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Artículo científico

A Phytochemical Analysis of *Espeletia nana* Cuatrec. a Midget Espeletiinae from Paramo Ortiz, Venezuela

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Resumen

La *Espeletia nana* Cuatrec. es una planta resinosa perteneciente a la Subtribu Espeletiinae. Es una especie de frailejón de pequeño tamaño que alcanza unos 25cm de altura y se encuentra en el Páramo de Ortiz. Estado Trujillo, Venezuela. Las hojas y las raíces fueron extraídas por separado con una mezcla de hexano- éter dietílico (3:1). Alícuotas de las dos fracciones ácidas fueron metiladas y analizadas mediante cromatografía de gases-masas y se determinó que las hojas contenían un 34,6% de ácido kaurénico (**1a**), 40,1% de ácido grandiflorénico (**2a**), 8% de ácido *ent*-15α-acetoxi-kaur-16-eno-19-oico (**3a**) y 13% de ácido *ent*-15α-hidroxi-kaur-16-eno-19-oico (**4a**). La fracción ácida proveniente de las raíces contenía 38% **1a**, 39,6% de **2b**, 8,5% de **3a** y 13,9% de **4a**. El análisis cromatográfico de la fracción neutra de las hojas permitió establecer que contenían 43% de kaurenal (**5**), 3% de kaurenol (**6**), 13% de ruilopeziol (**7a**), 7% de *epi*-ruilopeziol (**7b**), 25% de nonacontano y 8% de entriacontano. En cambio, en la resina de las raíces el kaurenal (**5**) constituye el 88%, hay pequeñas cantidades de kaurenol (7%), ruilopeziol (2,5%), epi-ruilopeziol (1,0%), y solamente 1,5% de ceras. La purificación mediante cromatografía flash de los extractos obtenidos permitió aislar e identificar todos los kaurenos mediante comparación con muestras auténticas.

Palabras clave: *Espeletia nana*, ácido grandiflorénico; ácido kaurénico; kaurenal; kaurenol; *epi*-ruilopeziol

Abstract

Espeletia nana Cuatrec is a resinous plant, member of the Espeletiinae Subtribe. It is a small size frailejón, 25cm high, found at Páramo Ortiz, Trujillo State, Venezuela. Leaves and roots were separately extracted with a 3:1 mixture of hexane-diethyl ether. Aliquotes of the acidic fractions were methylated and inspected by GC-MS. It was found that the resin from the leaves contained kaurenic acid (**1a**, 34.6%), grandiflorenic acid (**2a**, 40.1%), 15α- *ent*-acetoxy-kaur-16-en-19-oic acid (**3a**, 8%), and 15α-hidroxy- *ent*-kaur-16-en-19-oic acid (**3a**, 13%). The roots acid fraction contained 38% **1a**, 39,6% **2b**, 8.5% **3a**, and 13.9% **4a**. The GC-MS analysis of the leaves neutral fraction yielded 43% kaurenal (**5**), 3% kaurenol (**6**), 13% ruilopeziol (**7a**), 7% *epi*-ruilopeziol, 25% of nonacontane and 8% of entriacontane. On the other hand the roots resin contained 88%, 5.7% of **6**, 2.5% **7a**, 1.0% 7b, but only 1.5% of waxes. The bulk extracts were submitted to flash chromatography, leading to the isolation of pure kaurenes which were identified by direct comparison with authentic samples.

Keywords: *Espeletia nana*, grandiflorenic acid, kaurenic acid, kaurenal, kaurenol, *epi*-ruilopeziol

Introduction

Espeletia nana is a resinous plant that grows above 2900 m of altitude at Paramo Ortiz, Trujillo State (9° 14´ 3.8"N, 70° 24´ 22.7¨W) which is located NE of the city of Boconó. This plant, is a resinous herb about 25cm high with narrow leaves 9cm long) covered with a light green woolly indumentum. Its flowering stems end with a yellow capitulum about 2cm in diameter. It is one of eighty nine species of this genus described as part of the Subtribe Espelletiinae, popularly known as frailejon, and one of seventeen species that have been found in the Venezuelan Andes[1]. *Espeletia. nana* is an acaulescent rosette, that shares with other dwarf members of the genus like *Espeletia batata E. tenore*, and *E. weddellii* a tubercule

root with ellipsoidal shape 4-6cm in diameter[2,3]. The constituents of *E. nana* resin have not been previously reported.

