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Evaluation of antiplasmodial and antileishmanial activities of herbal medicine *Pseudelephantopus spiralis* (Less.) Cronquist and isolated hirsutinolide-type sesquiterpenoids

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ABSTRACT

Ethnopharmacological relevance: *Pseudelephantopus spiralis* (Less.) Cronquist is distributed in the Caribbean, Mesoamerica and Latin America. Preparations of the plant are traditionally used in Latin America for the treatment of various diseases including fever, malaria, and spleen or liver inflammations.
Materials and methods: Aerial parts of *P. spiralis* were extracted with either ethanol or distilled water. Seven hirsutinolide-type sesquiterpenoids were isolated: 8-acetyl-13-ethoxypiptocarphol (**1**), diacetylpiptocarphol (**2**), piptocarphins A (**3**), F (**4**) and D (**5**), (1S*,4R*,8S*,10R*)-1,4-epoxy-13-ethoxy-1,8,10-trihydroxygermacra-5*E*,7(11)-dien-6,12-olide (**6**), and piptocarphol (**7**). Extracts and isolated compounds (**2, 3, 5–7**) were screened for their in vitro antiplasmodial activity against the chloroquine-resistant *Plasmodium falciparum* strain FcM29-Cameroon and antileishmanial activity against three stages of *Leishmania infantum*. Their cytotoxicities were also evaluated against healthy VERO cell lines and J774A.1 macrophages, the host cells of the *Leishmania* parasites in humans.
Results: Aqueous extracts showed a greater inhibitory effect than alcoholic extracts, with IC_{50} on *P. falciparum* of 3.0 μg/mL versus 21.1 μg/mL, and on *L. infantum* of 13.4 μg/mL versus >50 μg/mL. Both extracts were found to be cytotoxic to VERO cells ($CC_{50}<3$ μg/mL). Sesquiterpene lactones **2** and **3** showed the best activity against both parasites but failed in selectivity. Carbon 8 hydroxylated hirsutinolides **5–7** presented the particularity of exhibiting two conformers observed in solution during extensive NMR analyses in CD_3OD and UHPLC-MS. The presence of a hydroxyl function at C-8 decreased the activity of **5–7** on the two parasites and also on VERO cells.
Conclusion: The antiplasmodial activity displayed by the aqueous extract explains the traditional use of *P. spiralis* in the treatment of malaria. This activity seems to be attributable to the presence of sesquiterpene lactones **2** and **3**, the most active against *P. falciparum*. Aqueous extract and compounds **2**, **3** and **6** were also active against *L. infantum* but lacked in selectivity due to their cytotoxicity towards macrophages. Exploring the safety and antiplasmodial efficacy of this traditional remedy will require further toxicological and in vivo studies in the light of the cytotoxicity towards healthy cell lines displayed by the aqueous extract and compounds **2** and **3**.

1. Introduction

Tropical protozoan diseases are currently a major public health problem throughout the world. Malaria, caused by *Plasmodium* species is the most devastating parasitic disease with 198 million cases estimated in 2013 and about 600,000 deaths mainly due to *Plasmodium falciparum* (WHO | Malaria Fact sheet N 94, 2014). Leishmaniasis, identified as a "Neglected Tropical Disease" by the World Health Organization, is endemic in 98 countries and territories, with a number of new cases estimated at 1.3 million

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per year (WHO | Leishmaniasis Fact sheet N 375, 2014). *Leishmania infantum,* the agent responsible for the lethal visceral form of Leishmaniasis (Romero and Boelaert, 2010) is present in Latin America and in the Old World (WHO Expert Committee on the Control of the Leishmaniases and Meeting, 2010). Many plants are traditionally used in South American folk medicine for the treatment of these two important parasitoses (Domingues Passero et al., 2014; Kvist et al., 2006; Valadeau et al., 2009; Willcox and Bodeker, 2004). Most of the drugs marketed for use against malaria are directly or indirectly derived from natural products (Cragg and Newman, 2013), and plant secondary metabolites still constitute a promising source of new antiprotozoal leads and/or drugs (Fournet and Munoz, 2002; Schmidt et al., 2012a, 2012b; Singh et al., 2014).

