

## Antileishmanial Activity of a New *ent*-Kaurene Diterpene Glucoside Isolated from Leaves of *Xylopi*a *excellens* R.E.Fr. (Annonaceae)

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**Abstract:** This work describes a new *ent*-kaurene diterpene glucoside, 7 $\beta$ -O- $\beta$ -D-glucopyranoside-*ent*-kaur-16-ene, from the leaves of *Xylopi*a *excellens* R.E.Fr. (Annonaceae). The compound showed high *in vitro* antileishmanial activity (IC<sub>50</sub> of 15.23  $\pm$  0.64  $\mu$ g/mL) towards promastigote forms of *Leishmania amazonensis*, and low cytotoxicity against J774A1 cells (SI of 1.96).

**Keywords:** Annonaceae; *ent*-kaurene diterpene; *Leishmania amazonensis*; *Xylopi*a *excellens*. © 2018 ACG Publications. All rights reserved.

### 1. Plant Source

Leaves of *X. excellens* R.E.Fr. were collected in the Campus of the Universidade Federal do Amazonas (UFAM), in July 2011. The specimen was identified by Dr. Antonio Carlos Webber from UFAM. A voucher specimen was deposited in the herbarium of UFAM under registration number 8249

### 2. Previous Studies

*Xylopi*a is one of the most representative genera of the Annonaceae family and comprises approximately 157 tree and shrub species distributed in the pantropical region [1]. Several species are

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employed in popular medicine with a remarkable set of purposes [2,3]. Previous phytochemical investigations report this genus as source of biologically active diterpenes [4,5]. However, *Xylopi* *excellens* R.E.Fr. (Annonaceae), popularly known as “envira amarela”, is an Amazon species which has not yet been chemically and biologically studied.

Leishmaniasis has been considered the second most important protozoal disease by the World Health Organization (WHO). The search for new model compounds for the development of alternative drugs that exhibit potential leishmanicidal activity is growing, once causative agents of its various clinical manifestations present increasing rates of resistance to conventional treatments. Beyond this, the number of commercially available drugs for the treatment of chronic diseases still is below a satisfactory number [6]. In our continuous search for new biologically active compounds from Amazonian plants [7-9], this work reports a new *ent*-kaurene diterpene glucoside from *Xylopi* *excellens* R.E.Fr. and its evaluation against promastigote forms of *Leishmania amazonensis*.

### 3. Present Study

*General procedure:* One-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy data were acquired with a Bruker AVANCE III 600 NMR spectrometer, operating at 14.1 T, observing  $^1\text{H}$  at 600 MHz and  $^{13}\text{C}$  at 150 MHz. All  $^1\text{H}$  and  $^{13}\text{C}$  NMR experiments were obtained in  $\text{CD}_3\text{OD}$  or  $\text{CDCl}_3$ . Chemical shifts ( $\delta$ ) are given in ppm relative to the TMS and coupling constants ( $J$ ) are given in Hertz. High resolution atmospheric pressure chemical ionization mass spectrometry (APCI-HRMS) measurements were recorded on a Waters Synapt HDMS instrument with quadrupole time-of-flight geometry. Optical rotation was determined on a Jasco P-1020 polarimeter. Fourier transform infrared (FTIR) spectra were measured on a FTLA2000-104 spectrophotometer. Silica gel 60 (70-230 mesh) was used for column chromatography, while silica gel 60 F254 was used for analytical (0.25 mm) thin layer chromatography (TLC). The spots were revealed with vanillin-sulphuric acid reagent.

*Extraction and Isolation:* The plant material was dried over an oven with air circulation at 50 °C for 24 h and powdered. The powdered material (1,000 g) was macerated for three days with *n*-hexane (solvent renewal every day), being the extracts concentrated under reduced pressure at 50 °C and combined (13.2 g). An aliquot (5 g) was partitioned with hexane/10% aqueous methanol (1:1), yielding the hydroalcoholic fraction (1.7 g). The hydroalcoholic fraction was fractionated over a silica gel column eluted initially with *n*-hexane, followed by an increasing polarity gradient of ethyl acetate and methanol, affording **1** (AcOEt/MeOH 1:1, 148.5 mg).

