BIOLOGY, MEDICINE, & NATURAL PRODUCT CHEMISTRY ISSN 2089-6514 (paper)
Volume 12, Number 1, April 2023 | Pages: 151-157 | DOI: 10.14421/biomedich.2023.121.151-157 ISSN 2540-9328 (online)

A New *ent*-kaurene Diterpenoid Isolated from Leaves of *Espeletia semiglobulata* Cuatrec. and its Potential Antimicrobial Activity

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Manuscript received: 03 December, 2022. Revision accepted: 16 January, 2023. Published: 24 January, 2023.

Abstract

The fraction of the neutral extraction of the leaves of *Espeletia semiglobulata* Cuatrec. was subjected to chromatographic separation, it yielded four *ent*-kaurene type diterpenoids: three known [*ent*-kaur-16-en-19-al **(I)**, *ent*-kaur-18-*nor*-16-en-4-ol **(III)**, *ent*-kaur-16-en-19-ol **(IV)**] and a new one elucidated as *ent*-kaur-3-acetoxy-15-ene **(II)**, based on the physicochemical and spectroscopic data of FTIR, GC-MS, and 1D and 2D NMR. These compounds were subjected to antimicrobial bioassay studies. This new *ent*-kaurene showed a significant inhibition potential against the growth of gram negative bacterial strains [*Escherichia coli* (ATCC 25922): 8 mm, *Klebsiella pneumoniae* (ATCC 23357): 10 mm, *Pseudomonas aeruginosa* (ATCC 27853): 8 mm], it also showed inhibition against the growth of fungal strain (*Candida krusei*: 8 mm), at a 2 mg/mL concentration. The compounds **(I)**, **(III)** and **(IV)** failed to show any significant results in the antimicrobial screening against five bacterial strains [*Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Klebsiella pneumoniae* (ATCC 23357), *Escherichia coli* (ATCC 25922) y *Pseudomonas aeruginosa* (ATCC 27853)] and one fungal strain [*Candida krusei* (ATCC 6558)]. These results reveal a remarkable natural structure-activity relationship of the *ent*-kaurene core regarding the C-3 position (A ring of perhydrophenanthrene unit), whose oxygenation or addition of a hydrogen bond acceptor or donor group, improves the antimicrobial activity.

Keywords: Diterpene; *ent*-kaurene; Asteraceae; Natural Products; Antimicrobial Activity.

Abbreviations: NMR – Nuclear Magnetic Resonance; ^{1}H NMR – Proton Nuclear Magnetic Resonance; ^{13}C NMR – Carbon Nuclear Magnetic Resonance; ^{13}C-APT – attached proton test; COSY- Correlation spectroscopy; HSQC – Heteronuclear single quantum correlation spectroscopy; HMBC – Heteronuclear multiple bond correlation spectroscopy; IR – Infrared spectroscopy; GC-MS – Gas chromatography–Mass Spectrometry; mp – melting point; MH – Müeller-Hinton; DMSO – Dimethyl Sulfoxide; EtOAc – Ethyl Acetate.

INTRODUCTION

Bioactive natural products have played an important role in treating and preventing human diseases. These are important sources for new drugs and also good lead compounds suitable for further modification during drug development (Atanasov *et al*., 2021). The terpenoids are among the most important natural compounds known for their medicinal value (Kamran *et al*., 2022; Proshkina *et al*., 2020); within them, *ent*-kauranes represent an important group of tetracyclic diterpenes that possesses a wide spectrum of biological and pharmaceutical activities such as antimicrobial, antiparasitic, insect antifeedant, cytotoxic, antitumour, anti-HIV, steroidogenic, antifertility, hypotensive, antiinflamatory, among others (Ghisalberti, 1997). This type of diterpene is extensively found in different species of Espeletiinae (Asteraceae), a family of resinous plants, popularly known as "Frailejon", that grow at an altitude of 2500 m in the Andes of Northern South America. These plants are used by the inhabitants of the high moors to treat asthma and rheumatic conditions (Usubillaga *et al*., 2003; Aparicio *et al*., 2013). *Ent*-kaurenes have been identified in some studies about the *Espeletia semiglobulata* Cuatrec. Species. It grows above an altitude of 3900 m at the "Piedras Blancas" Moor, which is part of Sierra La Culata, located near the city of Mérida, Venezuela. *ent*-kaur-16-en-19-oic acid has been identified on the fraction of acid extraction of the leaves of this plant as the most abundant component and known for its diverse biological properties (Sosa-Sequera *et al*., 1996; Cavalcanti *et al*., 2005; Kim *et al*., 2016; Zhang *et al*., 2017; Cotoras *et al*., 2004; Mongelli *et al*., 2002), and *ent*-kauran-19-oic acid, *ent*-kaur-9(11)-16-dien-19-oic acid, *ent*-kaur-15α-isovaleroxy-16-en-19-oic acid, *ent*-kaur-15α-hidroxy-16-en-19-oic acid, *ent*-kauran-16α-hidroxy-19-oic acid, and *ent*-kaur-15-en-19-oic acid have been identified as the less abundant components. On the other hand, *ent*-kaur-16-en-19-al and *ent*-kaur-

