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Original Article

Chemical characterization of two morphologically related *Espeletia* (Asteraceae) species and chemometric analysis based on essential oil components

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ABSTRACT

In this study, a comprehensive phytochemical characterization of two morphologically related species from the genus *Espeletia* Mutis ex Bonpl., namely, *Espeletia grandiflora* Humb. & Bonpl. and *Espeletia killipii* Cuatrec., Asteraceae, has been performed by gas chromatography coupled to flame ionization detection, gas chromatography coupled to mass spectrometry and ultra-high performance liquid chromatography coupled to ultraviolet and high-resolution mass spectrometry. Analysis of ethanol extracts (70%, v/v) from leaves and concomitant compound dereplication allowed the identification of major peaks, most of them new reports for the genus *Espeletia* or the subtribe Espeletiinae. Chemical characterization of resins essential oils indicated several similarities and differences between both species and from other members of the subtribe. Chemometric analysis (hierarchical clustering analysis and orthogonal partial least-squares discriminant analysis) applied to the essential oil composition of 31 species from Espeletiinae furthermore allowed the identification of three primary clusters correlated with the taxonomy. Hence, this study underscored qualitative and semiquantitative differences between the chemical composition of leaves and resins of *E. grandiflora* and *E. killipii*, provided information on chemotaxonomy and described the presence of different trends in the essential oil composition from species of Espeletiinae.

Introduction

The genus *Espeletia* Mutis ex Bonpl., Asteraceae, constitutes a neotropical group endemic to the northern Andean regions of Venezuela, Colombia, and Ecuador. With *ca.* 71 species, *Espeletia* is the most diverse genus within the subtribe Espeletiinae, which also comprises the genera *Espeletiopsis* Cuatrec., *Coespeletia* Cuatrec., *Ruilopezia* Cuatrec., *Libanothamnus* Ernst, *Carramboa* Cuatrec., *Tamania* Cuatrec., and *Paramiflos* Cuatrec. (Cuatrecasas, 2013; Diazgranados, 2012a).

Espeletia grandiflora Humb. & Bonpl. was the first species in Espeletiinae to be described and continues to be frequently studied in terms of its morphology, ecophysiology, distribution, and taxonomy (Fagua and Gonzalez, 2007; Cuatrecasas, 2013). However, gaps remain in the taxonomic delimitation of this species, which could possibly include different taxonomic entities, given its high polymorphism and broad geographic distribution (Cuatrecasas, 2013). Its shared morphological characteristics with *Espeletia killipii* Cuatrec. (*e.g.*, leaf shape, plant height, and overall appearance) moreover hinder its unequivocal identification. According to Cuatrecasas (2013), two primary morphological characteristics that distinguish *E. grandiflora* from *E. killipii* include the presence of at least one pair of sterile opposite leaves in the proximal part of the synflorescences in the former species—by contrast, *E. killipii* lacks such sterile leaves—and the length of the synflorescences, which are longer in *E. grandiflora*. However, the frequent hybridization of the two species in zones where overlapping populations occur (*e.g.*, La Chisacá páramo in Colombia) further complicates their unambiguous taxonomic delimitation (Cuatrecasas, 2013).

The secondary metabolite chemistry of *E. grandiflora* and *E. killipii* has been poorly investigated. Nevertheless, classic phytochemical studies of *E. grandiflora* resins have reported the presence of six *ent*-kaurane diterpenes—kaur-16-ene, kaur-16-en-19-al, kaur-16-en-19-ol, kaurenoic acid, grandiflorolic acid, and grandiflorenic acid (Piozzi et al., 1968, 1971, 1972)—whereas other chemical classes (*e.g.* flavonoids, sesquiterpene lactones, and triterpenes) have been reported from the leaves of *E. killipii* (Torrenegra et al., 1994; Torrenegra and Tellez, 1995, 1996). However, no comprehensive phytochemical characterization that includes the leaves and resins of both species has been performed, and the similarities

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and differences between the metabolic fingerprints of *E. grandiflora* and *E. killipii* remain unknown.

Consequently, this study aimed to perform a comprehensive phytochemical characterization of *E. grandiflora* and *E. killipii* leaves and resins by using modern and complementary analytical techniques such as GC–FID, GC–MS and UHPLC–UV-HRMS, all to determine similarities and differences between the species, as well as among EO from 31 species of Espeletiinae, as performed by chemometric methods involving multivariate statistical analysis.

Materials and methods

Plant material

Leaves and resins of *Espeletia grandiflora* Humb. & Bonpl. and *E. killipii* Cuatrec., Asteraceae, were collected in the Cruz Verde (N 4° 34′ 48.5″ W 73° 59′ 45.3″ – 3328 m.) and Sumapaz páramos (N 4° 17′ 24″ W 74° 12′ 24.2″ – 3717 m.), respectively, in Cundinamarca, Colombia, by Sandra L. Castañeda and Carlos I. Suárez in January 2015. Plant identification was performed by Dr. Mauricio Diazgranados and Carlos I. Suarez from Jardín Botánico de Bogotá (JBB), and a voucher sample of each species was deposited at JBB's herbarium under the collection numbers SLC-192 and SLC-207, respectively. Plant collections were made with JBB collection permits.

Leaves extraction and UHPLC–UV-HRMS analysis

From each species, 20 mg of dry leaves were powdered with liquid nitrogen and extracted with 2 ml of EtOH:H_2O (7:3, v/v, HPLC grade) in an ultrasonic bath for 15 min at 25 °C. Plant extracts were centrifuged at 19,975 × *g* for 10 min and partitioned with 0.5 ml of hexane. The aqueous layer was filtered through a 0.2 μm PTFE membrane and analyzed by UHPLC-UV-HRMS in an Accela UHPLC apparatus (Thermo Scientific, Carlsbad, CA, USA) coupled to an 80-Hz photodiode array detector (PDA) (Thermo Scientific) and an Exactive™ Plus Orbitrap mass spectrometer (MS) (Thermo Scientific).

