SHORT REPORT

ACG publications

Rec. Nat. Prod. X:X (2017) XX-XX

records of natural products

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

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(Received June 19, 2017; Revised August 21, 2017; August 31, 2017)

Abstract: This work describes a new *ent*-kaurene diterpene glucoside, 7*β*-*O*-*β*-D-glucopyranoside-*ent*-kaur-16-ene, from the leaves of *Xylopia excellens* R.E.Fr. (Annonaceae). The compound showed high *in vitro* antileishmanial activity (IC_{50} of 15.23 ± 0.64 µg/mL) towards promastigote forms of *Leishmania amazonensis*, and low cytotoxicity against J774A1 cells (SI of 1.96).

Keywords: Annonaceae*; ent*-kaurene diterpene; *Leishmania amazonensis*; *Xylopia excellens.*

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

Xylopia 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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The article was published by ACG Publications
www.acgpubs.org/RNP © Published online 08/25/2017 EISSN:1307-6167
DOI: http://doi.org/10.25135/rnp.16.17.06.111

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, *Xylopia 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 *Xylopia 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}H at 600 MHz and ^{13}C at 150 MHz. All ^{1}H and ^{13}C NMR experiments were obtained in CD_3OD or $CDCl_3$. Chemical shifts (δ) 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 (1×10^6 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 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 $5x10^5$ cells/mL in RPMI 1640 medium supplemented with 10% FBS in 96-well microplates at 37 °C in a 5% 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. Students 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 (δ_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 ([α] = -156.1°), based on NMR data and the spectroscopic pattern of diterpenes from the *Xylopia* genus [12-14]. The ^1H NMR spectrum shows typical methyl groups at δ_H 0.83 (H-19), 0.87 (H-18), and 1.07 (H-20), besides hydrogen signals of exocyclic double bond at δ_H 4.74 (H-17a) and 4.77 (H-17b) [15]. The hydrogen signal at δ_H 4.28 (1H, d, *J* = 7.7 Hz, H-1`) exhibits besides the characteristic chemical shift expected for β anomeric hydrogens, a significantly larger *J* value, which is consistent with the axial-axial coupling [15,16], supporting the presence of a β-D-glucose moiety. HMBC experiment shows correlations of the hydrogen signals at δ_H 4.74 (H-17a) and 4.77 (H-17b) with the carbon atoms at δ_C 34.8 (C-12), 45.3 (C-13), 46.7 (C-15), and 157.3 (C-16). The hydrogen signal at δ_H 2.37 (H-15) correlates with carbon signals at δ_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` (δ_C 106.6).

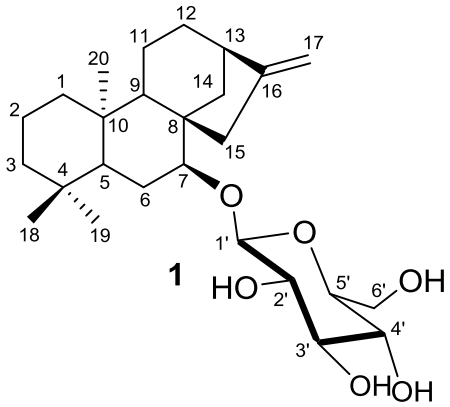

Figure 1. 7β-*O*-β-D-glucopyranoside-*ent*-kaur-16-ene isolated from *Xylopia excellens*.

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 δ_H 1.02 (H-20ax) shows mainly NOE enhancement on the signals at δ_H 1.90 (H-14ax), 1.71 (H-12ax) and 0.81 (H-19ax), which is compatible with *ent*-kaurane skeleton. The 7β position for the glucosidic unit was stablished by the selective irradiation of the resonance frequency of the hydrogen signal at δ_H 3.44 (H-7), which shows NOE enhancement on the signal at δ_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 (7β-*O*-β-D-glucopyranoside-*ent*-kaur-16-ene).

7β-O-β-D-glucopyranoside-ent-kaur-16-ene (***1***): Colorless amorphous solid. [α] = – 156.1 (MeOH, c 0.005). IR (KBr) υ_{max} = 3377, 2922, 2872, 1054 cm^{-1}. HRMS 451.3049 $[M+H]^+$ (calcd. *m/z* 451.3060). ^{1}H NMR (600 MHz, CD_3OD): δ (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 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 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 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 Hz, H-1`), 4.74 (1H, m, H-17a), 4.77 (1H, m, H-17b). ^{13}C NMR (150 MHz, CD_3OD): δ (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 CC_{50} value of 15.23 ± 0.64 μg/mL [17]. Additionally, low cytotoxicity against J774A1 macrophages was observed with CC_{50} and SI values (29.97 ± 2.72 μg/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.

Acknowledgments

The authors are grateful to to CAPES, CNPq, FINEP and FAPEAM for financial support.

Supporting Information

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

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