Pseudelephantopus spiralis (Less.) Cronquist belongs to the *Asteraceae* family and is one of the two species that make up the genus *Pseudelephantopus* (Pruski, 2011). The plant is distributed in the Caribbean, Mesoamerica and Latin America (Macbride, 1936), and is considered a weed (Canizales et al., 2010; Ochoa and Andrade, 2003; Tye, 2001). The species, named *Mata pasto* or *Pasto mula,* is traditionally used in Peru to treat menstrual cramps and as liver depurative (Internal report: base de datos de las plantas medicinales 2010, IIAP, 2014). In southern Equator the whole plant is cooked and used by oral administration to treat fever, high blood pressure, shivering fits and malaria (Bussmann and Sharon, 2006). In Colombia, infusions of roots or whole plant were dispensed as a beverage or in baths, in combination with other plants, to treat malaria, spleen and liver inflammations (Blair Trujillo and Madrigal, 2005). Moreover *Pseudelephantopus spicatus* (Juss. ex Aubl.) C.F. Baker, the second and only other species in the *Pseudelephantopus* genus, is also used in Peruvian Amazonia as a traditional antileishmanial remedy (Odonne et al., 2013, 2011). A previous phytochemical investigation of this latter species led to the isolation of two hirsutinolide-type sesquiterpene lactones exhibiting strong antileishmanial activities (Odonne et al., 2011). Based on this result, *P. spiralis* was expected to synthesize structurally related active compounds hence we decided to conduct the present phytochemical investigation of *P. spiralis* to evaluate its antiplasmodial and antileishmanial potential and thus confirm the relevance of its traditional use. In this context, aqueous and ethanolic extracts of the aerial parts of *P. spiralis* were prepared and bio-guided assay led to the isolation and identification of seven hirsutinolide-type sesquiterpenes lactones. They were identified as 8-acetyl-13-ethoxypiptocarphol (**1**), diacetylpiptocarphol (**2**), piptocarphins A (**3**), F (**4**) and D (**5**), (1S*,4R*,8S*,10R*)-1,4-epoxy-13-ethoxy-1,8,10-trihydroxygermacra-5*E*,7(11)-dien-6,12-olide (**6**), and piptocarphol (**7**). The extracts and pure compounds were tested for their in vitro antiprotozoal activities against two parasite species: a chloroquine-resistant strain of *P. falciparum* and the promastigote and axenic amastigote stages of *L. infantum*. The samples were also assessed for their cytotoxicity on VERO cells and macrophages in order to determine their selectivity indices.

2. Materials and methods

2.1. Plant material

Two batches of *P. spiralis* (Less.) Cronquist aerial parts were collected from a permanent plot in the Medicinal plant garden of the Reserva Allpahuayo-Mishana, Carretera Iquitos-Nauta, kilometre 28, Loreto, Peru, in May 2012 and May 2013. Plant authentication was confirmed during the collection by Elsa Rengifo, botanist at the Instituto de Investigaciones de la Amazonía Peruana (IIAP). A voucher is kept as reference in Université Paul Sabatier (Toulouse, France), faculty of pharmaceutical science, department of pharmacognosy under the number CG111.

2.2. General experimental procedure

The ^{1}H NMRand ^{13}C NMR spectra (Figs. S1–S19 in the supplementary data) were recorded on Bruker Avance 300 MHz or 500 MHz instrument with samples diluted in $CDCl_3$ or CD_3OD. Mass spectra were acquired using a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific). The system was equipped with an electrospray source operating in the positive ion mode. The apparatus was controlled by Xcalibur software version 2.0.7. Chromatography columns were filled with Merck silica gel 60A (40–63 μm). Reverse phase separations were performed on Agilent C18 Bond Elut SPE cartridge using a Varian Vac Elut SPS 24 Manifold system, and a ILMVAC LVS 101 Pump. Analytical TLC was achieved on precoated Kieselgel 60 F_{254} 20×20 cm^2 plates (Merck, 0.5 mm thin) using UV 254 nm and 1% vanillin/10% sulphuric acid reagent in EtOH for visualization. Chlorophylls were removed on Fisher Chemical activated charcoal. Freeze drying was performed on a Labconco™ Lyph-Lock 6™ 7753001 lyophilizer. Preparative HPLC were carried out on a LaChrom Merck Hitachi system, consisting of a LaChrom L-7100 Pump, a L-7455 DAD, a D-7000 Interface and using a Phenomenex Luna 5 μ C18(2) 100 Å, 250×10 mm^2 column.