A heated electrospray probe was used as an ionization source in MS analyses along with the following parameters: positive and negative ionization modes (full scan method) over a mass range of 150–2000 Da, resolution of 70,000 full width at half maximum (FWHM) (*m*/*z* 200), mass accuracy <3 ppm, scan time of about 2.5 scans/s, spray voltages of +3.6 kV and −3.2 kV for the positive and negative ionization modes, respectively, capillary and heater temperature of 300 °C, sheath gas of 30 arbitrary units, and S-lens of 55 arbitrary units.

Subsequently, 10 μl of each plant extract were injected and chromatographed in a C18 column (Hypersil Gold, 1.9 μm, 50 mm × 2.1 mm, Thermo Scientific) connected to a C18 Security Guard™ Ultra cartridge (Phenomenex, Torrance, CA, USA). The mobile phase involved purified water with 0.1% formic acid and acetonitrile, and separation was performed at a flow rate of 300 μl/min. The gradient program started with 5% acetonitrile for the first 3 min and increased the amount linearly to 100% in 30 min. Acetonitrile remained constant for 5 min before ultimately decreasing to 5% after 40 min. The column temperature was maintained at 30 °C, the tray temperature was fixed to 10 °C, and the PDA detector was set to record in the range of 200–600 nm.

Dereplication of leaf extracts

Extracts dereplication was performed based on spectral (UV and accurate mass) and retention time (Rt) comparisons with reference compounds previously isolated in our laboratory—namely, 3-*O*-(*E*)-caffeoylquinic acid, protocatechuic acid, 3,5-di-*O*-(*E*)-caffeoylquinic acid, 4,5-di-*O*-(*E*)-caffeoylquinic acid, quercetin, 3-methoxy quercetin, and *ent*-kaurenoic acid. Compounds whose reference standard was unavailable were tentatively identified by accurate mass and UV spectra comparisons with data from the literature, including the Dictionary of Natural Products (DNP) and the Asteraceae Database (AsterDB), the latter of which corresponds to an in-house database (www.asterbiochem.org) containing hundreds of chemical compounds previously reported in the Asteraceae family, including of the subtribe Espeletiinae. In-source fragmentation in the UHPLC–UV-HRMS (Orbitrap) (MS/MS) was additionally considered to propose some of the peak assignments.

Essential oil extraction

The resins of *E. grandiflora* (2.1 g) and *E. killipii* (16.8 g), both collected during anthesis, were hydrodistilled using a Clevenger-type apparatus for 3 h and stored at −20 °C for further analysis. Before gas chromatography analysis EOs were dissolved (1%, v/v) in ethyl acetate.

Essential oil analyses

EO from *E. grandiflora* and *E. killipii* resins were analyzed using a Hewlett–Packard 6890N Plus GC–FID (USA) apparatus where the percentage composition of individual components was determined. Identification of compounds was performed by comparison of the retention indices of each component relative to a series of *n*-alkanes, and by mass spectra comparisons with NIST 08 and Willey 7 databases based on GC–MS analyses on a Shimadzu QP-2010 system (Tokyo, Japan). To confirm identifications, the mass spectrum and retention index of each compound was further compared with published data (Adams, 2007).

During GC-FID analyses, 1 μl of each sample was injected (in triplicate) using a split ratio of 1:20 and separated on a HP-5 fused silica column (30 m; 0.32 mm I.D.; 0.25 μm film thickness). The carrier gas was hydrogen at 1.3 ml/min, the oven temperature was programmed from 60 to 240 °C at 3 °C/min, and the injector temperature was 240 °C. Retention indices were calculated relative to C_8–C_{40} *n*-alkanes. For GC–MS analyses, an EN5MS column was employed (30 m; 0.25 mm I.D.; 0.25 μm film thickness) with a source temperature of 250 °C, carrier gas adjusted to 41.6 cm/s, an ionization energy of 70 eV, and a scan range of 40–500 Da. The temperature program and remaining parameters were identical to those in GC–FID analyses.

Chemometric analyses

The relative amount of the EO components of 31 species from the subtribe Espeletiinae reported in the literature and in the present study (Table 1) were used to perform multivariate statistical methods in the software R 3.0.3 (R Foundation for Statistical Computing, Vienna, Austria) and SIMCA P 13.0.3.0 (Umetrics AB, Malmö, Sweden). Prior to analyses, the data matrix was scaled by using the arcsine method, which is appropriate and commonly used for data expressed as percentages. Hierarchical clustering analysis with bootstrap resampling (HCAbp) was performed in the R package pvclust (Suzuki and Shimodaira, 2006), using Ward's method as the clustering algorithm and Euclidian distance. To generate a quantitative measure of accuracy in the clusters obtained, approximately unbiased (AU) *p* values were considered in the analysis, calculated by multiscale bootstrap, which is more accurate than traditional bootstrapping, as Suzuki and Shimodaira (2006) have confirmed. In multiscale bootstrapping, since the size of the bootstrap sample varies according to the original matrix, it corrects the bias of the

Table 1
Species from subtribe Espeletiinae used to perform chemometric analyses based on essential oil compositions; species organized according to the three clusters identified by hierarchical clustering analysis with bootstrap resampling.