16-en-19-ol have been identified as majority components in the fraction of neutral extraction, and *ent*-kaur-16α-hidroxy-16-en-19-al, *ent*-kaur-18-*nor*-16-en-4-ol, *ent*-kaur-16-en-19-ol acetate have been identified as minority components without any biological activity reported on any of them (Usubillaga *et al*., 1988; Aparicio *et al*., 2013). In this study, we are reporting the isolation, structural identification, and antimicrobial activity of a new *ent*-kaurene-type terpenoid natural product **(II)** from neutral fraction of *Espeletia semiglobulata* Cuatrec. leaves, also, the evaluation of antimicrobial activity of some known *ent*-kaurenes coming from this neutral extraction is being reported for the first time.

MATERIALS AND METHODS

General methods

Melting points were determined on a Fisher-Johns melting point apparatus. IR spectra were measured on a Perkin Elmer Spectrum two, 10.03.06 version, as KBr disks. NMR spectra was recorded with a Bruker-UltraShield™ 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, 0.25 mm, film thickness 0.25 μm). 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

Leaves of *Espeletia semiglobulata* Cuatrec. were collected at 3900 m of altitude in "Piedras Blancas" Moor and along the road to Piñango, about 13 Km from the mountain's peak "Pico del Aguila". Voucher specimens (AU30 and AU21) were deposited at the Faculty of Pharmacy and Bioanalysis Herbarium, University of Los Andes (MERF Herbarium).

Extraction and neutral fraction obtainment

Espeletia semiglobulata Cuatrec. leaves were dried at 40 °C during 48 hours and they were grinded. The grinded material (200 g) was extracted at room temperature with a mixture of hexane/diethyl ether (3:1, v/v) for a period of two days. The solvents were removed under reduced pressure and the solid residues were dissolved in hexane/EtOAc, and shaken with 5 % aqueous NaOH. The aqueous phase was taken to pH 3 by careful addition of concentrated HCl and shaken with hexane to obtain the acid fraction. The original hexane-diethyl ether solution, left over after the alkaline treatment surplus, was mixed with active charcoal and boiled for 10 minutes. A neutral fraction of 25 g was obtained after an open column chromatography treatment.

Isolation of kaurene diterpenes

Most of the leaves neutral fraction was submitted to flash chromatography over silica gel. The column (A) was eluted with hexane and hexane/AcOEt mixtures and 100 mL fractions were collected, which were constantly motorized by TLC. Fractions 1-35 eluted with hexane yielded 840 mg of a mixture that contained plant waxes and **(I)**. Fractions 36-39 eluted with hexane rendered 500 mg of pure **(I)** as a white solid, mp 113-116 ºC, identical to an authentic sample of *ent*-kaur-16-en-19-al (mp,^{1}H-NMR, ^{13}C-NMR) obtained from *Espeletia semiglobulata* (Usubillaga *et al*., 1988; Aparicio *et al*., 2013). The elution continued with hexane (40-45 fractions), it yielded 150 mg of a mixture of **(I)** and **(II)**. The elution of the 46-61 fractions with hexane yielded 1000 mg of pure **(II)**, which was crystallized from hexane/Et_2O as white crystalline solid. This compound has a very similar polarity to **(I)**, however, they are not the same (mp, ^{1}H-NMR, ^{13}C-NMR). The elution of the 82-94 fractions with hexane/EtOAc 5 % yielded 300 mg of pure **(III)**, mp 146-148 °C, identical to an authentic sample of *ent*-kaur-18-*nor*-16-en-4-ol (mp,^{1}H-NMR, ^{13}C-NMR) obtained from *Espeletia nana* (Peña *et al*., 2012). The elution of the 101-106 fractions with hexane/EtOAc 10 % yielded 200 mg of pure **(IV)**, mp 142-144 °C, identical to an authentic sample of *ent*-kaur-16-en-19-ol (mp,^{1}H-NMR, ^{13}C-NMR) obtained from *Espeletia semiglobulata* Cuatrec. (Usubillaga *et al*., 1988; Aparicio *et al*., 2013). Finally, the elution using hexane/EtOAc 20 % yielded a complex mixture (21.4 g).

