. int): 361 $[M - 415]^+$ (55), 437 $[M - 339]^+$ (100%), 450 $[M - 326]^+$ (19), 647 $[M - C_3H_9Si - CH_3O]^+$ (65); *peak* **#2**; 450 $[M - 242]^+$ (22), 518 $[M - 174]^+$ (34), 575 $[M - 117]^+$ (100%), 590 $[M - 102]^+$ (43), 647 $[M - 45]^+$ (9); *peak* **#3**; 361 $[M - 331]^+$ (5), 450 $[M - 242]^+$ (58), 487 $[M - 205]^+$ (39), 559 $[M - 133]^+$ (29), 575 $[M - 117]^+$ (100), 590 $[M - 102]^+$ (63), 647 $[M - 45]^+$ (27); *peak* **#4**; 361 $[M - 287]^+$ (14), 437 $[M - 211]^+$ (30), 487 $[M - 161]^+$ (98), 503 $[M - 145]^+$ (64), 559 $[M - 89]^+$ (100), 575 $[M - 73]^+$ (40); *peak* **#5**; 487 $[M - 245]^+$ (22), 518 $[M - 214]^+$ (40), 575 $[M - 157]^+$ (100), 590 $[M - 142]^+$ (28); *peak* **#6**; 430 $[M - 548]^+$ (39), 437 $[M - 541]^+$ (62), 487 $[M - 491]^+$ (100), 502 $[M - 476]^+$ (42), 559 $[M - 419]^+$ (29); *E. n.* var. *novogranatense peak* **#1** flavonoid(s): (*fully derivatized product*: EIMS {probe}70 eV; rel. int): 362 $[M - 412]^+$ (38), 415 $[M - 359]^+$ (10), 590 $[M - 184]^+$ (100), 740 $[M - 34]^+$ (9); *peak* **#2**; 362 $[M - 416]^+$ (42), 415 $[M - 363]^+$ (14), 459 $[M - 319]^+$ (25), 474 $[M - 304]^+$ (12), 590 $[M - 188]^+$ (100), 740 $[M - 38]^+$ (32); *peak* **#3**; 362 $[M - 628]^+$ (100), 459 $[M - 531]^+$ (38), 474 $[M - 516]^+$ (41), 601 $[M - 389]^+$ (5), 740 $[M - 250]^+$ (11); *peak* **#4**; 362 $[M - 590]^+$ (100), 415 $[M - 537]^+$ (16), 740 $[M - 212]^+$ (9); *peak* **#5**; 362 $[M - 881]^+$ (100), 415 $[M - 828]^+$ (33), 487 $[M - 756]^+$ (14), 647 $[M - 596]^+$ (5), 740 $[M - 503]^+$ (14).

Results and Discussion

Leaf flavonoid chemistry

The methanolic extracts from *E. c.* var. *coca* and *E. n.* var. *novogranatense* leaves that were separated by semi-preparative HPLC (see Materials and Methods) contained six and five major distinct peaks respectively (Fig. 1a, b). Peaks 2 and 3 from the *E. c.* var. *coca* extract (Fig. 1a) did not give an ideal baseline separation (i.e., valley to valley) during the HPLC separation; therefore, fractions of those peaks were collected above peak junctions where well resolved. Peak separation of the methanolic extract from leaves of *E. n.* var. *novogranatense* was ideal, showing no co-elution (Fig. 1b). After collecting sufficient peak fractions from extracts of both *Erythroxylum* taxa (*ca* 200 mg of the flavonoid) the collected peak fractions were again separated by HPLC as above and the wavelength (λ_{min} and λ_{max}; Material and Methods) maintained to ensure that the eluting peaks were flavonoids (data not presented).

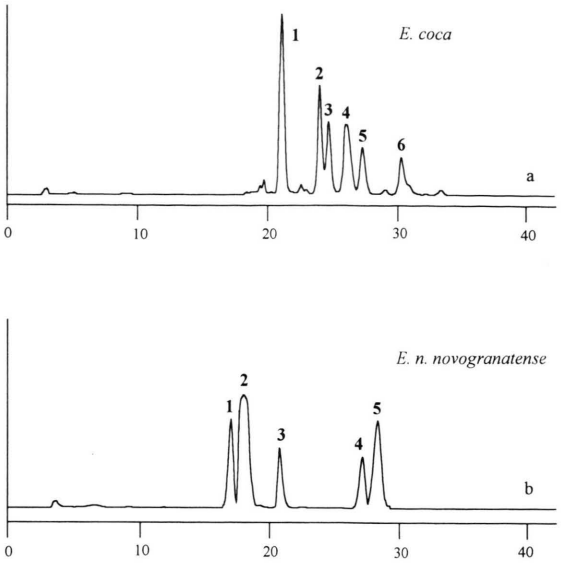


Fig. 1. HPLC profile of primary flavonoids in the methanolic extracts from dried leaves of *E. c.* var. *coca* (a) and *E. n.* var. *novogranatense* (b).