Cluster	Species	Locality of collection (Country)	Altitude m.a.s.l.	Date of collection	Plant stage	Plant part	Reference
1	*Co. moritziana 1*	La Culata (VEN)	3750	9/2/1998	Anthesis	Leaves	Aparicio et al. (2002)
1	*Co. moritziana 2*	Piñago (VEN)	3800	12/9/1999	Anthesis	Leaves	Aparicio et al. (2002)
1	*Co. moritziana 3*	Los Osos (VEN)	3700	5/25/2004	Anthesis	Leaves	Ibáñez and Usubillaga (2006a)
1	*Co. moritziana 4*	Pico del Águila (VEN)	4100	9/22/2002	Anthesis	Leaves	Ibáñez and Usubillaga (2006a)
1	*Co. spicata 1*	La Culata (VEN)	3500	11/22/1998	Anthesis	Leaves	Aparicio et al. (2002)
1	*Co. spicata 2*	Las Cruces (VEN)	3850	12/9/1999	Anthesis	Leaves	Aparicio et al. (2002)
1	*Co. timotensis*	Pico del Águila (VEN)	4000	n.r.	Anthesis	Aerial parts	Rojas et al. (1999)
1	*E. nana*	Paramo de Ortiz (VEN)	3085	5/1/2010	n.r.	Leaves	Peña et al. (2012)
1	*E. batata*	Piedras Blancas (VEN)	4200	11/1/1998	n.r.	Leaves	Usubillaga et al. (2001b)
1	*E. grandiflora*	Paramo de Cruz verde (COL)	3328	1/22/2015	Anthesis	Resin	Present study
1	*E. killipii*	Paramo de Sumapaz (COL)	3717	1/26/2015	Anthesis	Resin	Present study
1	*L. occultus* ssp *humbertii*	Laguna Negra (VEN)	3200	12/2/1999	Anthesis	Leaves	Usubillaga et al. (2001a)
2	*L. neriifolius*	El Batallón (VEN)	2800	11/6/1998	Anthesis	Leaves	Usubillaga et al. (2001a)
2	*L. occultus*	El Batallón (VEN)	2800	11/6/1998	Anthesis	Leaves	Usubillaga et al. (2001a)
2	*L. lucidus*	La Aguada (VEN)	3400	11/24/1999	Anthesis	Leaves	Usubillaga et al. (2001a)
2	*E. schultzii 1*	La Culata (VEN)	2800	9/12/2002	Anthesis	Leaves	Ibáñez and Usubillaga (2006b)
2	*E. schultzii 2*	Los Osos (VEN)	3700	9/22/2002	Anthesis	Leaves	Ibáñez and Usubillaga (2006b)
2	*E. schultzii 3*	Pico del Águila (VEN)	4100	10/22/2002	Anthesis	Leaves	Ibáñez and Usubillaga (2006b)
2	*E. weddellii*	Las Cruces (VEN)	4080	n.r.	Anthesis	Leaves	Khouri et al. (2000)
2	*E. semiglobulata*	Pico del Águila (VEN)	3800	n.r.	n.r.	Leaves	Usubillaga et al. (1999)
2	*E.* x *aurantia*	Pico del Águila (VEN)	n.r.	11/15/2003	Anthesis	Leaves	Ibáñez and Usubillaga (2008)
3	*R. marcescens 1*	El Batallón (VEN)	3000	6/22/1999	Anthesis	Leaves	Aparicio et al. (2001)
3	*R. marcescens 2*	El Batallón (VEN)	3000	1/27/2000	Anthesis	Leaves	Aparicio et al. (2001)
3	*R. atropurpurea 1*	El Batallón (VEN)	3400	11/6/1998	Anthesis	Leaves	Aparicio et al. (2001)
3	*R. atropurpurea 2*	El Batallón (VEN)	3400	6/22/1999	Anthesis	Leaves	Aparicio et al. (2001)
3	*R. lindenii*	San José (VEN)	3100	8/10/1999	Anthesis	Leaves	Aparicio et al. (2001)
3	*R. floccosa*	Pico del Águila (VEN)	3800	5/25/1999	Anthesis	Leaves	Aparicio et al. (2001)
3	*Ca. badilloi*	La Azulita (VEN)	1700	n.r.	n.r.	Leaves	Cordero et al. (2011)
3	*Ca. badilloi* var. *pittieri*	Bailadores (VEN)	1800	n.r.	n.r.	Leaves	Rojas et al. (2008)
3	*Co. thyrsiformis*	La negra (VEN)	3000	6/22/1999	Anthesis	Leaves	Aparicio et al. (2002)
3	*Es. angustifolia*	San José (VEN)	2870	6/1/2006	n.r.	Leaves	Meccia et al. (2007)

n.r., not reported. Genera abbreviations: Co., *Coespeletia*; E., *Espeletia*; L., *Libanothamnus*; R., *Ruilopezia*; C., *Carramboa*; Es., *Espeletiopsis*.
VEN, Venezuela; COL, Colombia.

traditional bootstrapping value caused by the constant sample size (Suzuki and Shimodaira, 2006).

To identify the compounds responsible for species clustering in the three primary clusters observed in the HCAbp, an orthogonal partial least-squares discriminant analysis (OPLS-DA) was performed with SIMCA P. This method separates the systematic variation in X into correlated (predictive) and uncorrelated (orthogonal) variables to Y (Eriksson et al., 2012). The variables responsible for the clustering pattern observed were identified according to a variable importance in the projection (VIP) value >1.0, a significant contribution to the loadings plot, and a high magnitude and high reliability confidence intervals in the coefficients plot.

