

SHORT REPORT

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publications

Rec. Nat. Prod. 11:3 (2017) 304-309

records of natural
products**Kaurane Diterpenes from the Fruits of *Zanthoxylum leprieurii* (Rutaceae)****Stephanie T. Guetchueng^{1,2}, Lutfun Nahar¹, Kenneth J. Ritchie¹, Fyaz M. D. Ismail¹, Jean D. Wansi^{1,3}, Andrew Evans¹ and Satyajit D. Sarker^{1*}**

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(Received January 09, 2017; Revised March 08, 2017; Accepted March 14, 2017)

Abstract: The fruits of *Zanthoxylum leprieurii* Guill. & Perr. (Rutaceae) are traditionally used in Africa, particularly in Cameroon, as a spice and in the treatment of sickle cell anaemia. Phytochemical investigation on the fruits of this plant afforded five kaurane diterpenes, *i.e.*, kaurenoic acid (**1**), xylopic acid (**2**), *ent*-kauran-16 β -ol (**3**), *ent*-16 β -hydroxykauran-19-al (**4**) and *ent*-16 β -hydroxykauran-19-oic acid (**5**). The structures of these diterpenes were determined comprehensively by spectroscopic means (1D and 2D NMR spectroscopy and MS analyses) and also by comparison with respective literature data. Among the isolated compounds, only kaurenoic acid (**1**) exhibited cytotoxicity against the PC3 cell line with an IC₅₀ value of 33.28 \pm 9.14 μ g/mL. To the best of our knowledge, this is the first report on the isolation of these kaurane diterpenes (**1-5**) from the genus *Zanthoxylum*.

Keywords: *Zanthoxylum leprieurii*; Rutaceae; kaurane; diterpene; fruit; traditional medicine. © 2017 ACG Publications. All rights reserved.

1. Plant Source

The fruits of *Zanthoxylum leprieurii* were procured from the Dschang market, Western Region of Cameroon in November 2015, and identified by Mr. Victor Nana, a taxonomist at the Cameroon National Herbarium, where a voucher specimen (HNC No 106669/SFRCAM) was deposited.

2. Previous Studies

Zanthoxylum leprieurii Guil. & Perr., also known as *Fagara leprieurii* Engl., a tree about 24 m tall from the family Rutaceae, is widespread throughout tropical Africa [1-3].

In Cameroon, the dried fruits are traditionally used as a spice in soups and their infusion is taken to treat sickle cell anaemia [2-4]. The leaves, barks and roots also have medicinal values, and are used as diuretics, laxatives and vermifuges, and to treat arthritis, leprosy, pains, rheumatism, stomach troubles and venereal diseases [3]. The plant is well-known as a large source of acridone and

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benzophenanthridine alkaloids, amides, coumarins and lignans [3-8]. A moderate level of anticancer and antimicrobial activities of the methanolic extract of the fruits have recently been reported [9].

3. Present Study

As a part of our continuing studies on the genus *Zanthoxylum* L. of the Cameroonian flora [1], we now report, for the very first time, on the isolation and structure determination of five kaurane diterpenes, *i.e.*, kaurenoic acid (**1**), xylopic acid (**2**), *ent*-kauran-16 β -ol (**3**), *ent*-kauran-16 β -ol-19-al (**4**) and *ent*-kauran-16 β -ol-19oic acid (**5**) from the fruits of *Z. leprieurii* Guill. & Perr. (Figure 1).