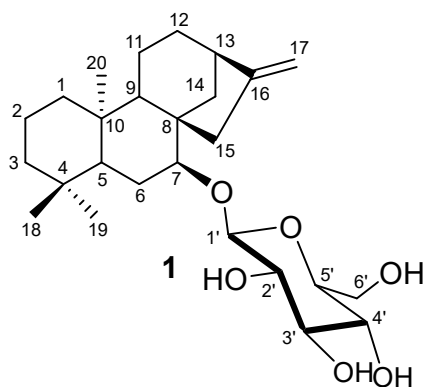
*Antileishmanial Activity Assay:* Promastigote forms of *Leishmania amazonensis* (MHOM/BR/75/JOSEFA) [10] were maintained by weekly transfers in Warren's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 25 °C in a tissue flask. *L. amazonensis* promastigotes ( $1 \times 10^6$  parasites/mL) with 48 h cultivated were inoculated in a 96-well plate containing Warren's medium supplemented with 10% inactivated fetal bovine serum with different concentrations of **1**, and incubated at 25 °C for 72 h. After treatment, the cells were incubated with XTT (0.5 mg/mL) and PMS (0.06 mg/mL) for 4 h at 25 °C. The viable cells were measured using a Bio-Tek Power Wave XS spectrophotometer, by absorbance at 450 nm. The results were expressed by  $\text{IC}_{50}$  (concentration that inhibited 50% parasite growth). Amphotericin was used as positive control.

*Cytotoxicity Assay:* J774A1 macrophages [11], obtained from confluent cultures, were cultured at  $5 \times 10^5$  cells/mL in RPMI 1640 medium supplemented with 10% FBS in 96-well microplates at 37 °C in a 5%  $\text{CO}_2$  atmosphere. After 24 h, the cells were treated with different concentrations of **1** and incubated for 48 h. The microplates were then washed with PBS, and incubated with MTT solution (2

mg/mL). The precipitate was solubilized in DMSO, and absorbance was read in a Bio-Tek Power Wave XS spectrophotometer at 492 nm. The cytotoxicity concentration for 50% ( $CC_{50}$ ) of the cells was determined. The selectivity index (SI) was calculated as  $CC_{50}/IC_{50}$ .

**Statistical Analysis:** The means and standard deviations were determined from at least three experiments. All tests were done in duplicate. Statistical analysis was performed with the program GraphPad Prism 4. Student's *t* test was applied, and a *p* value less than 0.05 was regarded as significant.

Compound diterpene glucoside was obtained as a colorless amorphous solid with molecular formula  $C_{26}H_{42}O_6$ , as determined by HRMS  $m/z$  451.3049  $[M+H]^+$  and NMR data. The IR spectrum of **1** shows typical absorptions of hydroxyl and C=C double bond groups. The  $^{13}C$  NMR spectrum showed 26 signals, including six typical signals for a glucose moiety ( $\delta_C$  63.2, 72.1, 76.1, 77.9, 78.4, and 106.6). Structure of the diterpene glucoside was identified as an *ent*-kaurene diterpene presenting the specific optical deviation ( $[\alpha] = -156.1^\circ$ ), based on NMR data and the spectroscopic pattern of diterpenes from the *Xylopi*a genus [12-14]. The  $^1H$  NMR spectrum shows typical methyl groups at  $\delta_H$  0.83 (H-19), 0.87 (H-18), and 1.07 (H-20), besides hydrogen signals of exocyclic double bond at  $\delta_H$  4.74 (H-17a) and 4.77 (H-17b) [15]. The hydrogen signal at  $\delta_H$  4.28 (1H, d,  $J = 7.7$  Hz, H-1') exhibits besides the characteristic chemical shift expected for  $\beta$  anomeric hydrogens, a significantly larger *J* value, which is consistent with the axial-axial coupling [15,16], supporting the presence of a  $\beta$ -D-glucose moiety. HMBC experiment shows correlations of the hydrogen signals at  $\delta_H$  4.74 (H-17a) and 4.77 (H-17b) with the carbon atoms at  $\delta_C$  34.8 (C-12), 45.3 (C-13), 46.7 (C-15), and 157.3 (C-16). The hydrogen signal at  $\delta_H$  2.37 (H-15) correlates with carbon signals at  $\delta_C$  40.0 (C-14), 45.3 (C-13), 89.7 (C-7), 103.6 (C-17), and 157.3 (C-16), confirming the *ent*-kaurene skeleton and suggesting the glucose presence at C-7 position. The glucose position was supported by the  $^1H$ - $^{13}C$  long-range correlation between H-1' ( $\delta_H$  4.28) and C-7 ( $\delta_C$  89.7), and correlations among H-7 ( $\delta_H$  3.47) and C-5 ( $\delta_C$  47.8), C-9 ( $\delta_C$  52.5), and C-1' ( $\delta_C$  106.6).