Antimicrobial Assay

- *Antibacterial activity*

The antibacterial activity was determined by the agar diffusion method with discs. The following microorganisms were tested: *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Klebsiella pneumoniae* (ATCC 23357), *Escherichia coli* (ATCC 25922) y *Pseudomonas aeruginosa* (ATCC 27853). An 18 hours culture of each microorganism was used in 2.5 mL Müeller-Hinton (MH) broth at 37 °C. The bacterial inoculum was adjusted with saline physiological solution to the Mc Farland Turbidity Standard N° 0.5 (10^{6-8} CFU/mL). Each inoculum was spread with a swab on the surface of a plate containing Müeller-Hinton agar and then a disc of filter paper (6 mm diameter) previously impregnated with 10 μL of the compound dissolved in DMSO was placed on the surface at a 2 mg/mL concentration for each one [**(I)**, **(II)**, **(III)**, **(IV)**]. A disc impregnated with DMSO was included as a negative control. In addition, the standard disc of the reference antibiotic was placed as a positive control for each of the microorganism (Oxacillin 1 μg

for *Staphylococcus aureus*; Vancomycin 30 µg for *Enterococcus faecalis*; Tobramycin 10 µg for *Escherichia coli*; Aztreonam 30 µg for *Klebsiella pneumoniae*; Cefepime 30 µg for *Pseudomonas aeruginosa*). After having placed the discs on the Petri plates, they were refrigerated at 4 °C for 24 h (Velasco *et al.*, 2007). The reading of the inhibition halos was performed at 24 h and it was measured (mm) around the disc. All the antibacterial activity trials were done twice. The measurement of the inhibition halos of the antibacterial activity was done thus: C= A-B (C= size of the inhibition halo; A= size of the halo plus the disk of filter paper; B= size of the filter paper disk, 6 mm).

- *Antifungal activity*

The antifungal activity was determined by the agar well diffusion method. The microorganism tested was *Candida krusei* (ATCC 6558), using Fluconazole 100 µg as positive control, and DMSO as negative control. The strain repacked 12 hours before, coming from a savory dextrose agar, it was used to prepare the inoculum which was compared using the Mc Farland Turbidity Standard N°0.5 (10^{6-8} CFU/mL). Each inoculum was spreaded with a swab on the surface of a plate containing Müeller-Hinton agar and then 6 mm diameter holes were opened in it, where 20 µL of the compounds dissolved in DMSO were placed at a 2 mg/mL concentration for each one [**(I)**, **(II)**, **(III)**, **(IV)**]. One well was included with the DMSO as a negative control and another well with the reference antibiotic as positive control. Then the Petri plates were refrigerated at 4 °C for 24 h (Velasco *et al.*, 2007). The reading of the inhibition halos was performed at 24 h and was measured (mm) around the well. All the antifungal activity trials were done twice.

RESULTS AND DISCUSSION

Phytochemical analysis and structure elucidation

The structures of the *ent*-kaurene diterpenoids isolated from neutral fraction of *E. semiglobulata* Cuatrec., leaves are presented on figure 1.

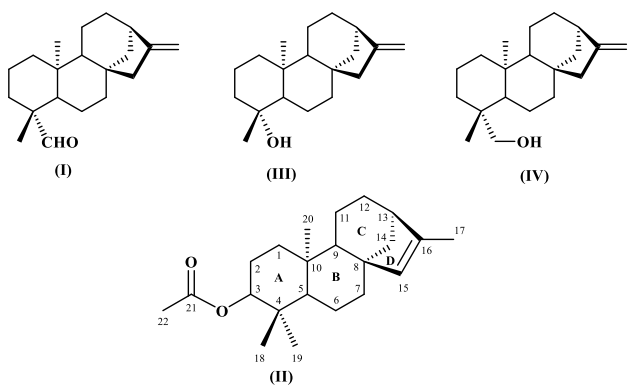

Figure 1. Molecular structure of *ent*-kaurenes from neutral fraction of aerial parts of *Espeletia semiglobulata* Cuatrec.