2.3. Drugs or reagents

Solvents CH_2Cl_2, CH_3CN, and MeOH (HPLC grade) were purchassed from Fisher Chemical (Fischer Scientific, France). Toluene, ethyl acetate and petroleum ether were analytical reagent grade from Fisher Chemical (Fisher Scientific, France). Formic acid (99 – 100%), 95% ethanol technical grade were from Prolabo (VWR Chemicals, France). Sulphuric acid 96% and vanillin 99% used for CCM development were from Acros Organics (Fisher Scientific, France). Extraction and separation used distilled water. High-purity water (18.2 MΩ cm; total organic carbon: 2 ppb) was obtained from a Milli-Q water purification system (Millipore, Merck, France) and used for analytical and preparative HPLC. DMSO used for biological assays was from Fisher Bioreagents (Fischer Scientific, France). Reference drugs amphotericin B, miltefosin, pentamidine, doxorubicin, chloroquine, artemisinin and antibiotics penicillin, streptomycin and geneticin as MTT reagent (> 97.5%) used for the biological assays were purchased from Sigma-Aldrich (France). *Media* RPMI 1640 and MAA-20, foetal calf serum (FCS) and L-glutamine used for the biological assays were purchased from Gibco (Fischer Scientific, France).

2.4. Preparation of the crude extracts

Aerial parts of the two batches of *P. spiralis* were dried in the dark, at room temperature before being reduced to powder.

Preparation of the aqueous extract: Powdered aerial parts (353 g) were extracted with boiling distilled water (4 L) for 10 min. The aqueous extract was then frozen at – 20 °C and lyophilized for 230 h to give 27.0 g of residue (yield 7.6% w/w). The extract was then stored in the dark in sterile universal tubes until use.

Preparation of the ethanolic extract: Powdered aerial parts (2.1 g) were extracted under stirring with 95% EtOH (80 mL) for 24 h at room temperature. Solvent was removed under reduced pressure at 40 °C to give 71 mg of dry residue (3.4% w/w). The extract was then stored as described above.

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2.5. Isolation and identification of pure compounds

Procedure A: isolation of compounds **1, 3, 4, 5, 6**, **7**: Powdered aerial parts (374 g) were extracted with EtOH (5 × 3 L) at room temperature for 48 h to give 30.7 g of dry residue (yield 8.2% w/w). The residue was suspended in 800 mL of a H_2O–MeOH 3:1 mixture and extracted with petroleum ether (6 × 600 mL). The lower phase was discarded and evaporation under reduced pressure of the upper phase gave rise to 4.32 g of residue which was chromatographed on a silica gel (200 g) column using a toluene gradient containing increasing amounts of ethyl acetate (0–100%) followed by ethyl acetate–MeOH 1:1 as eluting solvents. This afforded 12 fractions. In order to eliminate chlorophylls and other pigments, fractions 7–10 (80% toluene to 20%) were pooled (968.2 mg) and suspended in 500 mL MeOH containing 4 g of active charcoal for 1 h. The suspension was filtered on 0.45 μm nylon filter and a chlorophyll free residue (322 mg) was obtained after evaporation of the methanol. It was suspended in 2 mL of MeOH–H_2O 1:1 and subjected to SPE C18 cartridge (10 g) under vacuum (P=5 mm Hg) then eluted with increasing amounts of MeOH in H_2O (50–100%). This afforded 13 fractions (A–M). Fraction A (113 mg) was chromatographed on SPE C18 cartridge (10 g) with H_2O and increasing amounts of MeOH to give 15 fractions (A1–A15). Fraction A3 was identified as **7** (0.5 mg). Fractions A7 and A9 were identified as **5** (4.5 mg) and **6** (6.5 mg), respectively. Semi-preparative HPLC (MeCN-0.1% HCOOH in H_2O, 1:1, 3 mL/min) of the pooled fractions A12 (10.2 mg) and A13 (3.9 mg) afforded 2.3 mg of a mixture containing **2** (R_t=7.8 min) and 4.7 mg of **1** (R_t=9.9 min). Semi-preparative HPLC (MeCN-0.1 % HCOOH in H_2O, 11:9, 3 mL/min) performed on pooled fractions B (6.8 mg) and C (21.7 mg) gave **3** (7.2 mg, R_t=20.4 min) and a fraction containing **4** as a main compound. Finally, the latter fraction (3.2 mg) was pooled with fractions D (3.1 mg) and A15 (1.4 mg) and separated by semi-preparative HPLC (MeCN-0.1 % HCOOH in H_2O, 3:2, 3 mL/min) to yield **4** (2.8 mg, R_t=12.5 min).

