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Kaempferol (rhamnosyl) glucoside, a new flavonol from *Erythroxylum coca*. var.*ipadu*

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Abstract

A new flavonol, kaempferol rhamnosyl diglycoside, was isolated from leaf tissue of Amazonian field-grown coca *Erythroxylum coca* var. *ipadu* Plowman. The structure of the flavonol has been determined as kaempferol 4′-*O*-(rhamnosyl)glucoside by spectral analyses. The array of flavonoids present in *E. c.* var. *ipadu* currently under cultivation in Colombian fields is indicative of a recent cross, consistent with ancestralship to *E. c.* var. *coca* and the flavonol is useful as a chemotaxonomic marker for the taxon.
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1. Introduction

The geographical distribution, cultivation and use of *Erythroxylum coca* var. *ipadu* Plowman (*E. c.* var. *ipadu*) 'Amazonian coca,' was not delineated until the present century, hence, there is no archeological record to date its origin (Plowman, 1979, 1981, 1984; Schultes, 1981). *Erythroxylum coca* var. *ipadu* is marked as a recent development evolving from *E. c.* var. *coca* Lam, in the Andean foothills, through selective traits conducive to cultivation in Amazonia where it is geographically isolated from other coca varieties (Plowman, 1979, 1984). The leaf morphology of Colombian field-grown *E. c.* var. *ipadu* is morphologically different from the leaf of the taxon we obtained from the Colombian region in 1972 (Materials and methods section) and in our living collection. However, we found the cocaine alkaloid content in leaf tissue of Colombian field-grown *E. c.* var. *ipadu* and our living collection of *E. c.* var. *ipadu* to range from 0.38 to 0.42% which is slightly higher than the values reported by Plowman and Rivier (1983) for laboratory grown *E. c.* var. *ipadu*.

Leaf flavonoids have proven to be useful chemotaxonomic markers for the identity of both cultivated and feral taxons of *Erythroxylum* (Bohm et al., 1982, 1988; Johnson et al., 1997, 1998, Johnson and Schmidt, 1999). In the current study, leaves from Amazonian field-grown coca, *E. c.* var. *ipadu* (currently under cultivation in Colombia, South America) were harvested from several fields and our living collection of *E. c.* var. *ipadu*, to confirm their flavonoid profile. Of the eight flavonoids found in leaf tissue of Amazonian field-grown *E. c.* var. *ipadu*, one was distinctively different. The current study describes this flavonoid and its usefulness as a chemotaxonomic marker for Amazonian field-grown *E. c.* var. *ipadu*.

2. Materials and methods

2.1. Plant material

During the summer, fall and winter of 1997 through 1999, leaves, branches, and stems were harvested from field-grown coca plants (*E. c.* var. *ipadu* Plowman) throughout the Amazonian region of Colombia, SA for taxon identification and flavonoid profile determination. All leaves harvested from Colombian field-grown *E. c.* var. *ipadu*, lacked the characteristic pair of 'lateral lines' or 'folds' parallel to each side of the mid-vein commonly present on leaves of cultivated coca (Rury, 1981) and leaves of our living collection of *E. c.* var. *ipadu*. The harvested Amazonian field-grown coca was purported by the indigenous population of the region (i.e. Amazonian region of Colombia) and the local growers, to be *E. c.* var. *ipadu* Plowman. It was also authenticated by botanist, Dr Luis E. Parra Rodriguez, of Santafé de Bogota, Colombia, SA, as *E. c.* var. *ipadu* Plowman. Collected samples of *E. c.* var. *ipadu* (accession no. COL1997-099AMR) from Amazonian fields are stored in the Alternate Crops and Systems Laboratory at the Beltsville Agricultural Research Center, Beltsville, MD, USA.

Erythroxylum coca var. *ipadu* in our living collection, whose leaves possess the

pair of lateral lines, was collected during a 1972 taxonomic survey of Erythroxylaceae in the Amazonian region of Colombia, SA. It was transported to the Beltsville Agricultural Research Center, Beltsville, MD, USA, and cultured as a part of our living collection. Subsequently, *E. c.* var. *ipadu* in our living collection was authenticated by Drs T. Plowman, 1988, and P.M. Rury, 1992, as *E. c.* var. *ipadu* Plowman (voucher specimens, accession no. COL-1972AMR used in the current study were stored in the Alternate Crops and Systems Laboratory at Beltsville Agricultural Research Center, Beltsville, MD, USA). In 1994, vegetative propagates of our *E. c.* var. *ipadu* were transplanted to our Hawaiian field site for growth and development and designated as accession no. COL-1972AMR-HII. Both will be referred to in the current study as 'our collection.' Amazonian field-grown coca, *E. c.* var. *ipadu,* in this study should not be confused with 'Colombian coca,' *Erythroxylum novogranatense* var. *novogranatense* (Morris) Hieron (Plowman, 1979, 1984).