The air-dried and ground fruits (545.2 g) of *Z. leprieurii* were extracted with *n*-hexane using a Soxhlet extractor (800 mL, 10 cycles each). After evaporation at 35°C under reduced pressure, 32.7 g of an oily brown extract was obtained. A portion (20.0 g) of this extract was subjected to CC (6.5 cm x 50 cm) using a gradient system comprising *n*-hexane-ethyl acetate (EtOAc) (0-50%) and dichloromethane-methanol (0-50%) as eluents. A total of 53 sub-fractions (*ca.* 125 mL each) were collected and pooled on the basis of their analytical TLC profiles to six main fractions A-F. Fraction B (3.8 g, pooled sub-fractions 4-13) was further analysed with CC eluting with increasing amounts of EtOAc in *n*-hexane to afford kaurenoic acid (**1**, 120.3 mg) [10] and xylopic acid (**2**, 230.1 mg) [11]. Fraction C (1.9 g, pooled sub-fractions 14-17) was subjected to repeated CC as described above to yield *ent*-kauran-16 β -ol (**3**, 80.2 mg) [12] and *ent*-kauran-16 β -ol-19-al (**4**, 6.9 mg) [13]. Purification of fraction F (2.1 g, pooled sub-fractions 29-32) using CC with increasing amounts of EtOAc in *n*-hexane as an eluent afforded *ent*-kauran-16 β -ol-19-oic acid (**5**, 43.4 mg) [14]. The structures of the isolated diterpenes were elucidated unequivocally by spectroscopic means, mainly, ¹H NMR, ¹³C NMR, ¹H-¹H COSY, ¹H-¹³C HSQC, ¹H-¹³C HMBC, ¹³C DEPTQ, ¹H-¹H NOESY and mass spectrometry (MS). All spectroscopic data were also compared with respective literature data.

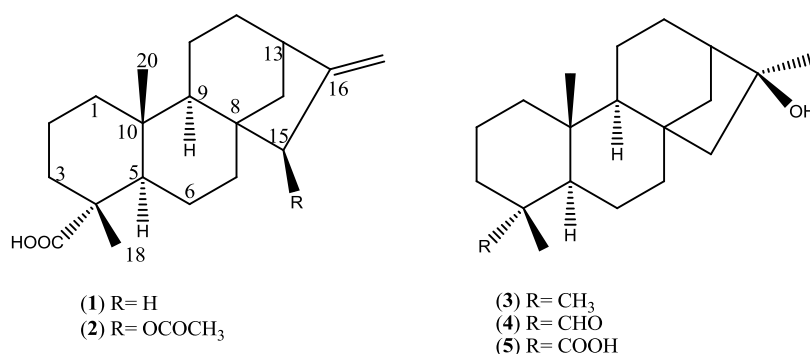


Figure 1. Kaurane diterpenes (**1-5**) from *Zanthoxylum leprieurii*

Kaurenoic acid (1): White crystals (120.3 mg); HRESIMS m/z 301.2170 [$M-H$]⁻ (calc. for C₂₀H₂₉O₂, 301.2173) in negative ion mode; ¹H NMR (300 MHz, CDCl₃) δ : 0.75 (1H, ddd, J = 13.0, 12.9, 5.5 Hz, H-1a), 0.92 (3H, s, H-20), 0.94 (1H, m, H-3a), 0.97 (1H, m, H-9), 1.03 (1H, m, H-5), 1.09 (1H, m, H-14a), 1.25 (3H, s, H-18), 1.36 (1H, m, H-2a), 1.38 (1H, m, H-12a), 1.40 (2H, m, H-7), 1.43 (2H, m, H-11), 1.47 (1H, m, H-12b), 1.71 (2H, m, H-6), 1.76 (1H, m, H-1b), 1.79 (1H, m, H-2b), 1.90 (1H, m, H-14b), 1.98 (2H, br s, H-15), 2.06 (1H, m, H-3b), 2.56 (1H, br t, H-13), 4.67 (1H, s, H-17a) and 4.72 (1H, s, H-17b); ¹³C NMR (75 MHz, CDCl₃) δ : 40.7 (C-1), 19.1 (C-2), 37.8 (C-3), 43.8 (C-4), 57.0 (C-5), 21.8 (C-6), 41.3 (C-7), 44.2 (C-8), 55.1 (C-9), 39.6 (C-10), 18.4 (C-11), 33.1 (C-12), 43.7 (C-13), 39.7 (C-14), 48.9 (C-15), 155.9 (C-16), 103.0 (C-17), 28.9 (C-18), 184.6 (C-19) and 15.6 (C-20).