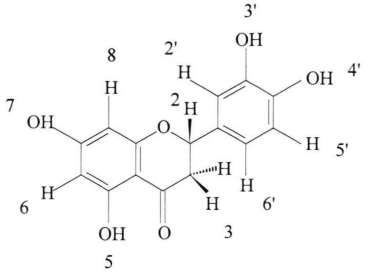
After separation, stored peak data were compared with those of authentic flavonoids in the Chem-library of the HPLC ChemStation and with those detailed by Mabry *et al.* (1970) and Markham (1982), using the classical shift reagents. Spectral analyses and wavelength comparisons showed that peaks **#1 – #6** of *E. c.* var. *coca* and peaks **#1 – #5** of *E. n.* var. *novogranatense* were flavonoids. After establishing that these peaks were flavonoids, peak fractions were collected from the methanolic extracts of both *Erythroxylum* taxa (semi-preparative) in amounts that yielded sufficient sample quantities (*ca* 2 mg/sample peak) for ^1H NMR spectroscopy.

The retention times for the methanolic extracted flavonoids of *E. c.* var. *coca* and *E. n.* var. *novogranatense* during semi-preparative HPLC are listed in Table I. *E. c.* var. *coca* flavonoids were six *O*-conjugates of **Eriodictyol** {(#**1**) (#**2**), (#**3**), (#4), (#**5**) and (#**6**)} [2- (3,4 – dihydroxyphenyl) - 5,7 – dihydroxy – 4 H – 1 benzopyran – 4 – one)] (Fig. 2). For *E. n.* var. *novogranatense*, two flavonoids were *O*-conjugates of **Luteolin** {(#**1**), (#**2**)} [(2 – (3,4 – dihydroxyphenyl) – 5, 7 – dihy-

Table I. Analytical HPLC retention times for flavonoids from MeOH leaf extracts.

	E. c. var. *coca*		*E. n.* var. *novogranatense*		
Compound	Peak number	R_t[min]	Compound	Peak number	R_t[min]
*Erio-3′,4′ OH-7-tri-†Ac§Rha	1	21.1	‡Lu-3′OH-4′H-3-tri-AcRha	1	16.5
Erio-3′-◇OEt-4′-AcRha	2	24.0	Lu-3′OEt-4′H-3-Rha	2	17.7
Erio-3′,4′-OH-7-AcRha	3	24.7	⊤K-3′H-4′OH-3-tri-AcRha-7-tri-AcGal⊗	3	21.0
Erio-3′-OEt-4′OH-7-AcRha	4	26.1	K-3′H-4′OEt-7-Gal	4	27.1
Erio-3′-OEt-4′OH-7-tri-AcRha	5	27.3	K-3′H-4′OH-3-Rha-7-Gal	5	28.2
Erio-3′,4′-OEt-7-tri-Ac-di-Rha	6	30.3			

* Erio = eriodictyol;
† Ac = acetyl;
§ Rha = rhamnosyl;
‡ Lu = luteolin;
◇ OEt = ethoxy;
⊤ K = Kaempferol;
⊗ Gal = galactosyl.



	3′	4′	7
E. c. var. *coca* **#1**	OH	OH	triacetylrhamnosyl
E. c. var. *coca* **#2**	OEt	acetylrhamnosyl	OH
E. c. var. *coca* **#3**	OH	OH	acetylrhamnosyl
E. c. var. *coca* **#4**	OEt	OH	acetylrhamnosyl
E. c. var. *coca* **#5**	OEt	OH	triacetylrhamnosyl
E. c. var. *coca* **#6**	OEt	OEt	triacetyldirhamnosyl

Fig. 2. Primary structure of the parent flavonoid, **Eriodictyol,** from the MeOH extract of dried leaves of *E. c.* var. *coca.* Numbers (#) correspond with the peak numbers in Figure 1a.