Compound **(I)** was isolated as a white powder, mp 113-116 ºC. GC-MS gave $[M]^+$ molecular ion at *m/z*: 286.1 (calcd. for $C_{20}H_{30}O$; *m/z:* 286). FT-IR, *v*max (cm^{-1}), functional group: 2729, *v*(C-H, aldehyde); 1711, *v*(C=O); 1655, *v*(C=C). ^{1}H-NMR (400 MHz, $CDCl_3$) δ_H: 9.73 (H_1-19, *s*, CHO), 4.74 (H_1-17a, *s*, =CH), 4.79 (H_1-17b, *s*, =CH), 2.64 (H_1-13, *s*, C-H), 2.05 (H_2-15, *m*, CH_2), 0.98 (H_3-18, *s*, CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$) δ_C: 205.94 (C-19, CHO), 103.24 (C-17, $=CH_2$), 43.75 (C-13, CH), 49.05 (C-15, CH_2), 24.29 (C-18, CH_3). This data completely matches with the spectral data reported in the bibliographic references (Usubillaga *et al*., 1988; Aparicio *et al*., 2013), this leads us to the conclusion, the compound **(I)** is an *ent*-kaurene-type diterpene called *ent*-kaur-16-en-19-al, commonly known as kaurenal. This compound was first isolated and characterized as a natural product of *Espeletia grandiflora* Humb. et Bompl. (Piozzi *et al*., 1971). It is particularly abundant in species of *Espeletia* genus (Morales *et al*., 1973; Bohlmann *et al*., 1980; Usubillaga *et al.*, 1988), and in fact, it has already been isolated and reported from *E. semiglobulata* Cuatrec. leaves (Usubillaga *et al*., 1988; Aparicio *et al*., 2013).

Compound **(II)** was obtained as a crystalline white powder. The analysis of its ^{13}C NMR and GC-MS data (*m/z* 330 $[M]^+$) deduced its molecular formula $C_{22}H_{34}O_2$, which suggested 6 degrees of unsaturation. The IR spectrum showed the absorption bands of ester group [1735 cm^{-1} (O−C=O) and 1243 cm^{-1} (O−C)] and typical bands of alkene group [3040 cm^{-1} (=C−H) and 1661 cm^{-1} (C=C)]. The ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR ($CDCl_3$, 100 MHz) data (Table 1) demonstrated the signals of thirty-four protons and twenty-two carbons sorted by attached proton test (^{13}C-APT) and heteronuclear single quantum coherence (HSQC) spectra, which can be classified as: A terminal ester group [δ_H/δ_C: 1.98 (H_3-22, *s*, CH_3) / 21.47 (C-22, CH_3); 171.17 (C-21, >C=O)], a trisubstituted alkene group [δ_H/δ_C: 5.06 (H_1-15, *s*, =CH) / 124.47 (C-15, =CH); 139.78 (C-16, >C<)], four methyl groups [δ_H/δ_C: 0.81 (H_3-18, *s*, CH_3) / 28.22 (C-18, CH_3); 0.80 (H_3-19, *s*, CH_3) / 22.70 (C-19, CH_3); 0.73 (H_3-20, *s*, CH_3) / 22.70 (C-20, CH_3)] including an olefinic methyl [δ_H/δ_C: 1.80 (H_3-17, *s*, CH_3) / 23.37 (C-17, CH_3)], a methinic proton bonded to a carbon bearing heteroatom (deshielded proton) [δ_H/δ_C: 4.50 (H_1-3, *t*, O−CH) / 80.84 (C-3, O−CH)] seven sp^3 methylene [δ_H/δ_C: 1.55 (H_2-1, *m*, CH_2) / 33.01 (C-1, CH_2); 1.57 (H_2-2, *t*, CH_2) / 23.52 (C-2, CH_2); 1.49 (H_2-6, *m*, CH_2) / 21.07 (C-6, CH_2); 1.30 (H_2-7, *m*, CH_2) / 36.69 (C-7, CH_2); 1.48 (H_2-11, *m*, CH_2) / 18.39 (C-11, CH_2); 1.54 (H_2-12, *m*, CH_2) / 26.75 (C-12, CH_2); 1.35 (H_2-14, *t*, CH_2) / 41.49 (C-14, CH_2)], three sp^3 methines [δ_H/δ_C: 1.51 (H_1-5, *m*, CH) / 52.51 (C-5, CH); 0.79 (H_1-9, *m*, CH) / 55.41 (C-9, CH); 1.86 (H_1-13, *m*, CH) / 47.79 (C-13, CH)], and three sp^3 quaternary carbon [δ_C: 37.86 (C-4); 40.17 (C-8); 39.68 (C-10)]. Detailed analysis of these NMR data suggested the presence of a typical *ent*-kaurene-type diterpenic

skeleton [perhydrophenanthrene unit (A, B and C rings) fused to a cyclopentane unit (D ring) formed by a two-carbon bridge between C-8 and C-13 (Figure 1)] (Batista *et al*., 2005).