Xylopic acid (2): White crystals (230.1 mg); HRESIMS m/z 361.2375 [$M+H$]⁺ (calc. for C₂₂H₃₃O₄, 361.2379) in positive mode; ¹H NMR (300 MHz, CDCl₃) δ : 0.91 (1H, ddd, J = 13.1, 8.8, 6.2 Hz, H-1a), 1.01 (3H, s, H-20), 1.08 (1H, m, H-3a), 1.04 (1H, m, H-5), 1.29 (1H, m, H-14a), 1.33 (3H, s, H-18), 1.35 (1H, m, H-9), 1.49 (1H, m, H-2a), 1.50 (2H, m, H-7), 1.55 (1H, m, H-11a), 1.61 (1H, m, H-

12a), 1.66 (1H, m, H-11b), 1.73 (1H, m, H-12b), 1.87 (1H, m, H-6a), 1.93 (1H, m, H-2b), 1.97 (1H, m, H-1b), 2.16 (1H, d, $J=3.5$ Hz, H-14b), 2.18 (1H, m, H-3b), 2.26 (3H, s, OCOCH₃), 2.26 (1H, m, H-6b), 2.68 (1H, br s, H-13), 4.88 (1H, br s, H-17a), 4.94 (1H, d, $J=2.6$ Hz, H-17b) and 5.16 (1H, t, $J=2.5, 4.9$ Hz, H-15); ¹³C NMR (75 MHz, CDCl₃) δ : 40.7 (C-1), 19.0 (C-2), 38.8 (C-3), 43.6 (C-4), 54.6 (C-5), 21.3 (C-6), 39.9 (C-7), 45.9 (C-8), 46.9 (C-9), 36.3 (C-10), 18.0 (C-11), 33.2 (C-12), 40.6 (C-13), 37.7 (C-14), 81.6 (C-15), 153.7 (C-16), 106.1 (C-17), 28.8 (C-18), 184.2 (C-19), 15.8 (C-20), 171.4 (OCOCH₃) and 21.3 (OCOCH₃).

ent-kauran-16 β -ol (**3**): White powder (80.2 mg); HRESIMS m/z 290.2843 [M-H₂O+NH₄]⁺ (calc. for C₂₀H₃₆N, 290.2848) in positive mode; ¹H NMR (300 MHz, CDCl₃) δ : 0.67 (1H, m, H-1a), 0.75 (1H, m, H-9), 0.78 (3H, s, H-19), 0.83 (3H, s, H-18), 0.97 (1H, m, H-5), 1.03 (3H, s, H-20), 1.08 (1H, m, H-3a), 1.28 (1H, m, H-6a), 1.31 (1H, m, H-7a), 1.38 (3H, s, H-17), 1.46 (1H, m, H-3b), 1.55 (2H, m, H-15), 1.57 (2H, m, H-11), 1.61 (1H, m, H-14a), 1.61 (2H, m, H-12), 1.63 (1H, m, H-2b), 1.65 (1H, m, H-7b), 1.59 (1H, m, H-6b), 1.76 (1H, m, H-2a), 1.76 (1H, m, H-1b), 1.84 (1H, m, H-13), and 1.93 (1H, m, H-14b); ¹³C NMR (75 MHz, CDCl₃) δ : 40.3 (C-1), 18.6 (C-2), 42.0 (C-3), 33.2 (C-4), 56.2 (C-5), 20.4 (C-6), 42.0 (C-7), 45.3 (C-8), 56.8 (C-9), 39.3 (C-10), 17.9 (C-11), 26.9 (C-12), 49.0 (C-13), 37.6 (C-14), 58.0 (C-15), 79.3 (C-16), 24.4 (C-17), 33.6 (C-18), 21.5 (C-19) and 17.7 (C-20).