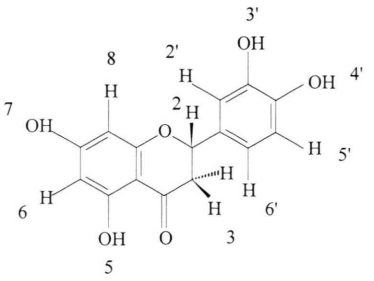
droxy – benzopyran – 4 – one)] and three, *O*-conjugates of **Kaempferol** {(#**3**), (#**4**), and (#**5**)} [(2 – (4 hydroxyphenyl) – 5, 7 – dihydroxy – benzopyran – 4 – one)] were identified (Fig. 3). Flavonoid structures were obtained by ^{1}H NMR and by comparison with structures detailed by Mabry *et al.* (1970) and Markham (1982).

In regards to peak elution time between taxa, it was noteworthy that peaks **#5** of *E. c.* var. *coca* and *E. n.* var. *novogranatense* had retention times that differed by only 0.9 min, (Table I) and probably would co-elute as a single peak, leading one to conclude that the flavonoids were identical for both *Erythroxylum* taxa. However, the ^{1}H NMR spectroscopy of both flavonoids (peaks **#5**; *E. c.* var. *coca* and *E. n.* var. *novogranatense*, Fig. 1a and b) showed that they were distinctly different. Flavonoid **#5** of *E.* c. var. *coca* was shown to be an *O*-conjugate of **Eriodictyol** whereas **#5** of *E. n. novogranatense* was an *O*-conjugate of **Kaempferol** (Figs. 2 and 3). Therefore, confirmation of chemical structure is required when retention time of flavonoids are selected as chemotaxonomic markers.

Chemistry

The primary differences in the flavonoid retention times among *E. c.* var. *coca* and *E. n.* var. *novogranatense* were due to the chemical composition of the *O*-conjugates on the parent compound and their location on the structure. Chemical shift data are presented in Table II, and chemical structures in Fig. 2 for *E. c.* var. *coca* and Fig. 3 for *E. n.* var. *novogranatense.*

The parent flavonoid structure of the two sets of samples are not identical. All six *E. c.* var. *coca* extract peaks were found to have a 2, 3 single bond, [in *E. n.* var. *novogranatense* the bond is replaced by a 2-hydroxyl allene (Figs. 2 and 3)]. The 2-H is a doublet of doublets of unequal intensities at 5.1 ppm and are coupled to the two 3-H at about 2.7 ppm. The presence of an alkyl group on 7-OH in *E. n.* var. *novogranatense* also resulted in a 0.03 ppm downfield chemical shift in 6-H and 8-H. 6-H and 8-H are singlets, but are meta-coupled (*ca.* 3Hz) to each other in *E. c.* var. *coca* (**#2**) and three *E. n.* var. *novogranatense* samples (**#1**, **#2** and **#5**; Fig. 3). Conjugation at the 7-OH position



	3′	4′	3	7
E. n. var. *novogranatense* **#1**	OH	H	triacetylrhamnosyl	OH
E. n. var. *novogranatense* **#2**	OEt	H	rhamnosyl	OEt
E. n. var. *novogranatense* **#3**	H	OH	triacetylrhamnosyl	triacetylgalactosyl
E. n. var. *novogranatense* **#4**	H	OEt	OH	galactosyl
E. n. var. *novogranatense* **#5**	H	OH	rhamnosyl	galactosyl

Fig. 3. Primary structure of one of the parent flavonoids, **Luteolin,** from the MeOH extract of dried leaves of *E. n.* var. *novogranatense.* Numbers (#) correspond with the peak numbers in Fig. 1b.

accounted for this change. Conjugation at the 5-OH position would not necessarily decouple 6-H and 8-H. Ethoxyl conjugation occurred on four *E. c.* var. *coca* samples (**#2, #4**, #**5**, and **#6**; Fig. 2) and on two *E. n.* var. *novogranatense* samples (**#2** and **#4**). The sites of conjugation were unambiguous because the chemical shift was largest closest to the binding site. Conjugation at 3'-OH resulted predominantly in chemical shifts with H-2', at 4'-OH with H-5' and at 7'-OH with H-8. No conjugation effecting primarily the 5'-OH was found. A methyl doublet at 1.10 ppm defines a rhamnosyl structure. The anomeric proton doublet at 5.90/5.86 ppm and the absence of a second rhamnosyl doublet at 1.10 ppm characterizes the second sugar as a galactosylic instead of rhamnosylic. The large coupling constant (J=12 Hz) verifies the H-1 anomeric proton and the adjacent H-2 proton are in a *cis* configuration. In glucosyl and rhamnosyl sugars, the corresponding protons are *trans* to each other.