Experimental

General methods

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. IR spectra were measured on a Perkin Elmer FT-1710 instrument, as KBr disks. NMR spectra were recorded with a Bruker Avance 400 MHz instrument for solutions in $CDCl_3$. GC-MS were performed on a Hewlett-Packard MSD 5973 instrument fitted with a 5% phenylmethyl polysiloxane fused-silica column (HP-5MS, 30 m, 0.25mm, film thickness 0.25µm). The initial analysis temperature was 250°C, which was increased at 5°C / min. to a final temperature of 300°C. Analytical thin-layer chromatography was performed on E. Merck aluminum-backed silica gel foils (F254). Flash chromatography was performed on silica gel E. Merck grade 60, 63-200 mesh, by gradient elution with hexane and hexane-EtOAc mixtures.

Plant Collection, extraction and kaurenes isolation

Leaves (650g) and roots (1.0kg) of *Espeletia nana* were collected at paramo Ortiz at 3085m of altitude on March 2010 (9° 14´ 39"N, 70° 24´22.7" W). The leaves were dried at 60°C during 48h and ground. The ground material (170g) was extracted at room temperature with a mixture of hexane/diethyl ether (3:1). Solvent evaporation yielded 11.4g of solids which were dissolved in hexane/EtOAc (2%) and shaken with 5% NaOH. The aqueous layer was acidified with diluted HCl and shaken with hexane to recover 4.5g of acid fraction which was treated with charcoal in boiling hexane/EtOAc, to get rid of some chlorophyll, yielding 4.4g of decolored mixture. A 10mg sample was methylated with diazomethane and analyzed by GC-MS- The TIC indicated that the mixture contained *ent*-kaur-16-en-19-oic acid methyl ester (**1b**, 34.6%), *ent*-kaur-9(11)16-dien-19-oic acid methyl ester (**2b**, 40.1%), 15α-acetoxy-*ent*-kaur-16-en-19-oic acid methyl ester (**3b**, 8%), and 15α-hydroxy-*ent*-kaur-16-en-19-oic acid methyl ester (**4b**, 13%). The bulk of the leaves´acidic fraction was then submitted to flash chromatography over silica gel. The column (A) was eluted with hexane and hexane/AcOEt mixtures and 100mL fractions were collected. Fractions 1-159 eluted with hexane yielded 2.02g of a mixture that contained **1a** and **2a**, which will be called for short kaurenic acid and grandiflorenic acid respectively.This mixture was separated on a new column which yielded 73mg of **1a**, mp 178-180°C, identical to an authentic sample of kaurenic acid (mp,^{1}H-NMR) obtained from *Espeletia semiglobulata*[5], and **2a** (55mg), mp 155-157°C, identical to an authentic sample of grandiflorenic acid (mp,^{1}H-NMR) isolated from *Coespeletia timotensis*[6]. Fractions 160-189 of column A, eluted with hexane/EtOAc 5%, rendered 20mg of a mixture of **1a**, **2a,** and **3a**. Elution was continued with hexane/EtOAc 10% yielding 95mg of pure **3a**, mp 172-174°C, identical to an authentic sample of 15α-acetoxy-*ent*-kaur-16-en-19-oic acid (mp, ^{1}H-NMR) isolated from *E. schultzii*[7]. Elution of column A was continued with hexane/EtOAc 20% yielding a mixture (678mg) of **3a**, **4a**, and another not identified compound. Finally elution with 50% EtOAc yielded a mixture (540mg) that contained **4a** and chlorophill, which was crystallized from C_6H_6,to yield 140mg of **4a** mp 224-226, identical to an authentic sample of 15α-hydroxy- *ent*-kaur-16-en-19-oic acid isolated from *Coespeletia timotensis*[6] (mp, ^{1}H-NMR). Figure 1 shows the molecular structure of the *ent*-kaurenes isolated from *E. nana* leaves and roots.