This partial structure was further confirmed by correlations spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC), which has been shown in Figure 2. By analysis of the COSY correlations, it was possible to identify six structural areas (*a* - *f*) of this compound. The fact that the terminal ester group [C-21 (δ_C 171.17, O-C=O)/C-22 (δ_C 21.47, CH_3); H_3-22(δ_H 1.98, *s*, CH_3)]; where the terminal arrangement was confirmed by the HMBC correlation between the methylic protons H_3-22 and carbonyl group C-21 (δ_C 171.17, C=O)] was on C-3 (δ_C 142.7) was confirmed by the HMBC correlation from methinic proton H_1-3 (δ_H 4.50, *t*, CH) to ester carbonyl group C-21 (δ_C 171.17, >C=O), as well as, the correlation of H_1-3 with methylene C-2 (δ_C 23.52, CH_2) and quaternary carbon C-4 (δ_C 37.86, >C<). The presence of two methyls on C-4 was confirmed by its HMBC correlation with H_3-18 (δ_H 0.81, *s*, CH_3) and H_3-19 (δ_H 0.80, *s*, CH_3), in addition to the HMBC correlation of these methylic prontons with methine C-3 (δ_C 80.84, O-CH), as well as, by the correlations: H_3-19 (δ_H 0.80, *s*, CH_3) / C-5 (δ_C 52.51, CH) and H_1-5 (δ_H 1.51, *m*, CH) / C-4 (δ_C 37.86, >C<). The mutual correlations of both methyls, both by HMBC [H_3-18 (δ_H 0.81, *s*, CH_3) / C-19 (δ_C 22.70, CH_3); H_3-19 (δ_H 0.80, *s*, CH_3) / C-18 (δ_C 28.22, CH_3)] and by COSY [H_3-18 (δ_H 0.81, *s*, CH_3) / H_3-19 (δ_H 0.80, *s*, CH_3)] also confirmed the vicinal arrangement of them on C-4. The existence of one methyl on C-10 was verified by the HMBC correlation from methylic protons H_3-20 (δ_H 0.73, *s*, CH_3) to quaternary carbon C-10 (δ_C 39.68, >C<) and methylene C-1 (δ_C 33.01, CH_2). On the other hand, the presence of an endocyclic C-C double bond between C-15 and C-16, and the presence of an olefinic methyl on C-16 (D ring) were confirmed by HMBC correlations between methylic prontons H_3-17 (δ_H 1.80, *s*, CH_3) with olefinic methine C-15 (δ_C 124.47, =CH) and methine C-13 (δ_C 47.79, CH), and at the same time, the methinic proton H_1-13 (δ_H 1.86, *m*, CH) correlates with quaternary carbon C-16 (δ_C 139.78, =C<) and methylene C-12 (δ_C 26.75, CH_2). This partial structure has also been confirmed by COSY correlation from olefinic methyl protons H_3-17 (δ_H 1.80, *s*, CH_3) to olefinic methine proton H_1-15 (δ_H 5.06, *s*, =CH), and vice versa, as well as, the mutual correlation between olefinic methyl protons H_3-17 with methinic proton H_1-13 (δ_H 1.86, *m*, CH), and this last, mutual correlated with methylenic ptotons H_2-14 (δ_H 1.35, *t*, CH_2) and H_2-12 (δ_H 1.54, *m*, CH_2). Finally, the central ring of *ent*-kaurene core (B ring) was confirmed by the HMBC correlations between methinic proton H_1-9 (δ_H 0.79, *m*, CH) with quaternary carbon C-10 (δ_C 39.68, >C<), C-8 (δ_C 40.17, >C<) and methylene C-11(δ_C 18.39, CH_2), as well as, this ring was confirmed by COSY correlations between methinic proton H_1-9 with H_1-5 (δ_H 1.51, *m*, CH), H_2-7 (δ_H 1.30, *m*, CH_2) and H_2-11 (δ_H 1.48, *m*, CH_2). Based on the spectroscopic analysis performed, the isolation of an *ent*-isokaurene-type diterpene is confirmed, especially *ent*-kaur-3-acetoxy-15-ene, which have also be called 3-acetoxy-isokaurene, this being a natural product reported for the first time.