Results and discussion

Leaves characterization by UHPLC–UV-HRMS

The metabolic fingerprinting of crude ethanol (70% v/v) extracts from *E. grandiflora* and *E. killipii* leaves by using UHPLC–UV-HRMS (Fig. 1) and concomitant dereplication allowed the identification of several similarities and differences between the

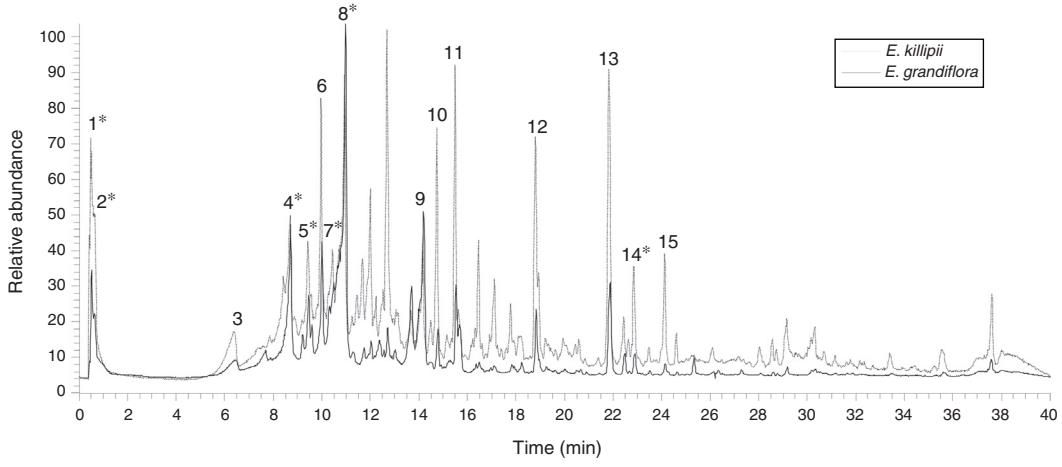

Fig. 1. Total ion current (TIC) chromatograms of *E. grandiflora* and *E. killipii* leaves extracts obtained in negative mode. (1) 3-*O*-(*E*)-caffeoylquinic acid, (2) protocatechuic acid, (3) di-caffeoylquinic acid isomer, (4) 3,5-di-*O*-(*E*)-caffeoylquinic acid, (5) 4,5-di-*O*-(*E*)-caffeoylquinic acid, (6) tri-caffeoylaltraric acid isomer, (7) quercetin, (8) 3-methoxy quercetin, (9) 8,8″-methylene-bisquercetin, (10) *ent*-12-oxo-kaura-9(11),16-dien-18-oic acid, (11) *ent*-12-hydroxy-kaura-9(11),16-dien-18-oic acid, (12) *ent*-15α-acetoxy-kaur-16-en-19-oic acid, (13) grandiflorenic acid, (14) *ent*-kaurenoic acid, (15) oleane triterpene. * identified by comparison with reference substances.

species. As observed in total ion current chromatograms (Fig. 1), both species have highly similar fingerprints with greater semi-quantitative instead of qualitative differences. All dereplicated compounds (Fig. 1) were detected in both species, though *E. killipii* showed a greater relative abundance of peaks 6 and 10–15 (Fig. 1). Four main chemical classes of secondary metabolites were identified in the leaves extracts of both species: *trans*-cinnamic acid derivatives, flavonoids, diterpenes, and one triterpene.

Among flavonoids, both species produce high quantities of 3-methoxy quercetin (peak 8, Fig. 1). This flavonoid, identified by its Rt, UV, and accurate mass comparison with a reference compound, has been previously reported only in the leaves of *E. killipii* (Torrenegra et al., 1994) and *E. barclayana* (Gutierrez et al., 1998). Its presence in *E. grandiflora* therefore constitutes a new report for this species. Quercetin (peak 7, Fig. 1), also identified in both species by its comparison with a reference substance, constitutes a new report for *E. grandiflora* as well. Peak 9 (Fig. 1) was tentatively proposed to be 8,8″-methylene-bisquercetin (flavonoid). This compound showed a deprotonated molecule at 615.07815 *m/z* and a protonated one at 617.09174 *m/z* for $C_{31}H_{20}O_{14}$. The mass spectrum in the negative mode also showed an intense peak at 299.01935 *m/z*, originated by in-source fragmentation of a quercetin unit and a methyl group in accordance with the literature (Martucci et al., 2014). Although this compound has not been previously reported in the subtribe Espeletiinae, Martucci et al. (2014) reported it in the genus *Vernonia* (Asteraceae).

Six *trans*-cinnamic acid derivatives were identified (Fig. 1). Among them, 3-*O*-(*E*)-caffeoylquinic acid (peak 1, Fig. 1), protocatechuic acid (peak 2, Fig. 1), 3,5-di-*O*-(*E*)-caffeoylquinic acid (peak 4, Fig. 1), and 4,5-di-*O*-(*E*)-caffeoylquinic acid (peak 5, Fig. 1) were identified based on spectral (UV and accurate mass) and Rt comparisons with reference substances. Peak 3 (Fig. 1) was tentatively proposed to be another dicaffeoylquinic acid isomer. This compound showed a deprotonated molecule at 515.11884 *m/z* and a protonated one at 517.13385 *m/z* for $C_{25}H_{24}O_{12}$. Its mass spectrum in the positive mode showed in-source fragmentation with two main peaks: one at 499.12311 *m/z*, which corresponds with the loss of a water molecule, and another at 163.03882 *m/z*, which corresponds with an ionized residue of caffeic acid (Santos et al., 2008). The UV spectrum of this peak exhibited two absorptions at approximately 300 and 325 nm, which suggest chlorogenic acid derivatives.

By contrast, peak 6 (Fig. 1) was proposed to be a possible tricaffeoylaltraric acid isomer based on accurate mass comparisons with compounds reported in AsterDB and maximum UV absorptions. This compound displayed a deprotonated molecule at 695.12531 *m/z* for $C_{33}H_{28}O_{17}$ and two UV maxima at approximately 300 and 329 nm. The presence of the previously reported *trans*-cinnamic acid derivatives in *E. grandiflora* and *E. killipii* constitutes the first report for this chemical class in the subtribe Espeletiinae. However, their presence is unsurprising, for they constitute a highly common class of secondary metabolites widespread in Asteraceae (Chagas-Paula et al., 2011).