Leaves harvested from *E. c.* var. *ipadu* from fields in the Amazonian region of Colombia, SA, and from our living collections (for comparison of flavonoid profiles) were oven dried in a circular air oven at 40° C as described by Johnson et al. (1998), for storage, flavonoid isolation and identification (i.e. spectral analyses).

2.2. *Isolation of leaf flavonoids*

Flavonoids were extracted from oven-dried leaves as described by Johnson et al. (1998). Briefly, for all samples harvested, two batches (0.025 kg/batch) of *E. c.* var. *ipadu* leaves were separately homogenized (anhydrously) in a Waring Blender for 30 s and placed in two glass beakers containing ca. 300 ml of 72% aqueous MeOH. Contents in each beaker were kept separate in subsequent steps. The beakers were capped and stored overnight (in darkness) at 21 °C for flavonoid extraction. The leaf homogenate was extracted twice more with 100 ml of 72% MeOH, combined, vacuum filtered and reduced en vacuo (45 °C) to ca. 5, and 25 ml of HPLC grade water 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. The pellet was re-extracted (2 ×) with 10 ml of HPLC grade H_2O and centrifuged as before. The resultant supernatants were combined and dried en vacuo to yield 7.00 mg of residue which was dissolved in 20 ml of 1% HOAc (v/v). This extract was then loaded, in 5 ml fractions, onto Supelclean ENVI-18, 1 g, SPE columns (Supelco, Inc., Bellefonte, PA) and subsequently washed with 1% HOAc (6 ml), 5% MeOH (4 ml) and 20% MeOH (4 ml). The flavonoids were eluted from the column with 70% MeOH (6 ml). This fraction was dried en vacuo (45 °C), redissolved in 10 ml of 1% (v/v) HOAc and filtered through a 0.2 μm PTFE Whatman filter for storage at 4 °C.

2.3. *HPLC resolution IR and flavonoid profile*

One-hundred μl from each of the stored flavonoid samples were injected into a Hewlett-Packard 1090M liquid chromatograph (equipped with ChemStation, Diode Array detector, ChemLibrary (Hewlett-Packard, Avondale PA, USA)) with a Gilson

FC 204 fraction collector (Gilson Inc., Middleton, WI, USA) attached to the outlet port of the HPLC. All injections were made onto a Phenomenex Columbus C-8, 25cm × 10mm (i.d.) 5 μm silica/spherisorb semi-prep/ 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 230–470 nm λ_{MeOH}; run time 105 min (0.01 min, 30% B; 85.00 min, 42% B; 95.00 min, 65% B; 95.01 min, 25% B; 105.00 min, 25% B). After equilibration, the flavonoid fractions (HPLC) were collected by peak elution times (Table 1) where repetitive flavonoid sample injections and peak separations afforded ca. 2 mg of each flavonoid. The flavonoid fractions (primary peaks) were dried en vacuo (45 °C) and subjected to ^1H NMR spectroscopy (Tables 1 and 2) and spectrum analyses (GC-MS, LC-MS, IR and UV). For UV analyses, shift reagents detailed by Mabry et al. (1970) and Markham (1982), were used with each flavonoid (i.e. peak fractions collected, #1 through #8) for *E. c.* var. *ipadu* for verification, and IR spectra recorded in KBr (data not presented). The kaempferol was further confirmed by comparison of IR spectra with an authentic sample. Unlabeled peaks in the HPLC chromatograph were not flavonoids.

