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Ent-Kaurene diterpenes and *n*-alkanes isolated from the leaves of *Coespeletia thyrsiformis* (A.C.SM.) CUATREC

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ABSTRACT

Coespeletia thyrsiformis is a resinous plant characteristic of the Andean highlands of Venezuela. The present work describes the extraction, from the leaves of this plant, of several biologically active diterpene compounds. An extract obtained by shaking the leaves with a 3:1 mixture of n-hexane-diethyl ether was treated with a 0.5 N NaOH solution to separate the acidic fraction. An aliquot of this acid fraction was methylated with diazomethane to submit the mixture to GC-MS analysis, which permitted to identify the following kaurene derivatives: *ent*-kaur-9(11),16-dien-19-oic acid (6.9 %), *ent*-kaur-16-en-19-oic acid (17.0 %), 15α-hydroxy-ent-kaur-16-en-19-oic acid (29.3 %), 15 α-isovaleroxy-ent-kaur-16-en-19-oic acid (7.6 %), and 15 α-*O*-isobutyroxy-*ent*-kaur-16-en-19-oic acid (6.1 %), as major components. On the other hand nonacosane (26.2 %) and 16-β-hydroxy-*ent*-kaurane (14.1 %) were found as major components in the neutral fraction.

Keywords: *Coespeletia thyrsiformis, Asteraceae, diterpenes, kaurenics acids.*

1. INTRODUCTION

Asteraceae (Compositae) have been the object of numerous studies because of their biological diversity. This family has more than 25.000 species distributed in about 1.500 genera. In Venezuela 210 genera and 760 species have been described [1]. The Coespeletia are resinous plants, popularly known as frailejon [3] that belong to the Espeletiinae subtribe, which was created by Cuatrecasas [2]. They have a rosette growth form and their leaves are covered by a heavy indumentum that protects them from freezing.

Seven species of *Coespeletia* have been described, six of them endemic to the venezuelan paramos: *C. albarregensis* Cuatrec., *C. elongata* (A. C. Sm.) Cuatrec., *C. moritziana* (Sch. Bip. ex Wedd.) Cuatrec., *C. spicata* (Sch. Bip. ex Wedd.) Cuatrec., *C. thyrsiformis* (A. C. Sm.) Cuatrec. (Included *C. thyrsiformis* f. *marcana* (Cuatrec.) Cuatrec.), *C. timotensis* (Cuatrec.) Cuatrec. Recently two new species have been reported: *C. laxiflora* native to Colombia (S. Díaz & Rodr. Cabeza) [4] and *C. palustris* (M. Diazgranados & G. Morillo, sp. nov.)[5], a new species for Venezuela.

Coespeletia thyrsiformis [2,6], known as "frailejon amarillo del batallon", grows at about 3.000 m.s.n.m. [4]. A previous study performed by Usubillaga *et al*. [7] identified by GC-MS as methyl esters the following acids: (-)-kaur-16-en-19-oic acid (46.5 %), (-)-kaur-9(11)16-dien-19-oic acid (16.4 %), 15*α*-hydroxy-kaur-16-en-19-oic acid (14.1 %), 15-*O*-isovaleroxy-kaur-16-en-19-oic acid (11.3 %), and 15-*O*-senecioxy-kaur-16-en-19-oic acid (10.8 %).

Aparicio *et al* [8] studied the composition of the essential oil obtained from the leaves of *C. thyrsiformis*, reported that *α*-pinene (20.4 %) and germacrene D (14.5 %) were the most abundant components, but also reported the presence in the oil of the diterpene (-)-Kaur-16-en-19-al (0.4 %). *Ent*-kaurene type compounds are present in all Espelletiinae species thus far studied. Kaurenic acid (*ent*- kaur-16-en-19-oic acid) is known for its diverse biological properties, which have been reported by several researchers [9-17]. Several other kaurene diterpenes have also been found to have a wide range of biological properties [18-32]. The object of the present study is to perform a detailed analysis of the neutral and acidic compounds present in an hexane/diethyl ether extract of the leaves of *C. thyrsiformis*.