In the leaves of *E. grandiflora* and *E. killipii*, five *ent*-kaurane diterpenes were also identified, among which *ent*-kaurenoic acid (peak 14, Fig. 1) was unambiguously identified by Rt and accurate mass comparison with a reference substance. The identities of the other four (peaks 10–13, Fig. 1) were tentatively proposed based only on accurate mass and chemotaxonomic information. All of these diterpenes have been previously reported in Espeletiinae (Bohlmann et al., 1980; Usubillaga et al., 2003).

Lastly, the identity of an oleane-type triterpene (peak 15, Fig. 1) was tentatively proposed based on accurate mass and database search. This compound showed a deprotonated molecule at 655.42114 *m/z* and a protonated one at 657.43524 *m/z* for $C_{39}H_{60}O_8$. An accurate mass search in DNP afforded three possible compounds: brevenal (CAS: 776331-34-1), olean-12-ene-3,16,21,22,28-pentol, 16,28-diacetate 22-(2-methyl-2-butenoate) (CAS: 124641-09-4), and olean-12-ene-3,16,21,22,28-pentol, 22,28-diacetate 21-[2(or 3)-methyl-2-butenoate] (CAS: 55949-26-3). Among them, only the two oleane-type triterpenes have previously been isolated from species belonging to plant families (Apiaceae and Polemoniaceae), whereas brevenal has been reported only in the marine dinoflagellate *Karenia brevis* (DNP). Further analyses are therefore necessary to unambiguously identify this compound in Espeletiinae.

Resin characterization by GC–FID and GC–MS

The hydrodistillation of *E. grandiflora* and *E. killipii* resins provided yellowish EOs with yields of 14.29% and 14.88% (v/w), respectively, based on their fresh weight. In both species, the identified substances constitute 95–97% of the total oil composition (Table 2).

A comparison of the EO composition of *E. grandiflora* and *E. killipii* resins revealed no significant differences; both species were characterized by a predominance of monoterpene hydrocarbons with α-pinene as the major component (Table 2), which represented more than 61% of the total oil composition (Table 2). However, important differences were observed. For example, β-pinene represented the second major compound (3.4%) in *E. grandiflora* resin EO, whereas sabinene constituted the second major peak in *E. killipii* (6.5%), as Table 2 shows. *E. grandiflora* possesses a greater proportion of oxygenated monoterpenes (18.1%) than *E. killipii* (12.5%), but a lesser proportion of sesquiterpene hydrocarbons (Table 2). Remarkably, only one sesquiterpene hydrocarbon was detected in *E. grandiflora* resin EO—namely, δ-cadinene—whereas several others, including α-copaene, β-bourbonene, δ-selinene, and γ-muurolene, were detected in *E. killipii* resin EO (Table 2). In general, *E. killipii* presents a more complex EO composition than *E. grandiflora*, since more compounds and different chemical classes were detected in this species (Table 2).

Monoterpene hydrocarbons constitute the main EO components from all species of Espeletiinae investigated thus far (Table 1). Among them, α-pinene usually constitutes the chief EO component in numerous species (Aparicio et al., 2002; Ibáñez and Usubillaga, 2006a, 2008; Meccia et al., 2007; Peña et al., 2012). However, some species produce other compounds such as limonene and α-phellandrene as the major peaks (Aparicio et al., 2001; Ibáñez and Usubillaga, 2006b). Based on this observation of different terpenoids predominance in EOs from Espeletiinae, chemometric analyses using GC data has been performed to identify trends in the EO composition of 31 species from Espeletiinae, as discussed below.

Chemometric analyses of essential oils

Percentage EO compositions of 31 species from Espeletiinae were used as input data to perform chemometric analyses (HCAbp and OPLS-DA). Table 1 shows the species names and their locality, altitude and date of collection, as well as the plant part and reproductive stage. In this study, we considered only species collected in the anthesis period, including a few cases in which that information was not specified (Table 1).

Results from HCAbp (Fig. 2) demonstrated trends in EO composition of species from Espeletiinae, and three primary clusters were determined based on chemical similarities and high support values (≥83%). As Fig. 2 also shows, intraspecies variation displayed by some species collected in the same or

Table 2
Percentage of essential oil components identified in *E. grandiflora* and *E. killipii* resins.

#	Compound name	RI	RI-lit	*E. grandiflora*	*E. killipii*
1	Santolina triene	908	906	0.3	0.9
2	α-Thujene	927	924	–	0.3
3	α-Pinene	937	932	69.7	61.8
4	Camphene	950	946	0.4	0.3
5	Thuja-2,4(10)-diene	955	953	1.2	0.5
6	Sabinene	975	969	0.6	**6.5**
7	β-Pinene	979	974	3.4	4.0
8	*o*-Cymene	1026	1022	2.1	0.6
9	Limonene	1029	1024	1.0	1.4
10	1,8-Cineole	1033	1026	0.8	2.1
11	γ-Terpinene	1058	1054	t	0.2
12	Fencholenic aldehyde[a]	1092	–	0.6	t
13	Linalool	1101	1095	t	0.1
14	α-Campholenal	1128	1122	2.8	1.2
15	*trans*-Pinocarveol	1141	1135	2.3	1.2
16	*cis*-Verbenol	1143	1137	0.8	0.3
17	*trans*-Verbenol	1147	1140	2.9	1.1
18	*trans*-Pinocamphone	1162	1158	0.2	t
19	Pinocarvone	1164	1160	0.6	0.2
20	*p*-Mentha-1,5-dien-8-ol	1169	1166	2.9	2.3
21	Terpinen-4-ol	1178	1174	t	0.8
22	*p*-Cymen-8-ol	1186	1179	–	0.2
23	α-Terpineol	1187	1186	t	0.4
24	Myrtenal	1188	1193	0.2	t
25	Myrtenol	1199	1194	1.6	1.0
26	Verbenone	1212	1204	1.4	1.2
27	*trans*-Carveol	1221	1215	0.8	0.2
28	Carvone	1246	1239	0.1	t
29	Linalool acetate	1253	1254	t	0.2
30	Lavandulyl acetate	1293	1288	0.1	t
31	α-Copaene	1376	1374	t	1.7
32	β-Bourbonene	1385	1387	t	0.7
33	γ-Muurolene	1477	1478	t	1.0
34	δ-selinene	1491	1492	t	0.2
35	α-Muurolene	1500	1500	–	0.3
36	δ-Cadinene	1524	1522	0.2	1.5
37	Spathulenol	1577	1577	t	0.3
38	Copaborneol	1604	1613	–	0.9
39	kaurane diterpene[b]	1981		t	0.3
40	kaurane diterpene[b]	1988		t	0.7
	Monoterpene hydrocarbons			78.7	76.5
	Oxygenated monoterpenes			18.1	12.5
	Sesquiterpene hydrocarbons			0.2	5.4
	Oxygenated sesquiterpenes			–	1.2
	Total identified			97.0	95.6