The roots (1.0kg) were cut in slices and dried at 60°C during 96h to yield 250g of dry and ground material. The ground roots were extracted with a mixture of hexane/diethyl ether (3:1) at room temperature. The hexane-ether extract was shaken with 5% NaOH. The aqueous layer was acidified with diluted HCl and shaken with hexane to recover 15.2g of acid fraction. A 10mg sample was methylated and analyzed by CG-MS which indicated that the roots acidic fraction contained 38.0% of **1b,** 39.6% of **2b**, 8.5% of **3b,** and 13.9% of **4b**. The bulk of the root acidic fraction was purified by flash chromatography over silica gel, 500mL fractions were taken and examined by TLC. Fractions 1-17 eluted with hexane yielded 3.97g of **1a** and **2a**. A 5mg sample was methylated and analyzed by GC-MS, the TIC indicated the presence of **1b** (retention time 3.83min, 45%, MS m/z 316) and **2b** (retention time 3.34min, 55%, MS m/z 314). Elution with hexane and 10% EtOAc rendered 1.2g of **1a** and **2a**. Fractions 17-26 eluted with 10% EtOAc yielded an additional amount of **1a** and **2a** (1.2g). Fractions 27-32 eluted with 10% and 20% EtOAc yielded 2.05g of **1a**, **2a**, and **3a**. Fractions 33-42 eluted with 50% EtOAc yielded 0.37g of **3a**. A 5mg sample was methylated and examined by GC-MS which indicated that if contained pure **3b**, retention time 5.78min, MW at m/z 374. Elution was continued with 50% EtOAc yielding 0.34g of a mixture of **3a** and **4a**. Finally fractions 44-98, eluted with 50% EtOAc and 100% EtOAc yielded 1.55g of **4a.** A 5mg sample was methylated to obtain **4b** which was inspected by GC-MS showing a single peak at 5.2min, MW at m/z 332.

After shaking the original hexane/diethyl ether extract with NaOH solution, the organic layer contained the neutral fraction. The roots neutral fraction was examined by GC-MS which showed that it contained mainly *ent*-kaur-16-en-19-al {**5**, 88% Retention Time (RT) 3,69min}, plus small amounts of *ent*-kaur-16-en-19-ol (**6**, 7%, RT 4,25min), ruilopeziol (**7b**, 2,5%, RT 3,33min), *epi*-ruilopeziol (**7a**, 1,0% RT 3,17min), and waxes (1.5%)