Table 1
Flavonoids in leaf tissue of *E. coca* var. *ipadu* from our living collection and Amazonian field-grown coca

Peak number	R_t (min)	Compound
E. coca var. *ipadu*[a]		
1	17.9	Tax-3′,4′,5-tri-OH-3H-7-Rham
2	30.3	Que-4′,3,5-tri-OH-3′,7-di-Rham
3	31.2	Tax-3′,3,5-tri-OH-4′,7-di-Rham
4	32.9	Tax-3′H-3,5-di-OH-4′,7-di-Rham
5	33.9	Tax-3′,5,7-tri-OH-4′,3-di-Rham
6	38.4	Tax-3′,5-di-OH-4′,3,7-tri-Rham
E. coca var. *ipadu*[b]		
1	29.3	Erio-3′OEt-5,7-di-OH-4′-Rham
2	31.3	Que-3′′,5,7-tri-OH-4′,3-di-Rham
3	33.2	Erio-3′,4′,5-tri-OH-7-Rham
4	33.9	Tax-3′,5,7-tri-OH-4′,3-di-Rham
5	38.2	Tax-3′,5-di-OH-4′,3,7-tri-Rham
6	45.4	K-3′H-4′,5-di-OH-3-Rham-7-Rutin
7	47.9	K-3′H-3,5,7,-tri-OH, 4′-Gluco-Rham
8	58.2	K-3′H-5,7-di-OH-4′,3-di-Rham

[a] Living collection, circa 1972.
[b] Amazonian field grown, 1997.

Table 2
^{1}H NMR data for leaf flavonol from *E. c.* var. *ipadu* (Amazonian coca) peak #7

Proton	Peak number #7
H2′, 6′	8.027
	8.021
	8.005
	7.999
H3′, 5′	6.924
	6.916
	6.901
	6.893
H6	6.200
	6.192
H8	6.396
	6.389
Sugar	
H-1	5.329
	5.314
H′-1	4.106
	4.092
	4.084
	4.070
CH3	0.965
	0.941
H2–H6	3.9–3.1

2.4. LC-MS procedures

Dried flavonoid fractions were redissolved in 1 ml of MeOH and were identified using a Micromass LC quattro mass spectrometer (Micromass, Beverly, MA) with an electrospray (ES) ionizer. Direct infusion injections were used in both (+) and (−) ion modes with the following parameters: capillary voltage: 2.80(+), −2.78 (−), cone voltage: 67 (+), −72 (−), source temperature (°C): 100(+), 120(−): desolvation temperature (°C): 199(+), 199(−). The Micromass LC quattro was a triple quadrupole with a single stage mass analyzer. The first quadrupole was scanned while the second and third were set to reference. All scans were acquired within 10 s. LC-MS data for flavonoid peak #7: 60 (M-519) (100), 63 (M-516) (95), 97 (M-482) (45), 220 (M-359) (22), 256 (M-323) (18), 284 (M-295) (15), 286 (M-293) (16), 326 (M-253) (12), 449 (M-130) (10), 552 (M-27) (25) 553 (M-26) (48).

3. Results and discussion

3.1. Leaf flavonoid chemistry

The polar fractions from leaves of *E. c.* var. *ipadu* currently growing in Colombian fields and *E. c.* var. *ipadu* from our living collection separated by HPLC afforded

six and eight discrete peaks of primary leaf flavonoids (Fig. 1A and B). The six flavonoids in leaf polar extracts of *E. c.* var. *ipadu* from our living collection (Johnson et al., 1998) and seven of the eight flavonoids from polar extracts of Colombian field-grown *E. c.* var. *ipadu* have been described (Johnson et al., 2002). However, peak #7 flavonoid (Fig. 1) of the of Colombian field-grown *E. c.* var. *ipadu* has not been delineated. Our examination of the peak #7 leaf flavonoid of *E. c.* var. *ipadu*, in Colombian field-grown coca, revealed a different and unique flavonoid (Fig. 1B; Tables 1 and 2). The flavonoid was a flavonol as determined by ^{1}H NMR, mass spectra and IR analyses to be kaempferol 4′-(rhamnosyl)glucoside. ^{1}H NMR was used to determine non-conjugation at the 3, 5, 7-OH positions of rings A and C and conjugation at the 4′-OH position of ring B (Fig. 1B; Table 2), whereas mass spectroscopy verified the orientation of the complex conjugate as a rhamnosyl-glucosyl-*O* ester as opposed to a glucosyl-rhamnosyl-*O* ester (Table 2). This finding was confirmed by making subsequent extracts from the original leaf tissue of *E. c.* var. *ipadu* and those harvested from several fields under cultivation throughout the Amazonian region of Colombia. The result obtained from subsequent leaf flavonoid extracts were identical to those previously obtained and displayed the flavonoid profile shown in Fig. 1B.