ent-kauran-16 β -ol-19-al (**4**): White powder (6.9 mg); ¹H NMR (600 MHz, CDCl₃) δ : 0.84 (1H, ddd, $J=4.1, 4.0, 3.9$ Hz, H-1a), 1.05 (1H, m, H-9), 9.75 (1H, d, $J=1.4$ Hz, H-19), 1.05 (3H, s, H-18), 1.17 (1H, dd, $J=2.5, 2.1$ Hz, H-5), 0.89 (3H, s, H-20), 1.05 (1H, m, H-3a), 1.72 (1H, m, H-6a), 1.52 (1H, d, $J=3.8$ Hz, H-7a), 1.39 (3H, s, H-17), 2.16 (1H, m, H-3b), 1.60 (2H, m, H-15), 1.60 (2H, m, H-11), 1.64 (1H, m, H-14a), 1.59 (1H, m, H-12a), 1.66 (1H, m, H-2b), 1.72 (1H, m, H-7b), 1.91 (1H, m, H-6b), 1.62 (1H, m, H-12b), 1.66 (1H, m, H-2a), 1.86 (1H, m, H-1b), 1.88 (1H, m, H-13), and 1.94 (1H, m, H-14b); ¹³C NMR (150 MHz, CDCl₃) δ : 39.7 (C-1), 18.3 (C-2), 34.2 (C-3), 48.4 (C-4), 56.6 (C-5), 20.0 (C-6), 41.9 (C-7), 45.1 (C-8), 55.4 (C-9), 39.3 (C-10), 18.0 (C-11), 26.6 (C-12), 48.8 (C-13), 37.7 (C-14), 57.6 (C-15), 79.3 (C-16), 24.5 (C-17), 24.2 (C-18), 205.9 (C-19) and 16.4 (C-20).

ent-16 β -hydroxykauran-19-oic acid (**5**): White powder (43.4 mg); HRESIMS m/z 319.2273 [M-H]⁻ (calc. for C₂₀H₃₁O₃, 319.2279) in negative ion mode; ¹H NMR (300 MHz, CDCl₃ + a few drops of CD₃OD) δ : 0.87 (1H, m, H-1a), 0.78 (1H, m, H-9), 0.82 (3H, s, H-18), 0.84 (1H, m, H-5), 0.87 (3H, s, H-20), 0.78 (1H, m, H-3a), 1.49 (2H, m, H-6), 1.16 (1H, m, H-7a), 1.08 (3H, s, H-17), 1.96 (1H, m, H-3b), 1.20 (2H, s, H-15), 1.29 (2H, m, H-11), 1.29 (1H, m, H-14a), 1.20 (2H, m, H-12), 1.57 (1H, m, H-2b), 1.23 (1H, m, H-7b), 1.16 (1H, m, H-2a), 1.50 (1H, m, H-1b), 1.50 (1H, m, H-13), and 1.51 (1H, m, H-14b); ¹³C NMR (75 MHz, CDCl₃ + a few drops of CD₃OD) δ : 40.3 (C-1), 18.6 (C-2), 37.6 (C-3), 48.3 (C-4), 56.4 (C-5), 21.6 (C-6), 42.7 (C-7), 43.0 (C-8), 55.6 (C-9), 39.3 (C-10), 17.7 (C-11), 26.2 (C-12), 48.8 (C-13), 36.6 (C-14), 58.0 (C-15), 78.5 (C-16), 23.2 (C-17), 28.2 (C-18), 180.4 (C-19) and 14.8 (C-20).

Although all these compounds are known diterpenes previously isolated from various other plants, especially from the family Rutaceae [15-17], they have never been reported from the genus *Zanthoxylum* before. Therefore, to the best of our knowledge, this is the first report on the occurrence kaurane diterpenoids (**1-5**) in this genus. Kaurane diterpenes are mainly found in the Asteraceae, Annonaceae and Euphorbiaceae families among others [16]. First occurrence of kaurane diterpenes in the Rutaceae was the isolation of kaurenoic acid and its 15 β -hydroxylated derivative from the genus *Phebalium* [17]. Several kauranes were later isolated from the genus *Fortunella* [18] and we here report on the isolation of kaurane diterpenes for the third time from the Rutaceae. Diterpenes are rather rare in the Rutaceae and only a few have been reported from the genera *Citrus*, *Evodia*, *Glycosmis* and *Pamburus* [16, 19, 20], in addition to the kauranes cited above. The co-occurrence of the kaurane diterpene (**1**) in various genera of the Rutaceae might have some chemotaxonomic significance.