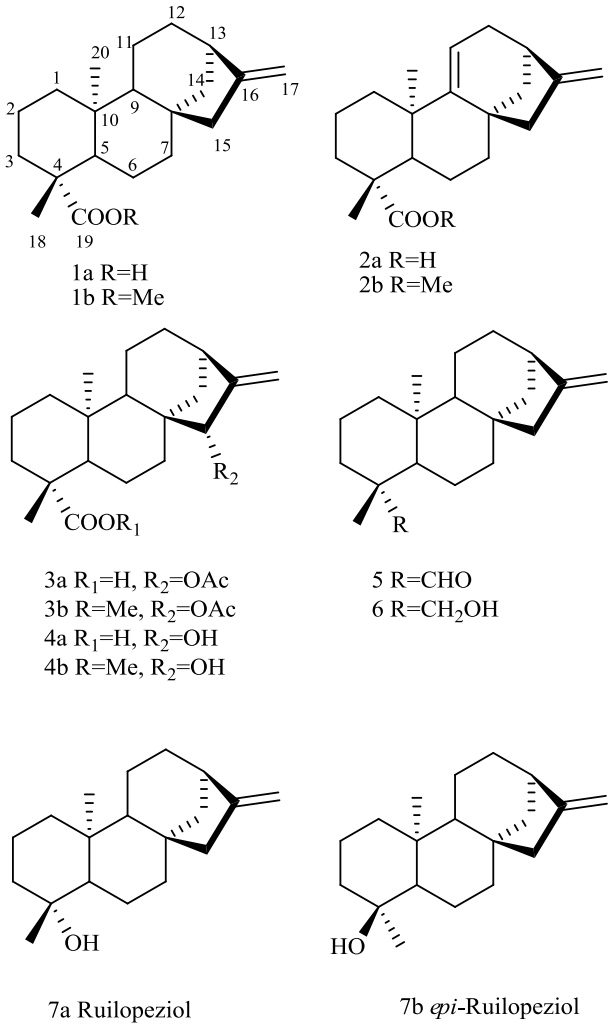

Fig. 1: Molecular structure of *ent*-kaurenes from aerial parts and roots of *Espeletia nana.*

A GC-MS analysis of the leaves neutral fraction showed that **5** was the main constituent (43%) while **6** (3%), **7b** (13%), **7a** (7%), nonacontane ($C_{29}H_{60}$, 25%), and entriacontane ($C_{31}H_{64}$, 8%) were also present. Since the neutral fraction from the roots (5g) contained a very small amount of chlorophyll it was concentrated to small volume, mixed with 5.0g of silicagel and submitted to flash chromatography This column was eluted with hexane and hexane/AcOEt mixtures; 180 fractions of 50mL were collected. Fractions 19-21 yielded 0.3g of a white solid, mp 113-116°C, which was identified as *ent*-kaur-16-en-19-al by direct comparison with an authentic sample isolated from *E. semiglobulata*[5] (mp, ^{1}H-NMR). GC-MS analysis of an aliquote of fractions 119-160 permited to identify the presence of *epi*-ruilopeziol (**7b**, MW 274g/mol, $C_{19}H_{30}O$), and *ent*-kaurenol (**6**, MW 288g/mol, $C_{20}H_{32}O$), in addition to traces of grandiflorenic acid (**2a**, MW 300g/mol). It was decided to methylate the mixture to separate through flash chromatography the methylated acid, which is less polar that **6** and **7b.** In this way it was possible to isolate 25mg of pure **6**, mp 140-141°C identical to an authentic sample of *ent*-kaur-16-en-19-ol isolated from *E. semiglobulata*[5] (mp, ^{1}H-NMR), and 30mg of a crystalline solid whose spectroscopic data correspond to **7b** previously reported by Bohlmann, *et al.*[4], in 1980 and named *epi*-ruilopeziol. Uni- and bi-dimentional NMR studies of *epi*-ruilopeziol were performed.

Table 2 presents proton and carbon 13 signal values of **7b**, as well as their COSY and HMBC correlations. Assignments of **7b** signals were determined by comparison with the spectra of kaurenol and kaurenic acid as well as through analysis of DEPT, HMQC, and HMBC experiments. The **7b** ^{1}H-NMR spectrum showed at δ 4.79 and 4.73, the signals corresponding to the geminals hydrogens at the exocyclic double bond (H-17a,b, 2H), most of the characteristic signal of Δ16-*ent*-kaurene nucleus are present in **7b**, as that of hydrogen at C-13 which appears at δ 2,63 (H-13, 1H, broad singlet). However, it is noticeable the absence of C-19. On the other hand, the C-4 carbon appears at 84.4ppm, which indicates

that the hydroxyl moiety is replacing the C-18 methyl, in other words it has a β-equatorial configuration. This is supported by the chemical shift of the H-20 methyl group which appears at δ 1.11 and is not greatly affected by the presence of the hydroxyl. According to Bohlmann H-20 should appear at δ 1.13 in *epi*-ruilopeziol (**7b**) and at δ 1.17 in ruilopeziol (**7a**). On the other hand polarity of the **7b** should be greater than that of its epimer because when the OH group is in equatorial position it is more available to interact with its environment. The GC-MS total ion chromatograph (TIC) of the neutral fractions showed a small peak at retention time of 3.17 minutes. The mass spectrum of this compound (figure 2c) showed a MW at m/z 274.3 and it is almost identical to the mass spectrum of **7b**. It was concluded that this substance was ruilopeziol (**7a**), which has the hydroxyl group in a 19α-axial position and it is less polar than epi-ruilopeziol (**7b**). That is the reason why **7a** elutes in a shorter time than **7b** on a phenyl (5%)-methyl-polysiloxane column (HP-5). Unfortunately it was not possible to isolate **7a** pure enough to submit it to NMR analysis