Compound **(III)** was isolated as a white powder, mp 146-148 °C. GC-MS analysis shown $[M]^+$ molecular ion at *m/z*: 274.3 (calcd. for $C_{19}H_{30}O$; *m/z:* 274). FT-IR, *v*max (cm^{-1}), functional group: 3500, *v*(O-H, alcohol); 1600, *v*(C=C). ^{1}H-NMR (400 MHz, $CDCl_3$) δ_H: 4.76 (H_1-17a, *s*, =CH), 4.82 (H_1-17b, *s*, =CH), 3.65 (H_1-18, *s*, OH), 2.66 (H_1-13, *t*, C-H), 2.16 (H_2-15, *m*, CH_2), 1.20 (H_3-18, *s*, CH_3), 1.11 (H_3-20, *s*, CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$) δ_C: 79.31 (C-4, >COH), 44.20 (C-13, CH), 49.40 (C-15, CH_2), 102.93 (C-17, $=CH_2$), 21.52 (C-18, CH_3), 17.74 (C-20, CH_3). This data completely matches with the spectral data reported in the bibliographic references (Bohlmann *et al*., 1980; Peña *et al*., 2012), this leads us to the conclusion that the compound **III)** is an *ent*-kaurene-type diterpene called *ent*-kaur-18-*nor*-16-en-4-ol, commonly known as ruilopeziol. This compound has only been isolated and reported as a natural product of *Ruilopezia linden*, *Coespeletia lutescens*, and *Espeletia nana* Cuatrec. (Bohlmann *et al*., 1980; Peña *et al*., 2012), therefore, its isolation and characterization from *E. semiglobulata* Cuatrec. leaves is a new discovery.

Table 1. ^{1}H-NMR ($CDCl_3$, 400 MHz), ^{13}C-NMR ($CDCl_3$, 100 MHz), COSY (^{1}H↔^{1}H) ($CDCl_3$, 600 MHz) and HMBC [^{1}H (600 MHz) → ^{13}C (100 MHz), $CDCl_3$] of compound **(II)** (*ent*-kaur-3-acetoxy-15-ene).

Position	δ ^{1}H (ppm), *mult.*	δ ^{13}C (ppm), Type	COSY (^{1}H↔^{1}H)	HMBC (^{1}H→^{13}C)
1	1.55, *m*	33.01, (>CH_2)	H_2-1 ↔ H_2-2	--
2	1.57, *t*	23.52,(>CH_2)	H_2-2 ↔ H_2-1	H_2-2 → C-3
3	4.50, *t*	80.84, (O-CH-)	H_1-3 ↔ H_2-2	H_1-3 → C-2, C-4, C-21
4	--	37.86, (>C<)	--	--
5	1.51, *m*	52.51, (>CH-)	H_1-5 ↔ H_3-19	H_1-5 → C-4, C-19, C-6
6	1.49, *m*	21.07, (>CH_2)	--	H_2-6 → C-5
7	1.30, *m*	36.69, (>CH_2)	--	H_2-7 → C-8
8	--	40.17, (>C<)	--	--
9	0.79, *m*	55.41, (>CH-)	H_1-9 → H_2-7, H_1-5, H_2-11	H_1-9 → C-10, C-11, C-8
10	--	39.68, (>C<)	--	--
11	1.48, *m*	18.39, (>CH_2)	--	H_2-11 → C-12
12	1.54, *m*	26.75, (>CH_2)	H_2-12 ↔ H_1-13	H_2-12 → C-13, C-11
13	1.86, *m*	47.79, (>CH-)	H_1-13 ↔ H_2-12, H_3-17	H_1-13 → C-17, C-16, C-12
14	1.35, *t*	41.49, (>CH_2)	H_2-14 ↔ H_1-13	--
15	5.06, *s*	124.47, (=CH)	H_1-15 ↔ H_3-17	H_1-15 → C-17
16	--	139.78, (=C<)	--	--
17	1.80, *s*	23.37, (-CH_3)	H_3-17 ↔ H_1-15, H_1-13	H_3-17 → C-13, C-15, C-16
18	0.81, *s*	28.22, (-CH_3)	H_3-18 ↔ H_3-19	H_3-18 → C-3, C-4, C-19
19	0.80, *s*	22.70, (-CH_3)	H_3-19 ↔ H_3-18, H_1-5	H_3-19 → C-3, C-4, C-5, C-18
20	0.73, *s*	17.66, (-CH_3)	--	H_3-20 → C-1, C-10
21	--	171.17, (>C=O)	--	--
22	1.98, *s*	21.47, (-CH_3)	--	H_3-22 → C-21

