Supporting Information to:

Anti-leishmanial Lindenane Sesquiterpenes from *Hedyosmum angustifolium*

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Materials and Methods

Solvents were of analytical or HPLC grade and were purchased from Fisher Scientific Bioblock. Silica gel (Si60, 40–63 μm; Merck) was used for flash chromatography. Preparative TLC plates (Merck) were coated with 1 mm of silica gel 60 F_{254}.

Semi-preparative reversed-phase HPLC was performed on an X-terra RP_{18} column (10 μm, 19 × 250 mm; Waters). Semi-preparative normal-phase HPLC was performed on a Lichrosphere column (5 μm, 10.5 × 250 mm; Merck).

The typical procedure for flash column chromatography was as follows. The size of the column and fractions were chosen according to the quantity of extract: more than 4 g of extract: diameter 6 cm, fraction size 30 mL; between 1 g and 4 g of extract: diameter 4 cm, fraction size 20 mL; between 0.5 g and 1 g of extract: diameter 3 cm, fraction size 10 mL; between 200 mg and 500 mg of extract: diameter 2 cm, fraction size 7 mL; between 40 mg and 200 mg of extract: diameter 1 cm, fraction size 2–4 mL; <40 mg of extract: SPE Macherey–Nagel, 1 g SiO_2, fraction size 1 mL.

The quantity of silica corresponded to a 15-cm column height.

For the cyclohexane–ethyl acetate or cyclohexane–dichloromethane solvent systems, the polarity was increased by steps of 10% of the more polar solvent, unless otherwise stated. The volume of each eluent was twice the volume of the silica. Fractions were pooled together according to their TLC profile.

Extraction and isolation

The air-dried and powdered bark of *H. angustifolium* (200 g) was subjected to successive extraction with cyclohexane (2 L), ethyl acetate (2 L), and methanol (2 L). The isolation process was guided throughout by the results of the anti-leishmanial activity against cultured axenic amastigotes of *L. amazonensis.* The anti-leishmanial activity was found mainly in the ethyl acetate extract (IC_{50} 17 μg mL^{-1}). This extract (8.0 g) was fractionated by flash chromatography on silica gel using cyclohexane containing increasing amounts of ethyl acetate (10–70%). The anti-leishmanial activity was concentrated in fractions F2 (IC_{50} < 10 μg mL^{-1}) and F3 (IC_{50} < 10 μg mL^{-1}) eluted with 20–30% ethyl acetate.

Isolation of 1 and 3

Fraction F3 (1.1 g) was fractionated by flash chromatography on silica gel eluted with cyclohexane containing increasing amounts of ethyl acetate (10%, 20%, 30%, 35%, 50%, and 100%). The fractions eluted with 20% and 30% ethyl acetate (210 mg), which showed the best anti-leishmanial activity, were then submitted to flash column chromatography on silica gel eluted with cyclohexane containing increasing amounts of ethyl acetate (10%, 12%, 50%, and 100%). The fraction eluted with 12% ethyl acetate (140 mg) was then submitted to flash column chromatography on silica gel eluted with cyclohexane containing 10% ethyl acetate to give three fractions (F3.2.3.1–F3.2.3.3). F3.2.3.2 (91 mg) was purified on preparative thin-layer silica chromatography using a mixture of dichloromethane–ethyl acetate (90:10). Two fractions of 15 mg and 24 mg were obtained and finally purified by reversed-phase semi-preparative HPLC (gradient: water–acetonitrile, 70:30 to 40:60 in 40 min, flow rate 20 mL min^{-1}) to give compounds **1** (4 mg) and **3** (11 mg), respectively.

Isolation of 4

Fraction F2 (0.9 g) was fractionated by flash chromatography on silica gel using cyclohexane containing increasing amounts of ethyl acetate (3%, 4%, 8%, 10%, 15%, 25%, and 100%) to afford 7 fractions (F2.1–F2.7). The anti-leishmanial activity was concentrated in fraction F2.2 (IC_{50} 8 $\mu g\ mL^{-1}$) eluted with 4% ethyl acetate. Fraction F2.2 (163 mg) was further purified by flash chromatography on silica gel using a cyclohexane–ethyl acetate gradient (1%, 2%, 5%, 10%, 15%, 50%, and 100% ethyl acetate), yielding eight fractions (F2.2.1–F2.2.8). F2.2.3 (40 mg, eluted with 2% ethyl acetate) was pooled together with F2.2.4 (9 mg, eluted with 5% ethyl acetate). This mixture was further purified by flash chromatography on silica gel eluted with 99:1 cyclohexane–ethyl acetate, and four fractions were obtained (F2.2.3.1–F2.2.3.4). From F2.2.3.4 (24 mg) compound **4** (2.5 mg) was obtained after normal-phase semi-preparative HPLC (isocratic method dichloromethane–cyclohexane, 80:20, at 5 mL min^{-1} during 40 min).