GC-MS was used to confirm the chemical structures of the conjugated flavonoids. TMSi derivatization enabled flavonoid volatilization and GC-MS separation into component structures. The number of hydroxyl groups conjugated and/or derivatized depends upon both the parent compound, the number of sugar molecules and whether the saccharides are glucosyl or rhamnosyl sugars. The location of the specified conjugated groups at specific molecular sites cannot be deciphered from the mass spectra data without knowing which sites are more reactive/stable with which conjugate. The fragment formed from the loss of a rhamnosyl group from the 3-position in *E. n.* var. *novogranatense* for example has the same mass loss as the rhamnosyl group from the 5-position. The loss of C_3H_9Si- from the 4'-position in *E. c.* var. *coca* resulted in the same mass ion as a loss of C_3H_9Si- from a glucosyl conjugate. The relative intensity of the mass ion could be different between the two, but ascertaining which intensity corresponds to which chemical structure for either compound remains ambiguous.

Elution patterns of flavonoids from *E. c. var. coca* and *E. n.* var. *novogranatense* were consistent and reliable. Each flavonoid collected from both species had minimum baseline noise (HPLC) and clean ^{1}H NMR spectra. To circumvent potential oxidation of the flavonoids and to prevent water absorption by the NMR, samples were dissolved in MeOD-d_3 (99.95 +% D) and heat sealed in NMR tubes. Peak separation of flavonoid fractions collected from the methanolic extract (Materials and Methods) enabled flavonoids present in dried leaves of *E. c.* var. *coca* and *E. n.* var. *novogranatense* to be separated and individually identified.

Related flavonoid chemistry

A biosystematic study of cultivated *Erythroxylum* taxa by Bohm *et al.* (1982) showed the presence of quercetin and kaempferol in leaf extracts

Table II. ^{1}H NMR data for leaf flavonoids of *E. c.* var. *coca* **#1, #2, #3, #4, #5, #6** and *E. n.* var. *novogranatense* **#1, #2, #3, #4, #5** in MeOD-d_3 + 99.5% D.

	E. c. var. *coca*						*E. n.* var. *novogranatense*				
PROTON	**#1**	**#2**	**#3**	**#4**	**#5**	**#6**	**#1**	**#2**	**#3**	**#4**	**#5**
2′	7.55	7.52	7.51	7.53	7.57	7.57	7.63	7.63	6.88 6.85d*	6.88 6.85d	6.88 6.85d
3′	–	–	–	–	–	–	–	–	8.05 8.02d	8.04 8.02d	8.05 8.01d
4′	–	–	–	–	–	–	–	–	–	–	–
5′	6.85 6.82d	6.84 6.81d	6.84 6.81d	6.86 6.83d	6.87 6.84d	6.86 6.89d	6.86 6.83d	6.86 6.85d	6.88 6.85d	6.88 6.85d	6.88 6.85d
6′	7.94 7.92	7.91 7.88 7.78	8.02 7.89 7.94 7.76	8.04 7.92 8.01 7.90	8.04 8.01 7.92 7.90	7.99 7.92 7.96 7.90	7.93 7.91	7.90 7.93	8.05 8.02d	8.04 8.02d	8.05 8.01d
2	5.13 5.08	5.09 5.07	5.11 5.07	5.11 5.07	5.11 5.07	5.12 5.10	–	–	–	–	–
3	2.65 2.33	2.78 2.37	2.78 2.38	2.72 2.34	2.71 2.36	2.71 2.96	–	–	–	–	–
6	6.16	6.16	6.15	6.15	6.16	6.16	6.19 6.18	6.18 6.17	6.19	6.18	6.19 6.18
8	6.35	6.36 6.35	6.35	6.35	6.37	6.36	6.38 6.37	6.37 6.36	6.38	6.38	6.39 6.38
OCH_2CH_3	–	3.57q*	–	3.56q	3.56q	3.56q	–	3.57q	3.57q	3.57q	–
OCH_2CH_3	–	1.14t*	–	1.14t	1.13t	1.13t	–	1.14t	1.14t	1.14t	–
Sugar H-1	5.48 5.46	5.47 5.44	4.86 4.83	5.45 5.42	5.42 5.40	5.28 5.26 4.89 4.86	4.82 4.81	4.49 4.48	5.90 5.86 4.49 4.48	5.90 5.86 4.49	5.90 5.86 4.49 4.48
Sugar CH_3	1.12 1.10	1.12 1.10	1.12 1.10	1.12 1.10	1.12 1.10	1.12 1.10	1.10 1.08	1.10 1.08	1.10 1.08	1.10 1.08	1.10 1.08
Sugar H2–H6	4.10 3.04	4.10 3.04	4.10 3.12	3.90 3.13	3.79 3.13	3.82 3.15	3.78 3.19	3.78 3.19	3.92 3.19	3.78 3.19	3.78 3.19
Acetyl	1.92	1.93	1.95	1.92	1.91	1.93	1.94	1.95	1.94	1.95	1.91

d* = doublet;
t* = triplet;
q* = quartet.