2. EXPERIMENTAL SECTION

2.1. Material vegetal. Fresh leaves of *C. thyrsiformis* were collected at paramo "El Batallon", at 3200 m.s.n.m, in Táchira State. A voucher specimen (RA03) was deposited at MERF, the herbarium of Facultad de Farmacia y Bioanálisis, Universidad de Los Andes. Professor Gilberto Morillo (Facultad de Ciencias Forestales y Ambientales, Universidad de Los Andes, identified the botanical material.

2.2. Extraction of the leaves of *Coespeletia thyrsiformis* with hexane-diethyl ether. Leaves of *C. thyrsiformis* were dried at 40 ºC for 5 days. The dried material was ground yielding 118 g of solids which were submitted to extraction during 4 days, at ambient temperature, with a mixture of *n*-hexane: diethyl-ether (3:1). The extract was taken to dryness in a rotavapor yielding 5.2 g of solid residue.

2.3. Isolation of the acidic and neutral fractions. The crude extract was dissolved in hexane/diethyl-ether (3:1) and it was shaken with a 5 % NaOH solution. The aqueous phase was taken to pH 3 by careful addition of conc. HCl and shaken with

hexane/diethyl-ether (3:1) to yield 2.3 g of acid fraction. The original hexane-ether solution, left over after alkaline treatment, was mixed with active charcoal and boiled for 10 minutes. After open column chromatography, treatment a neutral fraction of 3.5 g was obtained.

2.4. GC-MS analysis. A small amount (10 mg) of acid fraction was methylated with diazomethane [33,34] and then submitted to CG-MS analysis. A Hewlett-Packard 6890 gas chromatograph fitted with an HP 5973 mass detector was used. A 30 m long (0.25 mm d.i and 0.25 μm film) HP-5MS capillary column was used. The ionization energy was 70 eV, the analysis mass range was 40:500 amu at 3.9 scans/s. Each sample (acid or neutral) was dissolved in diethyl ether (3 mg/mL) and 1 μL was injected using a Hewlett-Packard ALS injector, applying a 30:1 split ratio. Helium at 0.9 mL/min was used as carrier gas. The following temperature program was used: initial temperature 100 °C, which was increased at 10 °C/min to 200 °C. A second temperature increase of 8 ºC/min was applied to a final temperature of 300 ºC to obtain a total analysis time of 30 min.

The compounds present in both fractions were identified by their retention times, direct comparison of their mass spectra with the spectra of authentic samples available in the laboratory and by computer comparison with the spectra of Wiley and Nist libraries.

3. RESULTS SECTION

Figure 1 presents the total ion chromatogram of the acid fraction. In this fraction it was possible to identify ten *ent*-kaurene derivatives as well as hexadecanoic acid and octadecanoic acid. All these compounds made up 82.9 % of the acidic fraction. Table 1 shows the retention times, relative abundances and masses of the methyl esters of these compounds. Their structures are shown on Figure 2**.**

The most abundant identified compounds were: *ent*-kaur-9(11),16-diene-19-oic acid methyl ester (6.9 %, **1**); *ent*-kaur-16-en-19-oic acid methyl ester (17.0 %, **2**); *ent*-kaur-15α-hydroxy-16-en-19-oic acid methyl ester (29.3 %, **5**); *ent*-kaur-15α-*O*-isovalerioxy-16-en-19-oic acid methyl ester (7.6 %, **10**) and *ent*-kaur-15α-*O*-isobutiloxy-16-en-19-oic acid methyl ester (6.1 %, **9**). It was not possible to identify 17.1 % of the mixture of methyl esters.

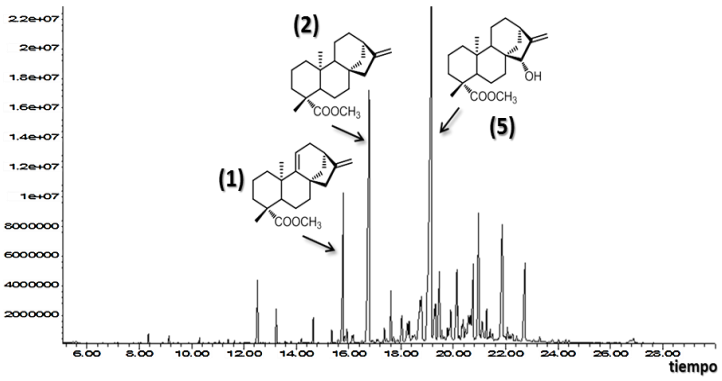

Figure 1. TIC chromatogram of the methylated acid fraction of *C. thyrsiformis*.