Isolation of 2

The same purification protocol was used to give 1.2 g of F2, 343 mg of F2.3, and 278 mg of F2.5, starting from 13 g of ethyl acetate extract. Fraction F2.3 (343 mg) was further purified by flash chromatography on silica gel eluted with a mixture of cyclohexane–ethyl acetate

(5%, 20%, 25%, 35%, 50%, and 100% ethyl acetate), leading to six fractions (F2.3.1–F2.3.6). F2.3.4 (54 mg, eluted with 35% ethyl acetate) was further purified by flash chromatography on silica gel eluted with a mixture of cyclohexane–ethyl acetate (10–60% ethyl acetate), leading to five fractions (F2.3.4.1–F2.3.4.5). From F2.3.4.3 (12 mg, eluted with 40% ethyl acetate), compound **2** (6 mg) was purified on normal-phase SPE eluted with a mixture of cyclohexane and ethyl acetate (90:10).

Isolation of 5

Fraction F2.5 (278 mg) was further purified by flash chromatography on silica gel eluted with a mixture of cyclohexane–dichloromethane (20%, 22%, 25%, 30%, 50%, and 100% dichloromethane), leading to six fractions (F2.5.1–F2.5.6). F2.5.4 (43 mg, eluted with 30% dichloromethane) was further purified by flash chromatography on silica gel eluted with a mixture of cyclohexane–dichloromethane (30–70% dichloromethane), leading to three fractions (F2.5.4.1–F2.5.4.3). From F2.5.4.2 (30 mg), compound **5** (13 mg) was purified on normal-phase SPE eluted with a mixture of cyclohexane–ethyl acetate (97:3).

Anti-leishmanial assay

For the bioassay, the fractions were resuspended in DMSO at a concentration of 10 mg mL^{-1}. The anti-leishmanial activity was evaluated against axenic amastigotes of *L. amazonensis* (MHOM/BR/76/LTB-012) and the luciferase-expressing parasites of *L. infantum* (MHOM/MA/67/ITMAP-236). Axenically grown amastigote forms of *L. amazonensis* were maintained at 32 ± 1 °C, while *L. infantum* was maintained at 37 ± 1 °C with 5% CO_2 by weekly subpassage in a cell-free medium called MAA/20 (medium for axenically grown amastigotes) in 25-cm^2 flasks [1]. The *in vitro* anti-leishmanial activity against axenic amastigotes of *L. amazonensis* was determined after 72-h incubation by a colorimetric method based on the reduction of tetrazolium salt (MTT; Sigma) [2]. To measure the luciferase activity, we used axenic amastigotes of *L. infantum.* Dilutions of compounds were put in contact with the parasites and incubated for 72 h. Then the parasites were lysed with luciferase assay buffer, and the light output was quantified with a VICTOR luminometer. Amphotericin B (IC_{50} 0.23 ± 0.06 μM) and pentamidine (IC_{50} 1.7 ± 0.26 μM) were used as reference drugs.

Intracellular amastigotes of *L. infantum* recombinants stably expressed the firefly luciferase gene in a human leukemia monocyte cell line (THP-1 cells) [3]. The THP-1 cells in the log phase of growth were differentiated by incubation for 2 days in medium containing 40 ng mL^{-1} PMA (Sigma). After infection with stationary-phase extracellular amastigotes, with a parasite/macrophage ratio of 16:1 for 4 h at 36 ± 1 °C with 5% CO_2, non-internalized parasites were washed with RPMI medium. Serial dilutions of each drug were made in the RPMI medium supplemented with 10% fetal calf serum (FCS; Cambrex) and were dispensed in wells. After different incubation periods (24, 48, 72, and 96 h) of drug exposure, luciferase activity was determined as described.

Antiplasmodial and cytotoxic assay

P. falciparum was cultured according to the method described by Trager and Jensen [4] with modifications [5]. Cultures were synchronized by 5% D-sorbitol lysis (Merck). FcB1-Columbia was considered a chloroquine-resistant strain (chloroquine IC_{50} 145 ± 11.2 nM). *In vitro* anti-malarial activity was performed by [^{3}H]-hypoxanthine (ICN) incorporation as described by Desjardin et al. [6]. The incubation time between parasite culture and the drugs was 48 h. The cytotoxicity of the drugs was estimated on human breast cancer cells (MCF-7), African green monkey kidney epithelial cells (VERO), and a human leukemia monocyte cell line (THP-1 cells). These three cell lines were cultured in the same conditions as *P. falciparum*, except for the 5% FCS, and incubated under standard conditions (36 ± 1 °C, 5% CO_2). After addition of drugs at various concentrations, cell growth was estimated by [^{3}H]-hypoxanthine incorporation after 48 h incubation.

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