– not detected; t, traces.

[a] Identification based only on mass spectra comparison with Willey 7 database.

[b] Tentative identification based on fragmentation pattern comparison with Adams (2007) and Usubillaga and Nakano (1979); The two most abundant compounds in *E. grandiflora* and *E. killipii* are emphasized in bold.

different localities (*e.g.*, *Coespeletia moritziana* and *Espeletia schultzii*) was not significant enough to promote species grouping in a different cluster, which provides support for the proposed groups.

Notably, the three clusters identified should not be considered to be different chemotypes, since we analyzed different species, and by definition, a chemotype corresponds to "organisms categorized under same species, subspecies or varieties having differences in quantity and quality of their components in their whole chemical fingerprint that is related to genetic or genetic expression differences" (Polatoglu, 2013).

Supervised OPLS-DA displayed a good separation ($R^2Ycum = 0.985$; $Q^2cum = 0.789$), in which 22% of the variation in X relates to the separation between the three clusters (Fig. 3) and afforded the identification of discriminating substances. Cluster 1 was characterized by high α-pinene, β-pinene, and β-phellandrene contents, cluster 2 by high α-phellandrene, α-thujene, *p*-cymene, and sabinene contents, and cluster 3 mainly by high germacrene D, limonene, and β-caryophyllene contents (Fig. 3).

Additional OPLS-DA analyses considering species locality or elevation range as Y variables (Table 1) revealed no correlation with EO compositions (data not shown, but are evident from Table 1). This result suggests that intraspecies variation due to geography or elevation, as reported by Aparicio et al. (2002) and Ibáñez and Usubillaga (2006b), is less than interspecies variation in Espeletiinae. Therefore, when considering several species from different genera in a single analysis, even species collected at different localities cluster according to their taxonomic relatedness rather than their geographic origin (Fig. 2). As shown in Table 1, a certain correlation between species EOs and their generic classifications was evident. For example, cluster 1 is composed primarily of species belonging to the genus *Coespeletia* and a few species of *Espeletia*, whereas cluster 2 presents species mostly of *Espeletia* and a few of *Libanothamnus*. By contrast, cluster 3 is primarily composed of species of *Ruilopezia* and a few of *Carramboa* (Table 1). Nevertheless, the clustering of Espeletiinae species based on their EO components does not follow strict generic boundaries (based on morphology), since no evident correlation was observed when considering the six genera as Y variable in OPLS-DA (data not shown).

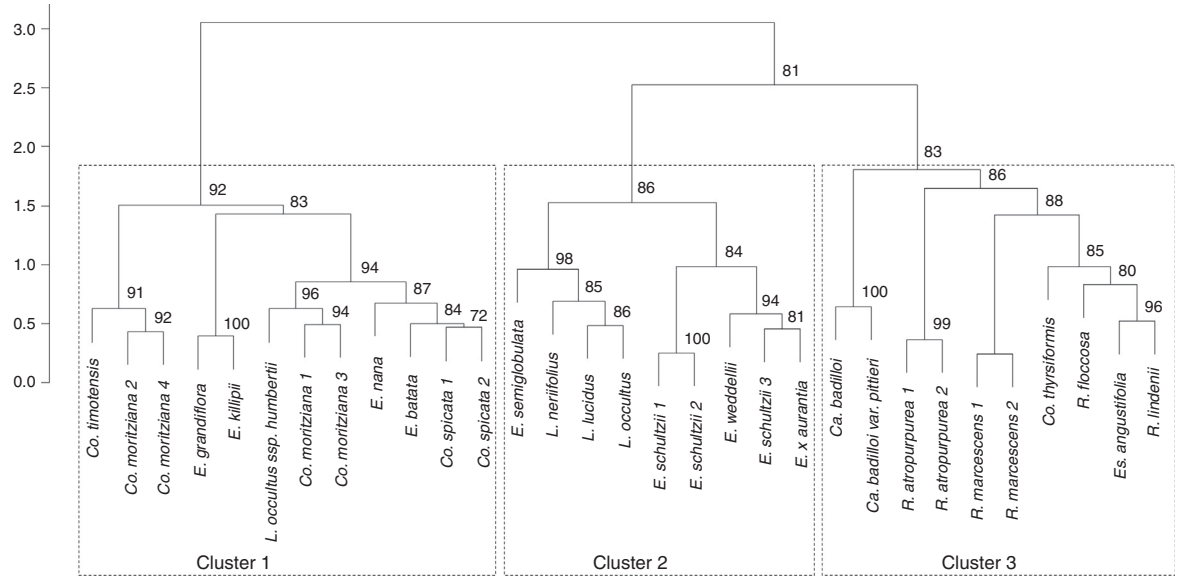

Fig. 2. Hierarchical clustering analysis with bootstrap resampling of 31 species of the subtribe Espeletiinae based on percentage of essential oil compositions; dotted boxes correspond to the three primary clusters.