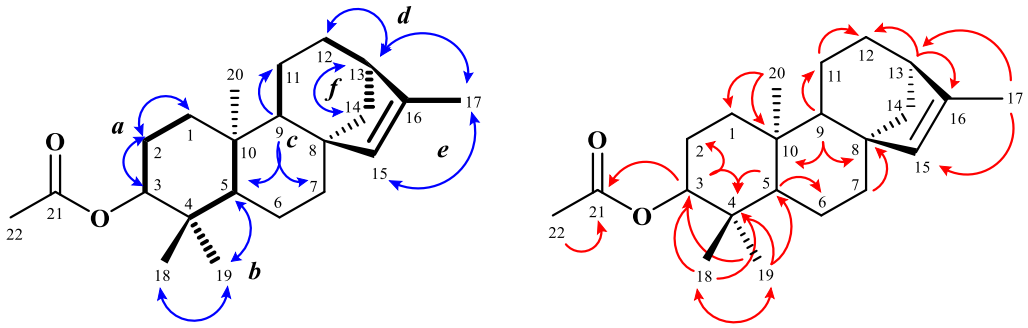

Figure 2. Selective ^{1}H↔^{1}H COSY (▬) and ^{1}H→^{13}C HMBC (▬) correlations of **(II)**.

Compound **(IV)** was isolated as a white powder, mp 142-144 °C. GC-MS analysis shown $[M]^+$ molecular ion at *m/z*: 288.3 (calcd. for $C_{20}H_{32}O$; *m/z:* 288). FT-IR, *v*max (cm^{-1}), functional group: 3406, *v*(O-H, alcohol); 1026, *v*(C-O); 1600, *v*(C=C). ^{1}H-NMR (400 MHz, $CDCl_3$) δ_H: 4.73 (H_1-17a, *s*, =CH), 4.79 (H_1-17b, *s*, =CH), 3.44 (H_1-19a, *dd*, CH_2), 3.74 (H_1-19b, *dd*, CH_2), 2.63 (H_1-13, *t*, C-H), 2.06 (H_2-15, *m*, CH_2), 1.01 (H_3-18, *s*, CH_3), 0.96 (H_3-20, *s*, CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$) δ_C: 65.60 (C-19, CH_2OH), 155.90 (C-16, >C=), 103.00 (C-17, $=CH_2$), 27.10 (C-18, CH_3), 18.21 (C-20, CH_3), 44.20 (C-13, CH), 49.10 (C-15, CH_2). By comparing this experimental data with the spectroscopic data reported in the bibliography (Bohlmann *et al*., 1980; Piozzi *et al*., 1971), it is evident that this compound is an *ent*-kaurene-type diterpene called *ent*-kaur-16-en-19-ol, commonly known as kaurenol. Currently, this diterpene is considered a widely distributed product in the Asteraceae family, since it has been isolated in different species, including the species of the *Espeletia* genus (Seaman *et al*., 1990), like *Espeletia semiglobulata* Cuatrec. (Usubillaga *et al*., 1988; Aparicio *et al*., 2013).

Antimicrobial Activity

The isolated compounds, *ent*-kaur-16-en-19-al **(I)**, *ent*-kaur-3-acetoxy-15-ene **(II)**, *ent*-kaur-18-*nor*-16-en-4-ol **(III)** and *ent*-kaur-16-en-19-ol **(IV)** were evaluated for *in vitro* growth inhibitory potential against different bacterial [*Staphylococcus aureus* (ATCC 25923),

Enterococcus faecalis (ATCC 29212), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 23357), *Pseudomonas aeruginosa* (ATCC 27853)] and fungal [*Candida krusei* (ATCC 6558)] strains, according to the previously described procedure to agar diffusion method with discs (strain of bacteria), and agar well diffusion method, (strain of fungi). The results in Table 2, shows that all the compounds are inactive againt the bacterial and fungal strains tested, except compound **(II)**, which showed growth inhibition against gram-negative bacterial strains (*Escherichia coli*: 8 mm, *Klebsiella pneumoniae*: 10 mm, *Pseudomonas aeruginosa*: 8 mm), as well as it showed growth inhibition against the fungal strain (*Candida krusei*: 8 mm), at a concentration of 2 mg/mL.

These results reveal a remarkable natural structure-activity relationship of the *ent*-kaurene core with regarding the C-3 position (A ring of perhydrophenanthrene unit), whose oxygenation or structural modification apparently, improves the antimicrobial activity. Similar results have been found synthetically, by preparing derivatives of hydroxylated *ent*-kaurenes at C-3, improving their biological activity (Ohkoshi *et al*., 2004). Moreover, the evaluation of the relationship between structure and antimicrobial activity of some diterpenes suggested that the presence of a substituted decalin skeleton (essential hydrophobic portion for crossing membranes), and a hydrophilic region bearing a donor or acceptor group able to interact with hydrogen-bond acceptor/donor groups on the membrane were necessary for their insertion in a phospholipid bilayer model. This feature enables the diterpenes to cross the bacterial cell membrane causing cell damage (Urzúa *et al.*, 2008).

