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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 resinos a 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 raíces fueron extraídas por separado con una mezcla de hexano-éter dietílico (3:1). Aliquotas 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-en-19-0ico (3a) y 13% de ácido ent-15α-hidroxi-kaur-16-en-19-0ico (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 nonacantone 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-dithyl ether. Aliquots 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%), grandiflorenc acid (2a, 40.1%), 15α-ent-acetox-kaur-16-en-19-0ic acid (3a, 8%), and 15α-hidroxy-ent-kaur-16-en-19-0ic 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 nonacantone 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*, grandiflorenc 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 wooly 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 frailejón, and one of seventeen species that have been found in the Venezuelan Andes. *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 4-6 cm in diameter. 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 CDCl₃. 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 (1.0 kg) of *Espeletia nana* were collected at paramo Ortiz at 3085 m 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 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-ol (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%)
A GC-MS analysis of the leaves neutral fraction showed that 5 was the main constituent (43%) while 6 (3%), 7a (13%), 7b (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 5.0 g 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-ol 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 permitted 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 grandiflorene 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 \(\delta\) 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 \(\Delta^{16}\)-ent-kaurene nucleus are present in 7b, as that of hydrogen at C-13 which appears at \(\delta\) 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

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

Table 1: GC-MS analysis of the methylated acidic fractions of E. nana.

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

R_T Retention 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 kaurennes present in the neutral fraction of leaves and roots of
d E. nana.

Ruiilopeziol (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 Bohlmann4 from
Ruiilopezia lindeni and Coespeletia lutescens. They were
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 frailejón. Since Bohlmann reported
only the IR, 1H-NMR, and mass spectrum of these
compounds, the 13C-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.

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

<table>
<thead>
<tr>
<th>Peaks</th>
<th>Compound</th>
<th>R_T (min)</th>
<th>Leaves area (%)</th>
<th>Roots area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ruilopeziol [7a]</td>
<td>3.17</td>
<td>13.0</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td><em>epi</em>-ruilopezio [7b]</td>
<td>3.33</td>
<td>7.0</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td><em>ent</em>-kaure-16-en-19-al [5]</td>
<td>3.69</td>
<td>43.0</td>
<td>88.0</td>
</tr>
<tr>
<td>5</td>
<td>nonacontane</td>
<td>7.62</td>
<td>25.0</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>entriacontane</td>
<td>9.57</td>
<td>8.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>99.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

R_T Retention time

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

<table>
<thead>
<tr>
<th>Position</th>
<th>^1^H NMR (CDCl₃, 400 MHz)</th>
<th>^13^C NMR (CDCl₃, 100 MHz)</th>
<th>COSY</th>
<th>HMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>aH(axi): 0.78 td</td>
<td>41.4</td>
<td>H-1/H-2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>bH(εcu): 1.88 td</td>
<td></td>
<td>H-2/H-3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>aH(axi): 1.55 m</td>
<td>20.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>bH(εcu): 1.75 m</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>aH(axi): 1.06 td</td>
<td>40.9</td>
<td>C-3/H-18</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>bH(εcu): 2.01 td</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>84.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.05 dd</td>
<td>56.1</td>
<td>H-5/H-6</td>
<td>C-5/H18</td>
</tr>
<tr>
<td>9</td>
<td>aH(axi): 1.50 m</td>
<td>19.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>bH(εcu): 1.75 m</td>
<td>40.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>aH(axi): 1.55 m</td>
<td>40.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>bH(εcu): 1.60 m</td>
<td>44.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>0.87 m</td>
<td>55.4</td>
<td>C-9/H-20</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>39.2</td>
<td>C-10/H-1</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>2.04 m</td>
<td></td>
<td>C-10/H-11</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td></td>
<td>35.2</td>
<td>H-13/H-14</td>
<td>C-13/H-17</td>
</tr>
<tr>
<td>17</td>
<td>2.63 s ancho</td>
<td></td>
<td>H13/H12</td>
<td>C-13/H-14</td>
</tr>
<tr>
<td>18</td>
<td>1.28 s</td>
<td>44.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td></td>
<td>156.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>103.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>1.11 s</td>
<td>24.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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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