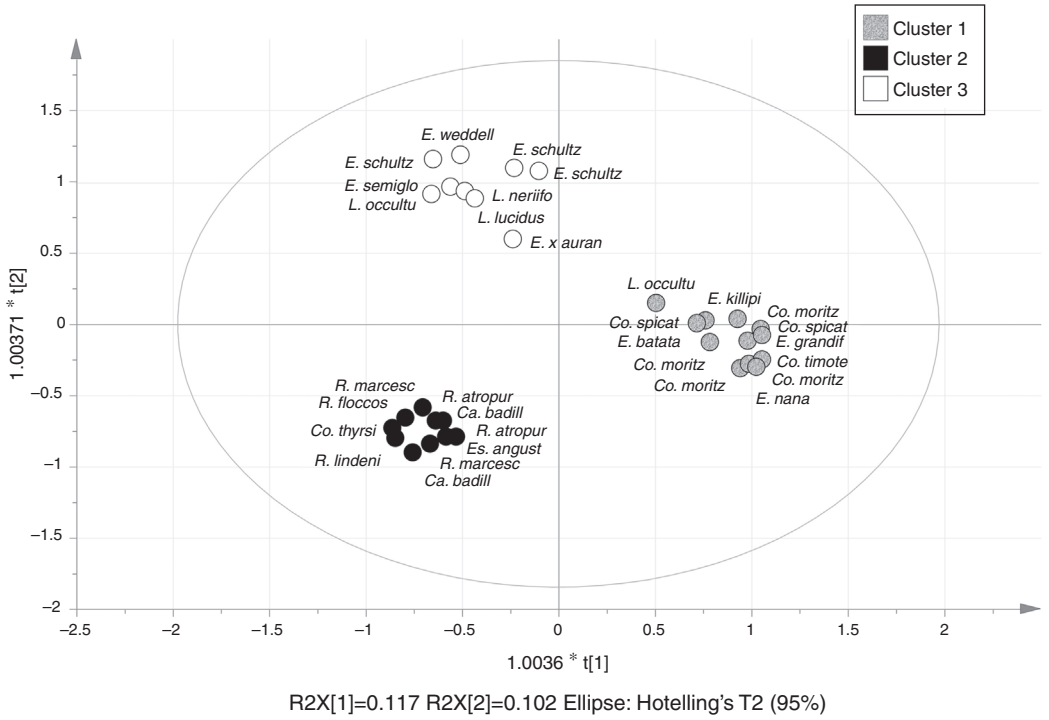

Fig. 3. Orthogonal partial least-squares discriminant analysis score plot of 31 species of the subtribe Espeletiinae based on percentage of essential oil compositions; Clusters 1–3, determined by hierarchical clustering analysis with bootstrap resampling, as the Y variable. Discriminating variables of each cluster in order of relevance as follows, Cluster 1: α-pinene, β-pinene, β-phellandrene; cluster 2: α-phellandrene α-thujene, *p*-cymene, and sabinene; cluster 3: germacrene D limonene, β-caryophyllene and *ent*-kaur-16-en-al.

Conclusions

This research represents the first comprehensive study to report the chemical composition of a *Espeletia* species by modern and complementary analytical techniques, followed by proper chemometric analyses. The similarities observed between the chemical fingerprints of *E. grandiflora* and *E. killipii* agree with their morphological and phylogenetic relatedness. However, important qualitative and quantitative differences were identified in the leaves and resins of both species, which suggest that chemical markers can be potentially used to assist in their taxonomy. Among the fifteen dereplicated compounds, ten correspond to new reports for either or both species, whereas eight correspond to new reports for the genus *Espeletia* and subtribe Espeletiinae, indicating that the secondary metabolite chemistry of this group of plants remains largely unexplored. This study moreover afforded the identification of different trends in the accumulation of EOs from 31 species of Espeletiinae, which seem to correlate with their taxonomy and thus offer a basis for future studies.

Authors contributions

GFPG (Ph.D. student) contributed in collecting plant samples and identification, running the laboratory experiments, analysis of the data and drafted the paper. JAAM contributed to laboratory work and critical reading of the manuscript and FBDC designed the experiments, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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References

Adams, R., 2007. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, 4th ed. Allured Business Media.

Aparicio, R., Romero, M., Khouri, N., Rojas, L.B., Usubillaga, A., 2002. Volatile constituents from the leaves of three *Coespeletia* species from the Venezuelan Andes. J. Essent. Oil Res. 14, 37–39.

Aparicio, R., Romero, M., Rojas, L.B., Khouri, N., Usubillaga, A., 2001. Composition of the essential oil of four species of *Ruilopezia* from the Venezuelan Andes. Flavour Fragr. J. 16, 172–174.

Bohlmann, F., Suding, H., Cuatrecasas, J., Robinson, H., King, R.M., 1980. Tricyclic sesquiterpenes and further diterpenes from *Espeletiopsis* species. Phytochemistry 19, 2399–2403.

Chagas-Paula, D.A., De Oliveira, R.B., da Silva, V.C., Gobbo-Neto, L., Gasparoto, T.H., Campanelli, A.P., Faccioli, L.H., Da Costa, F.B., 2011. Chlorogenic acids from *Tithonia diversifolia* demonstrate better anti-inflammatory effect than indomethacin and its sesquiterpene lactones. J. Ethnopharmacol. 136, 355–362.

Cordero, Y.R., Rojas, L.B., Usubillaga, A., 2011. Chemical composition of the essential oil from *Carramboa littlei* (Asteraceae). Nat. Prod. Commun. 6, 127–128.

Cuatrecasas, J., 2013. A Systematic Study of the Subtribe Espeletiinae: Heliantheae, Asteraceae. The New York Botanical Garden Press, New York, USA.