Table 1. Methylated *ent*-kaurane diterpenic acids and esterified fatty acids identified in the acid fraction from the resin of *C. thyrsiformis.*

Nº	Components	m/z	RT min	Area %
1	hexadecanoic acid methyl ester	270	12.52	2.4
2	hexadecanoic acid ethyl ester	284	13.23	1.2
3	Octadecanoic acid methyl ester	298	14.84	0.9
4	*ent*-kaur- 9(11),16-dien-19-oic acid methyl ester (1)	314	15.79	6.9
5	*ent*-kaur-16-en-19-oic acid methyl ester (2)	316	16.78	17.0
6	*ent*-kauran-16α-hydroxy-19-oic acid methyl ester (3)	334	18.73	3.2
7	*ent*-kauran-19-oic acid-methyl ester (4)	318	18.78	1.8
8	*ent*-kaur-15α-hydroxy-16-en-19-oic acid methyl ester (5)	332	19.15	29.3
9	*ent*-kaur -15-en- 17-oxo-19-oic acid methyl ester (6)	330	19.32	1.2
10	*ent*-kaur-15-en-17-hydroxy -19-oic acid methyl ester (7)	332	19.47	3.8
11	*ent*-kaur-15 α-*O*-acetoxy- 16-en-19-oic acid methyl ester (8)	374	20.66	1.5
12	*ent*-kaur-15α-*O*-isobutiloxy-16en-19-oic acid methyl ester (9)	402	20.95	6.1
13	*ent*-kaur-15α-isovalerioxy-16-en-19-oic acid methyl ester (10)	416	21.87	7.6
			Total	82.9

These results agree with those reported by Usubillaga *et al.*, 2003 [7], even though at different concentrations, since they reported the *ent*-kaur-16-en-19-oic acid (**1**) as the major component (45.5 %), as well as *ent*-kaur-9(11),16-dien-19-oic acid (**2**)(16.4 %) and *ent*-kaur-15α-hidroxy-16-en-19-oic acid (**5**) (14.1 %) present with 16.4 % and 14.1 % respectively. In our investigation the same three acids were found but *ent*-kaur-15α-hydroxy-16-en-19-oic acid (29.3 %, **5**), was the most abundant. Since Usubillaga *et al.*, collected *C. thyrsiformis* at the same paramo the differences are probably do to age of the plants or weather conditions at the moment of collection.

Kaurene diterpenes have interesting biological properties. It has been reported that *ent*-kaur-15α-hydroxy-16-en-19-oic acid (**5**), has an effect against B16F1 melanoma in mice [35]. On the other hand this compound is very useful because it is used as starting compound to obtain 15-oxo-kaurénic acid, a substance that causes apoptosis on the epithelial cells of prostatic cancer [30].

The analysis of the neutral fraction led to the identification of 88.4 % of its compounds. The main constituents of these fraction were: 16-β-kauranol (**16**) 14.1 %; *ent*-kaur-16-en-19-hydroxy-16-ene (**17**) 3.5 %; *ent*-kaur-19-nor-16-ene (**12**) 1.5 %; *ent*-kaur-16-ene (**13**) 1.2 %; *ent*-kaur-18-nor-16-ene (**11**) 1.1 %. Other, non-kaurenic components were also present in the neutral fraction: nonacosane (26.2 %) and heptacosane (11.4 %) (Table 2). Structure of *ent*-kauranes isolated from the neutral fraction, are also shown on Figure 2.