In this context, the *ent*-kaurenes tested have the structural characteristics described above: a substituted decalinic system that confers a lipophilic character, and a hydrophilic region bearing a hydrogen-bond donor or acceptor group. However, the compounds **(I)**, **(III)** and **(IV)**, which possess these polar groups in the C-4 position, they are sterically limited to form hydrogen-bond with acceptor/donor groups on the microorganism membrane, and therefore cannot cause damage to it. On the contrary, compound **(II)** has this moderately hydrophilic group (ester) in C-3 position, whose steric hindrance is low and this confers the advantage to interact and establish hydrogen bonds with acceptor/donor groups on the microorganism membrane, being able to cross and cause damage to it. The position and steric hindrance of this hydrogen-bond donor or acceptor group on decalinic system are important characteristics for the biological activity of *ent*-kaurenes (Urzúa *et al.*, 2008).

Table 2. Antimicrobial activity of natural compounds **(I)-(IV)** expressed as diameters of the inhibition zone (mm).

Microorganism	Compound				Positive control					
	(I)	**(II)**	**(III)**	**(IV)**	**OX**	**VA**	**TO**	**AZ**	**CF**	**FL**
***Staphylococcus aureus* (ATCC 25923)**	IA	IA	IA	IA	*23	-	-	-	-	-
***Enterococcus faecalis* (ATCC 29212)**	IA	IA	IA	IA	-	*19	-	-	-	-
***Escherichia coli* (ATCC 25922)**	IA	8	IA	IA	-	-	*26	-	-	-
***Klebsiella pneumoniae* (ATCC 23357)**	IA	10	IA	IA	-	-	-	*34	-	-
***Pseudomonas aeruginosa* (ATCC 27853)**	IA	8	IA	IA	-	-	-	-	*32	-
***Candida krusei* (ATCC 6558)**	IA	8	IA	IA	-	-	-	-	-	*15

IA: inactive; OX: Oxacillin®; VA: Vancomycin®; TO: Tobramycin®; AZ: Aztreonam®; CF: Cefepime®; FL: Fluconazole®; *: Inhibition halo in mm, for control groups (CLSI, 2020).

CONCLUSIONS

Ent-kaurene-type diterpenes, *ent*-kaur-16-en-19-al **(I)**, *ent*-kaur-3-acetoxy-15-ene **(II)**, *ent*-kaur-18-*nor*-16-en-4-ol **(III)**, *ent*-kaur-16-en-19-ol **(IV)**, were isolated from the neutral fraction of *Espeletia semiglobulata* Cuatrec. leaves, being *ent*-kaur-3-acetoxy-15-ene **(II)** a natural product reported for the first time. Compound **(II)** was the only one that showed antimicrobial activity, but only against gram-negative bacterial strains (*Escherichia coli*: 8 mm, *Klebsiella pneumoniae*: 10 mm, *Pseudomonas aeruginosa*: 8 mm) and fungal strain (*Candida krusei*: 8 mm), at of 2 mg/mL concentration. These results reveal a remarkable natural structure-activity relationship of the *ent*-kaurene core with regarding the C-3 position (A ring of perhydrophenanthrene unit), whose oxygenation or addition of a hydrogen-bond acceptor or donor group, improves the antimicrobial activity.

***Acknowledgements*:** All authors are grateful to the Research Institute "Dr. Alfredo Nicolás Usubillaga del Hierro", Faculty of Pharmacy and Bioanalysis, University of Los Andes, Mérida 5101, Venezuela for the support. The Research Institute authors are grateful to the Prof. Freddy Ramos from the Department of Chemistry, Faculty of Science, National University of Colombia, Bogotá D.C. 111321, Colombia, for NMR and MS equipment. Besides, all authors are grateful to the MSc. Ana Deixy Flores from the "Instituto Clínico Santa Filomena", Microbiology Laboratory, Mérida 5101, Venezuela, for the antimicrobial assays.

***Competing Interests*:** The authors declare that there are no competing interests.

Funding This research was supported by Research Institute "Dr. Alfredo Nicolás Usubillaga del Hierro", Faculty of Pharmacy and Bioanalysis, University of Los Andes, Mérida 5101, Venezuela.

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