Diazgranados, M., 2012a. A nomenclator for the frailejones (Espeletiinae Cuatrec., Asteraceae). PhytoKeys 16, 1–52.

Eriksson, L., Rosén, J., Johansson, E., Trygg, J., 2012. Orthogonal PLS (OPLS) modeling for improved analysis and interpretation in drug design. Mol. Inform. 31, 414–419.

Fagua, J.C., Gonzalez, V.H., 2007. Growth rates, reproductive phenology, and pollination ecology of *Espeletia grandiflora* (Asteraceae), a giant Andean caulescent rosette. Plant Biol. 9, 127–135.

Gutierrez, S.R., Fuentes, O., Tellez, A.N., Torrenegra, R., 1998. Active antibacterial principle from *Espeletia barclayana*. Rev. Latinoam. Química 26, 71–74.

Ibáñez, J., Usubillaga, A., 2006a. Analysis of the essential oil of two different altitudinal populations of *Coespeletia moritziana* (Sch Bip.ex Wedd) Cuatrec. Flavour Fragr. J. 21, 760–763.

Ibáñez, J., Usubillaga, A., 2006b. The essential oil of *Espeletia schultzii* of different altitudinal populations. Flavour Fragr. J. 21, 286–289.

Ibáñez, J., Usubillaga, A., 2008. Estudio de la composición del aceite esencial de un frailejón híbrido entre *Espeletia schultzii* y *Coespeletia moritziana* (Espeletiinae). Rev. Fac. Farm. Univ. Los Andes 50, 16–19.

Khouri, N., Usubillaga, A., Rojas, L.B., Galarraga, F., 2000. The essential oil of *Espeletia weddellii* Sch Bip. ex Wedd. Flavour Fragr. J. 15, 263–265.

Martucci, M.E.P., De Vos, R.C.H., Carollo, C.A., Gobbo-Neto, L., 2014. Metabolomics as a potential chemotaxonomical tool: application in the genus *Vernonia* Schreb. PLoS ONE 9, e93149.

Meccia, G., Rojas, L.B., Velasco, J., Diaz, T., Usubillaga, A., 2007. Composition and antibacterial screening of the essential oils of leaves and roots of *Espeletiopsis angustifolia* Cuatrec. Nat. Prod. Commun. 2, 1221–1224.

Peña, A., Rojas, L., Aparicio, R., Alarcon, L., Baptista, J.G., Velasco, J., Carmona, J., Usubillaga, A., 2012. Chemical composition and antibacterial activity of the essential oil of *Espeletia nana*. Nat. Prod. Commun. 7, 661–662.

Piozzi, F., Passanna, S., Paternos, M.P., Sprio, V., 1971. Kauranoid diterpenes in *Espeletia grandiflora*. Phytochemistry 10, 1164–1166.

Piozzi, F., Passannanti, S., Marino, M.L., Sprio, V., 1972. Structure of grandiflorenic acid. Can. J. Chem. 50, 109–112.

Piozzi, F., Sprio, V., Passannanti, S., Mondelli, R., 1968. Structure of grandiflorolic acid. Gazz. Chim. Ital. 98, 907–910.

Polatoglu, K., 2013. Chemotypes – a fact that should not be ignored in natural product studies. Nat. Prod. J. 3, 10–14.

Rojas, L.B., Gutierrez, R., de Rojas, Y.C., Usubillaga, A., 2008. Chemical composition of the essential oil from *Carramboa pittieri* (Cuatrec.) Cuatrec. (Asteraceae). Nat. Prod. Commun. 3, 1739–1740.

Rojas, L.B., Usubillaga, A., Galarraga, F., 1999. Essential oil of *Coespeletia timotensis* Cuatrec. Phytochemistry 52, 1483–1484.

Santos, M.D., Lopes, N.P., Iamamoto, Y., 2008. HPLC–ESI–MS/MS analysis of oxidized di-caffeoylquinic acids generated by metalloporphyrin-catalyzed reactions. Quim. Nova 31, 767–770.

Suzuki, R., Shimodaira, H., 2006. Pvclust: an R package for assessing the uncertainty in hierarchical clustering. Bioinformatics 22, 1540–1542.

Torrenegra, R.D., Tellez, A.N., 1995. Chemotaxonomic value of melampolides in *Espeletia* species (Asteraceae). Biochem. Syst. Ecol. 23, 449–450.

Torrenegra, R.D., Tellez, A.N., 1996. Phytochemistry of *Espeletia killipii* Cuatr. and gibberellic activity of some of the isolated compounds. Rev. Latinoam. Química 24, 2–6.

Torrenegra, R.D., Tellez, A.N., Garcia, G., 1994. Chemical investigation of the species of the genus *Espeletia*. Part I. Chemistry of *Espeletia kilipii–Espeletia tunjana*. Rev. Colomb. Química 23, 29–35.

Usubillaga, A., Aparicio, R., Romero, M., Rojas, L.B., Khouri, N., 2001a. Volatile constituents from the leaves of four *Libanothamus* species from the Venezuelan Andes. Flavour Fragr. J. 16, 209–211.

Usubillaga, A., Khouri, N., Rojas, L.B., Morillo, M., 2001b. Essential oil of the leaves from *Espeletia batata* Cuatrec. J. Essent. Oil Res. 13, 450–451.

Usubillaga, A., Khouri, N., Visbal, T., 1999. Volatile constituents from the leaves of *Espeletia semiglobulata* Cuatrec. J. Essent. Oil Res. 11, 757–758.

Usubillaga, A., Nakano, T., 1979. Kauranoid diterpenes in *Ruilopezia margarita*. Planta Med. 35, 331–338.

Usubillaga, A., Romero, M., Aparicio, R., 2003. Kaurenic acids in Espeletiinae. Acta Hortic. 597, 129–130.