from *E. c.* var. *coca* and *E. n.* var. *novogranatense*, *E. n.* var. *truxillense*, *E. c.* var. *ipadu,* artificial crosses, and previously, in leaves of *E. rufum* and *E. ulei* (Bohm *et al.*, 1981). Subsequently, Bonefeld *et al.* (1986) characterized flavin-3-ols an additional flavonol in stems of *E. n.* var. *novogranatense* and Chávez *et al.* (1996) several flavonoids in *E. leal costae.* Noteworthy, in a latter investigation of 13 species of *Erythroxylum* from Brazil, Bohm *et al.* concluded that all exhibited profiles of flavonol glycosides, where the predominate flavonols were kaempferol, quercetin and 7,4′ dimethylquercetin. The investigators used absorption chromatography and TLC for separation and purification of flavonoids. In our methanolic extract from *E. c.* var. *coca* leaves, no quercetin was detected. The six flavonoids detected were *O*-conjugates of **Eriodictyol** and aceylated rhamnosyl sugars (**#1, 2, 3, 4, 5, 6**; Fig. 2). We do not refute the presence of quercetin in leaf extracts previously reported for *E. c.* var. *coca* and other *Erythroxylum* taxa (Bohm *et al.*, 1981; 1982; 1988; Bonefeld *et al.*, 1986; Chávaz *et al.*, 1996). However, we consider its presence in *E. c.* var. *coca* leaf extracts, potentially the result of the oxidation of **Eriodictyol**. Structurally, **Eriodictyol** may undergo oxidation during long-term exposure to atmospheric conditions. Related dihydroflavonols are subject

to such oxidation during extraction and workup (J. B. Harborne, personal communication). It is unknown whether previous investigators used techniques to prevent the potential for oxidation during absorption chromatography. It is noteworthy that Hradetzky *et al.* (1987) in their investigation of flavonoids in aerial part extracts of *Gutierrezia sarothrae* (Pursh) Britton (*Asteraceae*) after the use of thin layer chromatography and absorption chromatography (Sephadex LH-20) reported the presence of a trace of **Eriodictyol-7-Me**, a flavonoid similar to the predominate *O*-conjugated flavonoids present in the methanolic leaf extract of *E. c.* var. *coca* in the current study. Whether the trace of **Eriodictyol-7-Me** observed by Hradetzky *et al.* (1987) was a remnant of non-oxidized **Eriodictyol** is unknown. Therefore, we are currently investigating conditions under which flavonoids oxidize. The current flavonoid extraction procedure prevented long-term exposure of leaf extracts to atmospheric conditions, thus, preventing potentiality for oxidation.

In terms of flavonoid extracts from leaves of *E. n.* var. *novogranatense*, two of the flavonoids (**#1**, **#2**; Fig. 2) were **Luteolin** conjugates and three **Kaempferol** conjugates (**#3**, **#4**, **#5**; Fig. 3). The presence of **Kaempferols** were previously reported in leaves of *E. n.* var. *novogranatense*, *E. rufum* and *E. ulei* and several *Erythroxylum* species from Brazil (Bohm *et al.*, 1981, 1982; 1988). Using the current procedure, flavonoids **#1**, **2**, **3**, **4**, **5** and **6** (the primary leaf flavonoids) are considered as distinct chemotaxonomic markers for *E. c.* var. *coca* (Fig. 2) and **#1**, **2**, **3**, **4** and **5** for *E. n.* var. *novogranatense* (Fig. 3). Our procedures provide a refined and efficient method for extracting and determining the flavonoid profile of the methanolic extract from leaves of *E. c.* var. *coca* and *E. n.* var. *novogranatense*. Long-term exposure of flavonoid extracts to oxidation that may occur during absorption chromatography and TLC is precluded. It avoids the degradation of flavonoids and/or labile compounds that are subjected to high temperature when extracted with hot methanol and/or ethanol and those used in GLC procedures. It was noteworthy in our preliminary study of leaf flavonoid extracts from the two taxa, that different flavonoid profiles were observed when the leaf tissue was extracted with hot methanol and those soaked over night in methanol (21 °C). The flavonoid profile of leaf tissue soaked overnight was more consistent than tissue extracted with hot methanol (E. L. Johnson, unpublished data). The procedure also enables differentiation of peaks that may otherwise co-elute during HPLC separation.

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