Results and Discussion

The structures of the kaurenic acids, their methyl esters, and neutral kaurenes isolated from *E. nana* roots and leaves are presented on figure 1. The total ion chromatogram (TIC) indicated that the leaves´ acidic fraction contained kaurenic acid methyl ester (**1b**, 34.6%), grandiflorenic acid methyl ester (**2b,** 40.1%**),** 15α-acetoxy-kaur-16-en-19-oic acid methyl ester (**3b**, 8%), 15α-hydroxy-kaur-16-en-19-oic acid methyl ester (**4b**, 13%), and 4.2% of a compound with molecular weight of 344 g/mol which was not identified. The bulk of the leaves´ acidic fraction was then submitted to flash chromatography over silica gel. A similar GC-MS analyses of a 10 mg sample of the roots methyl ester mixture indicated that it contained 38.0% of **1b,** 39.6% of **2b**, 8.5% of **3b,** and 13,9% of **4b**. The compound with MW of 344g/mol was not found in the roots´ acidic fraction. The bulk of leaves and roots´ acidic fraction was purified by flash chromatography. It was found that the most abundant constituent of *Espeletia nana* was grandiflorenic acid followed by kaurenic acid. On the other hand 15α-acetoxy-kaur-16-en-19-oic acid (**3a**) and 15α-hydroxy-kaur-16-en-19-oic acid (**4a**) made about 20% of the acidic fraction, but **4a** was more abundant on the leaves while **3a** was more abundant on the roots. These four kaurenes made up about 99% of the roots´ acid fraction and about 95% of the leaves´ acid fraction, which sets *Espeletia nana* aside from other species of Espeletiinae thus far studied, which normally contain a more complex mixture of diterpene acids with kaurenic structure. Table 1 presents the retention times and percentage composition observed on the TIC spectrum of the GC-MS analysis of the acid fraction of leaves and roots of *E. nana*.

Table1: GC-MS analysis of the methylated acidic fractions of *E. nana*.

Peak	Compound	R_T (min)	Leaves área (%)	Roots area (%)
1	*ent*-kaur-9(11),16-dien-19-metil éster [**2b]**	3.34	40.14	39.60
2	*ent*-kaur-16-en-19-metil éster [**1b]**	3.81	34.56	37.97
3	Not identified (m/z 344 (100%), 257, 215, 121, 91)	4.07	4.25	-
4	15*α*-hidroxi- *ent*-kaur-16-en-19-metil éster [**4b]**	5.25	13.08	8.51
5	15*α*-acetoxi- *ent*-kaur-16-en-19-metil éster [**3b]**	5.76	7.97	13.91
	Total		99.94	99.99

R_T Rentention time

A GC-MS analysis of the neutral fractions of leaves and roots indicated that its most abundant component is *ent*-kaur-16-en-19-al (**5**), which made up 88% of the neutral fraction. Minor components are kaurenol (**6**), ruilopeziol (**7a**), *epi*-ruilopeziol (**7b**), and waxes. On this respect *E. nana* is also different from other Espeletiinae which usually contain large quantities of waxes. Table 2 shows the retention times and percentage composition of kaurenes present in the neutral fraction of leaves and roots of *E. nana*.