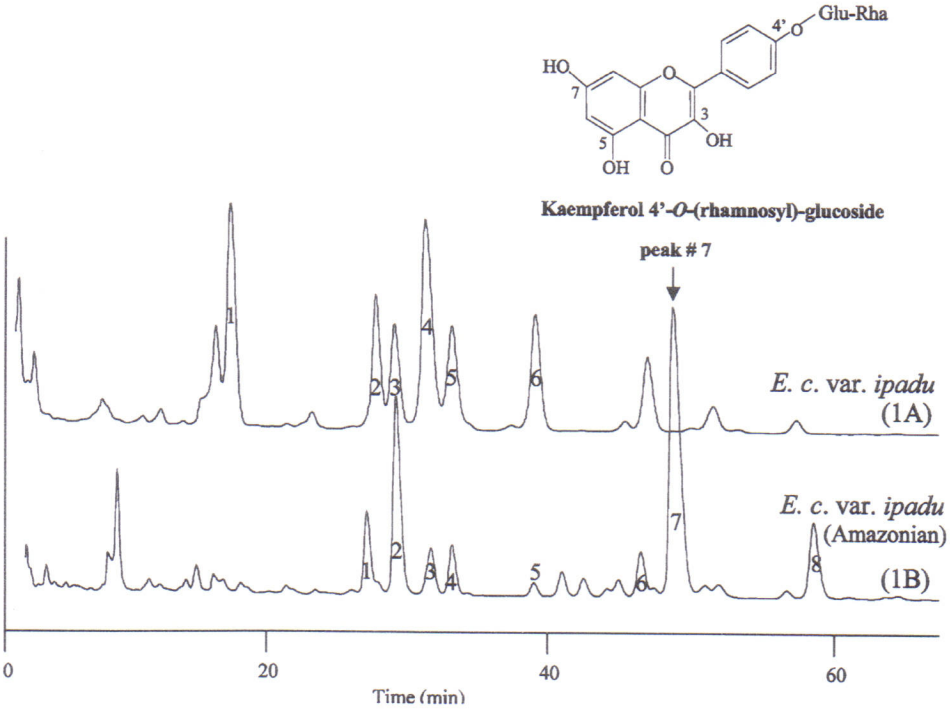

Fig. 1. A and B. Overlap of the HPLC chromatographs of flavonoids from the polar leaf extracts showing peak elution times in *E. c.* var. *ipadu* (1A living collection, circa 1972) and *E. c.* var. *ipadu* (1B, Amazonian field-grown). Note the presence of peak #7 and its structure in B which was not found in A.

In a previous study of flavonoids of *E. c.* var. *ipadu* in our living collection from the same region of Colombia, SA (obtained in 1972) we identified the flavonoids in leaf tissue grown under greenhouse, controlled environmental and field conditions, no kaempferols or eriodictyols were found (Johnson et al., 1998). We concluded that flavonoids in this taxon of *E. c.* var. *ipadu* were derivatives of, coincided with and were indicative of kinship to *E. c.* var. *coca* due to the presence of taxifolin and quercetin in its leaf tissue (Johnson et al., 1998). In addition to the flavonol at peak #7 of the HPLC chromatograph, Fig. 1 also shows two additional kaempferols present in Colombian field-grown *E. c.* var. *ipadu* that were not present in *E. c.* var. *ipadu* from our living collection (Table 1; Johnson et al. (1998).

We previously showed that the flavonoid profile in leaf tissue of *E. c.* var. *coca* was not affected by differences in environmental conditions regardless of the region in which the taxon was grown (Johnson et al., 1997). We also showed that the flavonoids in our living collection of *E. c.* var. *ipadu* obtained from Colombia, SA, did not contain kaempferol-based flavonoids (Johnson et al., 1998). Therefore, to obtain the current array of flavonoids present in leaf tissue of Colombian field-grown *E. c.* var. *ipadu* would suggest a latter cross between *E. c.* var. *coca*×*E. c.* var. *truxillense*. The latter cross would account for the presence of eriodictyol, taxifolin, quercetin and kaempferol in the leaf tissue of Colombian field-grown *E. c.* var. *ipadu* (Johnson et al., 1998). We suggest the above cross since crosses between *E. c.* var. *coca* and *E. n.* var. *novogranatense* were determined incompatible (Bohm et al., 1982). Hybridization between *E. c.* var. *coca*×*E. c.* var. *truxillense* would be consistent with the views of Rieseberg and Ellstrand (1993); also a phenomenon addressed earlier by Harborne (1975) who noted that the expression of chemical traits in hybrids are considered additive. Thus, hybrids often combine their parental chemical characteristics, an apparition reported for several taxons (Mikanagi et al., 1994; Raymond et al., 1995).