Cell viability assay: The *in vitro* cytotoxic activity of the isolated kaurane diterpenes (**1-5**) from *Z. lepreurii* was assessed against the human prostate cancer cell line PC3 and the human normal prostate

epithelium cell line PNT2. Both cell lines were grown in RPMI medium supplemented with L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 µg/mL) and 10% foetal bovine serum (FBS). The cells were cultured at 37°C in a 5% CO₂ atm., seeded into 24 wells plate (5×10⁴/well) and incubated for 24 h. Cells were treated with crude extract or isolated compounds (0 to 100 µg/mL) for PC3 cell line and (0 to 15 µg/mL) for PNT2 cell line. After treatment, plates were again incubated for 24 h and the cell viability was measured using the MTT assay [15]. The formazan crystals formed were dissolved in DMSO and optical density was read at 570 nm in a ClarioStar plate reader. Three individual wells were assayed per treatment; the assay was repeated three times and cytotoxic activity was determined as percentage of control cells [(absorbance of treated cells/absorbance of untreated cells) × 100]. Etoposide as used as positive control and the IC₅₀ value of each test sample was calculated using the software GraphPad Prism 7.02.

The cells were treated with the crude extract and all compounds for 24 h and the percentage of cell viability for both cell lines after treatment was compared with that of untreated control cells. The IC₅₀ value of each compound was calculated and results are presented in Table 1. Among the isolated compounds, only kaurenoic acid (**1**) exhibited cytotoxicity against the PC3 cell line killing nearly 92% of cancer cells at the concentration of 100 µg/mL with an IC₅₀ value of 33.28 ± 9.14 µg/mL with no effect on the PNT2 cells at the highest concentration tested. This result was in agreement with the work by Cuca *et al.* [21], where this compound was shown to possess a lethal cytotoxic effect on PC3 cell lines after 48 h of treatment [21]. Xylopic acid (**2**) did not show any activity for concentration below 100 µg/mL suggesting that the presence of the acetyloxy group on position C-15 might have modified the activity of the molecule. This result was similar to the findings for kaurenoic acid (**1**) and its 15β-hydroxy derivative in the tests for gibberellin activity [16].

Table 1. Antiproliferative activity of the isolated compounds (**1-5**) on human prostate cancer (PC3) and human normal prostate epithelium (PNT2) cell lines

| Compounds | IC ₅₀ (µg/mL) | |
|----------------------|--------------------------|--------------|
| | PC3 | PNT2 |
| 1 | 33.28 ± 9.14 | > 15 |
| 2 | > 100 | > 15 |
| 3 | > 100 | > 15 |
| 4 | > 100 | > 15 |
| 5 | > 100 | 10.66 ± 3.83 |
| Crude extract | 114 ± 0.008 | 135.5 ± 39.9 |

Kaurenoic acid (**1**) and xylopic acid (**2**) were the major compounds isolated from the *n*-hexane extract of *Z. leprieurii* in the present study. Both diterpenes were reported to have trypanocidal and antimicrobial activities [22-23]. Kaurenoic acid (**1**) is the raw material for the synthesis of other biologically active diterpenes [23] and possesses a wide range of interesting biological properties including antidiabetic, analgesic, anti-inflammatory, antioxidant and neurological activities [24]. This diterpene has also been shown to possess cytotoxic and antitumoral activities, inhibiting the growth of CEM-leukemic cells *in vitro* [25]. Kaurene diterpenes were reported to have antiproliferative properties against various cancer cell lines [26]. Thus, the presence of these diterpenes, especially **1** and **2**, might provide some scientific evidence in favour of the usage of *Z. leprieuri* in traditional medicine.

Statistical analysis: Data were expressed as means ± standard error of the mean (SEM). The graph was plotted using non-linear regression with the use of GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA).

Acknowledgements

Mass spectrometry data were acquired at the EPSRC UK National Mass Spectrometry Facility at Swansea University. The authors gratefully acknowledge the award of a Commonwealth PhD Scholarship from the UK Department for International Development to Stephanie T. Guetchueng (CMCS-2015-107).

Supporting Information

Supporting Information accompanies this paper on <http://www.acgpubs.org/RNP>

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