Ruilopeziol (**7a**) and *epi*-ruilopeziol (**7b**) are two epimeric kaurenic derivatives where either the α-axial C-19 carbon has been replaced by an OH (**7a**), or the equatorial C-18 carbon has suffered the same transformation (**7b**). These compounds were first isolated by Bohlmann[4] from *Ruilopezia lindeni* and *Coespeletia lutescens*. They were named to honor Luis Ruiz Terán and Manuel Lopez Figueiras, two botanists, members of the staff of the Faculty of Pharmacy at the University of Los Andes, who contributed with José Cuatrecasas to the discovery of more than 30 new species of frailejon. Since Bohlmann reported only the IR, ^{1}H-NMR, and mass spectrum of these compounds, the ^{13}C-NMR spectrum of *epi*-ruilopeziol is reported in this study (table 3). The bidimensional NMR experiments made on ruilopeziol confirmed the structure proposed by Bohlmann.

Table 1: GC-MS analysis of neutral fractions of *E. nana*.

Peaks	Compound	R_T (min)	Leaves area (%)	Roots area (%)
1	Ruilopeziol [**7a**]	3.17	13.0	1.0
2	*epi*-ruilopeziol [**7b**]	3.33	7.0	2.5
3	*ent*-kaur-16-en-19-al [**5**]	3.69	43.0	88.0
4	*ent*-kaur-16-en-19-ol [**6**]	4.25	3,0	7.0
5	nonacontane	7.62	25.0	1.0
	entriacontane	9.57	8.0	0.5
	Total		99.0	100.0

R_T Rentention time

Table 3: ^{1}H and ^{13}C NMR data of *epi*-ruilopeziol (δ in ppm, J in Hz).

Position	^{1}H NMR ($CDCl_3$, 400 MHz)	^{13}C NMR ($CDCl_3$ 100 MHz)	COSY	HMBC
1	$a^{\beta}_{(axi)}$: 0.78 *td* $b^{\alpha}_{(ecu)}$: 1.88 *td*	41.4	H-1/H-2	
2	$a^{\beta}_{(axi)}$: 1.55 *m* $b^{\alpha}_{(ecu)}$: 1.75 *m*	20.2	H-2/H-3	
3	$a^{\beta}_{(axi)}$: 1.06 *td* $b^{\alpha}_{(ecu)}$:2.01 *td*	40.9		C-3/H-18
4	-	84.4		
5	1,05 *dd*	56.1	H-5/H-6	C-5/H18 C5/H20
6	$a^{\beta}_{(axi)}$: 1.50 *m* $b^{\alpha}_{(ecu)}$: 1.75 m	19.4		
7	$a^{\beta}_{(axi)}$: 1.55 *m* $b^{\alpha}_{(ecu)}$:.1.60 *m*	40.2		
8	-	44.2		
9	0.87 *m*	55.4		C-9/H-20
10	-	39.2		C-10/H-1 C-10/H-11
11	$a^{\beta}_{(axi)}$: 1.45 *m* $b^{\alpha}_{(ecu)}$:1.59 *m*	18.2		
12	$a^{\beta}_{(axi)}$: 1.48 *m* $b^{\alpha}_{(ecu)}$:1.62 *m*	33.2		
13	2,63 *s ancho*	44.2	H-13/H-14 H13/H12	C-13/H-17 C-13/H-14
14	$a^{\beta}_{(axi)}$: 1.17 *d* $b^{\alpha}_{(ecu)}$: 2.05 *d*	35.2		
15	2,04 *m*	49.4	H-15/H-14	C-15/H-17
16	-	156.1		
17	$a^{\beta}_{(axi)}$: 4.70 *d* $b^{\alpha}_{(ecu)}$:4.79 *d*	103.1		
18	1.28 *s*	24.9		
20	1.11 *s*	17.3		

Usually the percentage composition of volatile compounds is measured using a flame ionization detector. In this case, however, since the mass spectra of the kaurene derivatives found in this "frailejon" have similar fragmentation patterns, as can be appreciated in the mass spectra of **1b**, and **2b** (figure 2) the TIC percentage composition was taken as a good approximation to the real composition of the resin.

Conclusions

The major constituents of acid fraction of the leaves and roots of *Espeletia nana* Cuatrec. were grandiflorenic acid and kaurenic acid, while kaurenal was the most abundant component of the neutral fraction. Waxes were present in the leaves but were not as abundant as usually found in

Espeletiinae thus far studied, on the other hand, the roots contained only 1.5%.

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