**Figure 1.**  $7\beta$ -*O*- $\beta$ -D-glucopyranoside-*ent*-kaur-16-ene isolated from *Xylopi*a *excellens*.

The relative stereochemistry of the diterpene moiety was based on 1D NOE NMR experiments, obtained in  $CDCl_3$ . The selective irradiation of the resonance frequency of the methyl hydrogen at  $\delta_H$  1.02 (H-20ax) shows mainly NOE enhancement on the signals at  $\delta_H$  1.90 (H-14ax), 1.71 (H-12ax) and 0.81 (H-19ax), which is compatible with *ent*-kaurane skeleton. The  $7\beta$  position for the glucosidic unit was established by the selective irradiation of the resonance frequency of the hydrogen signal at  $\delta_H$  3.44 (H-7), which shows NOE enhancement on the signal at  $\delta_H$  1.52 (H-6ax), 1.99 (H-6eq), 1.16 (H-14eq), and 2.26 (H-15), in the aglicone moiety. Considering the NMR data and comparison with literature data [14], the structure of **1** corresponds to a new diterpene glucoside ( $7\beta$ -*O*- $\beta$ -D-glucopyranoside-*ent*-kaur-16-ene).

*7β-O-β-D-glucopyranoside-ent-kaur-16-ene (1)*: Colorless amorphous solid.  $[\alpha] = -156.1$  (MeOH, c 0.005). IR (KBr)  $\nu_{\max} = 3377, 2922, 2872, 1054 \text{ cm}^{-1}$ . HRMS 451.3049  $[M+H]^+$  (calcd.  $m/z$  451.3060).  $^1\text{H NMR}$  (600 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  (ppm) = 0.80 (1H, m, H-1a), 0.83 (3H, s, H-19), 0.87 (3H, s, H-18), 1.07 (3H, s, H-20), 1.16 (1H, m, H-14a), 1.19 (1H, m, H-3a), 1.38 (1H, m, H-3b), 1.40 (1H, m, H-2a), 1.45 (1H, m, H-12a), 1.47 (1H, m, H-9), 1.52 (1H, dt,  $J = 13.0, 1.8 \text{ Hz}$ , H-6a), 1.58 (2H, m, H-11), 1.60 (1H, m, H-5), 1.68 (1H, m, H-2b), 1.74 (1H, d,  $J = 11.9 \text{ Hz}$ , H-12b), 1.81 (1H, m, H-1b), 1.93 (1H, m, H-14b), 2.10 (1H, m, H-6b), 2.37 (2H, m, H-15), 2.62 (1H, m, H-13), 3.44 (1H, m, H-4'), 3.46 (1H, m, H-3'), 3.47 (1H, m, H-7), 3.53 (1H, dd,  $J = 9.7, 7.7 \text{ Hz}$ , H-5'), 3.65 (1H, m, H-6'a), 3.72 (1H, m, H-6'b), 3.87 (1H, m, H-2'), 4.28 (1H, d,  $J = 7.7 \text{ Hz}$ , H-1'), 4.74 (1H, m, H-17a), 4.77 (1H, m, H-17b).  $^{13}\text{C NMR}$  (150 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  (ppm) = 18.4 (C-20), 18.9 (C-11), 20.0 (C-2), 22.5 (C-19), 27.6 (C-6), 34.2 (C-4), 34.2 (C-18), 34.8 (C-12), 40.0 (C-14), 40.4 (C-10), 41.7 (C-1), 43.5 (C-3), 45.3 (C-13), 46.7 (C-15), 47.8 (C-5), 50.1 (C-8), 52.5 (C-9), 63.2 (C-6'), 72.1 (C-2'), 76.1 (C-5'), 77.9 (C-4'), 78.4 (C-3'), 89.7 (C-7), 103.6 (C-17), 106.6 (C-1'), 157.3 (C-16).

Compound **1** was active against promastigote forms of *L. amazonensis*, being observed  $\text{CC}_{50}$  value of  $15.23 \pm 0.64 \mu\text{g/mL}$  [17]. Additionally, low cytotoxicity against J774A1 macrophages was observed with  $\text{CC}_{50}$  and SI values ( $29.97 \pm 2.72 \mu\text{g/mL}$  and 1.96, respectively). According literature parameters [17], SI values lower than 1 indicate that the analyte is more toxic to peritoneal macrophages than to the parasites. SI values greater than 1 indicate higher selectivity against the parasites than against the peritoneal macrophages.

Several terpenoids, such as sesquiterpenes, diterpenes, and triterpenes, have *in vitro* antileishmanial activity. However, *ent-kaurane* type and *kaurene* diterpenoids, including those which have sugar moiety, did not show significant activities on previous studies [18]. The activity recorded for **1** suggest that the position of glycoside moieties at C-7 can play an important role on the leishmanicidal activity. Further investigation is required in order to confirm this observation.

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## Supporting Information

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

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