We will be examining the F_1 progeny of the above cross for genetic variability within the taxon to assess the genetic additives of flavonoids, especially the rare hydroxylated kaempferol 4′-*O*(-rhamnosyl-)glucoside (peak #7). In a preliminary comparative examination between *E. c.* var. *ipadu* currently under cultivation in Colombian fields and *E. c.* var. *ipadu* from our living collection, using amplified restriction fragment length polymorphism (AFLP), we observed variation among the taxon under cultivation in Colombian fields that were not present in our living collection. The variations observed among Colombian field-grown *E. c.* var. *ipadu* will be characterized in a subsequent paper. Our AFLP data strongly suggest that both taxa are indicative of *E. c.* var. *coca* as the ancestral taxon and further suggests the latter cross between the taxa indicated before.

3.2. Cognate flavonoid chemistry

Examination of the flavonoid literature revealed few monosaccharide 4′-*O* flavonoids in tropical plant species but one related flavonol triglycoside rhamnustrioside, was found in fresh fruit of *Rhamnus nakaharai* (Lin et al., 1982) which possessed a kaempferol 4′-*O*-rhamnosyl-(1-2)-*O*[rhamnosyl-(1-6) galactoside].

However, it differs from the 4′-kaempferol found in leaf tissue of Colombian field-grown *E. c.* var. *ipadu* because of the presence of the 1-2 and 1-6 *O*-rhamnosyl galactoside linkages which were not found in leaf extracts from Colombian field-grown *E. c.* var. *ipadu* (Fig. 1; Table 1). We refer to the two flavonols because of their 4′-glycosylation which apparently is rare for woody taxa. The question now arises as to whether flavonoid metabolism in leaf tissue of Colombian field-grown *E. c.* var. *ipadu* is genetically influenced by several metabolites (i.e. enzymes), whereas those of *E. c.* var. *ipadu* in our living collection to lesser; or are the differences observed in the array of flavonoids for the taxa a result of additivity because of the cross suggested above. This is currently unknown. However, because of the suggested cross, i.e. *E. c.* var. *coca*×*E. c.* var. *truxillense*, the flavonoid metabolism for Colombian field-grown *E. c.* var. *ipadu* when compared to our collection may parallel the complexity of metabolism described for *O*-methylated flavonols of *Chrysosplenium americanum* (Collins et al., 1981). *Chrysosplenium americanum* flavonoids were shown to be influenced by several *O*-methyltransferases that exhibits a high degree of position (i.e. ring) specificity (Collins et al., 1981; De Luca and Ibrahim, 1982, 1985; Ibrahim et al., 1987; Khouri and Ibrahim, 1987; Khouri et al., 1988; Ibrahim, 1992; Gauthier et al., 1996.

We summarized before that Colombian field-grown *E. c.* var. *ipadu* appears to be the result of a latter cross between *E. c.* var. *coca*×*E. c.* var. *truxillense*, thus yielding an array of complimentary (i.e. those present in *E. c.* var. *coca* and *E. n.* var. *truxillense*) as well as different flavonoids not present in our collection of *E. c.* var. *ipadu* (Johnson et al., 1997, 1998). The 4'(-rhamnosyl-)glucosyl-kaempferol present in leaf tissue of Colombian field-grown *E. c.* var. *ipadu* (peak #7; Fig. 1) differs from any of the flavonoids we have found in the four cultivated taxons of *Erythroxylum* to date (Johnson et al., 1997, 1998). We therefore consider 4′(-rhamnosyl-)glucosyl-kaempferol a definitive flavonol for the taxon and a distinct chemotaxonomic marker for field-grown Amazonian coca, *E. c.* var. *ipadu*.

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