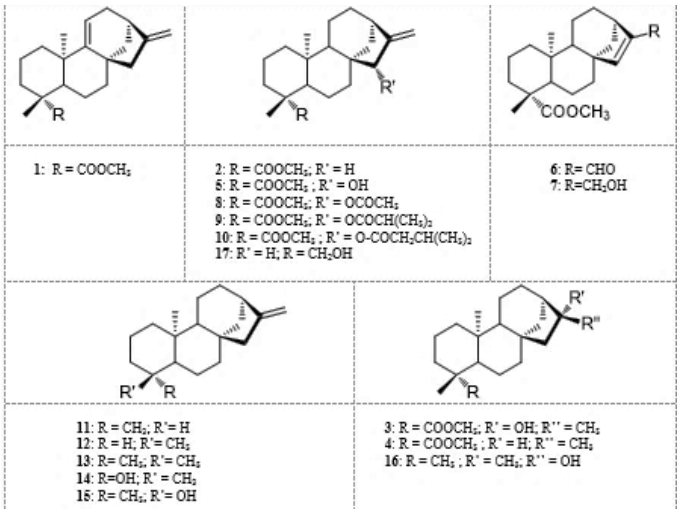

Figure 2*: Ent*-kaurane diterpenes identified in the resin of *C. thyrsiformis*.

Hydrocarbons found in the neutral fraction agree with previous studies which indicate that higher plants produce abundant long chain *n*-alkanes (C27 a C35). Generally odd carbon number n-alkanes are found, C27, C29 and C31 are usually the most abundant ones [36], which is related to important physiological processes [37]. C29 and C31 n-alkanes also play an important function because they contribute to the impermeability of the surface of the leaves of the plants, which has some connection to paleoecology and quimiotaxonomy [38-40].

Table 2. Neutral fraction components of *Coespeletia thyrsiformis*.

N°	Components	m/z	RT Min	Area %
1	Spathulenol	220	9.12	2.0
2	*ent*-kaur-18-nor-16-ene (11)	258	13.5	1.1
3	*ent*-kaur-19-nor-16-ene (12)	258	13.89	1.5
4	*ent*-kaur-16-ene (13)	272	14.34	1.2
5	Ruilopeziol (14)	274	15.39	4.7
6	*epi*-ruilopeziol (15)	274	15.82	9.5
7	16-β-kauranol (16)	290	16.41	14.1
8	*ent*-kaur-19-hydroxy-16-ene (17)	288	17.61	3.5
9	Pentacosane ($C_{25}H_{52}$)	352	18.58	1.9
10	Heptacosane ($C_{27}H_{56}$)	380	20.43	11.4
11	Octacosane ($C_{28}H_{58}$)	394	21.31	1.2
12	Nonacosane ($C_{29}H_{60}$)	408	22.18	26.2
13	Triacontane ($C_{30}H_{62}$)	422	23.01	1.6
14	Hentriacontane ($C_{31}H_{64}$)	436	23.97	8.5
			Total	88.4

4. CONCLUSIONS

Analysis of the methylated acidic fraction of the resin from the leaves of *C. thyrsiformis* (A. C. Sm.) Cuatrec, led to the identification of 10 *ent*-kaurenic acids which account for 78.4 % of the fraction. The most abundant compounds identified were: *ent*-kaur-15α-hydroxy-16-en-19-oic methyl ester (29.3 %, **5**); *ent*-kaur-16-en-19-oic acid methyl ester (17.0 %, **2**); *ent*-kaur-9(11),16-dien-19-oic acid methyl ester (6.9 %, **1**); *ent*-kaur-15α-*O*-isovalerioxy-16-en-19-oic acid methyl ester (7.6 %, **10**) and *ent*-α-*O*-isobutiloxy-16-ene-*ent*-19-oic acid methyl ester (6.1 %, **9**).

In the neutral fraction seven *ent*-kaurane derivatives were identified, 16-β-kauranol (**16,** 14.1 %), and *epi*-ruilopeziol (**15,** 9.5 %) were the most abundant ones. A mixture of C_{25} to C_{32} hydrocarbons were also found, which represent 50.8 % of the total fraction, where C_{27} (11.4 %) and C_{29} (26.2 %) hydrocarbons were the most abundant.

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