Procedure B: isolation of **2** *and* **3**: The entire crude aqueous extract (27.0 g) was suspended in H_2O (200 mL) and extracted by CH_2Cl_2 (6 × 200 mL). Evaporation of the CH_2Cl_2 layer under reduced pressure gave rise to 0.62 g of residue which was chromatographed on SPE C18 cartridge (10 g) under vacuum (P=5 mm Hg) with H_2O and increasing amounts of MeCN to give 11 fractions (F1–F11). Fraction F8 (95 mg) was chromatographed on a 30 × 1.5 cm column, containing silica gel (10 g) and eluted with CH_2Cl_2 with increasing amounts of MeOH (0–100%). This afforded 7 fractions (F81–F87). Fraction F81 (38 mg) was pure and contained **3.** Fractions F6 (185 mg) and F7 (135 mg) were pooled and chromatographed on a 30 × 1.5 cm silica gel (10 g) column eluted with CH_2Cl_2 with increasing amounts of MeOH (0–100%) to give 8 fractions (I–VIII). Fraction III (125 mg) was re-chromatographed on the same column and gave 6 sub-fractions (III-1 to III-6). Fraction III-4 was identified as compound **2** (50.8 mg).

2.6. Biological tests

2.6.1. Antileishmanial evaluation

2.6.1.1. Antileishmanial activity on promastigotes. The effects of the various samples on the growth of promastigotes of *L. infantum* (MHOM/MA/67/ITMAP-263, CNR *Leishmania*, Montpellier, France) were assessed using promastigotes expressing luciferase activity. Briefly, promastigotes in log-phase in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin and 50 μg/mL geneticin), were incubated at an average density of 10^6 parasites/mL in sterile 96-well plates with various concentrations of compounds dissolved in DMSO (final concentration less than 0.5% v/v), in triplicate. Appropriate controls treated by DMSO and amphotericin B, miltefosine and pentamidine (reference drugs purchased from SigmaAldrich) were added to each set of experiments. After a 72 h incubation period at 24 °C, each plate well was microscope-examined to detect the possible formation of precipitate. To estimate the luciferase activity of promastigotes, 80 μl of each well were transferred to white 96-well plates, Steady Glow reagent (Promega) was added according to the manufacturer's instructions, and the plates were incubated for 2 min. The luminescence was then measured in a Microbeta Luminescence Counter (PerkinElmer). Inhibitory concentration 50% (IC_{50}) was defined as the concentration of drug required to inhibit by 50% of the metabolic activity of *L. infantum* promastigotes compared to the control.

2.6.1.2. Antileishmanial activity on axenic amastigotes. L. infantum promastigotes in log phase were centrifuged at 900*g* 10 min, cell medium was then replaced by MAA-20 medium and maintained at 37 °C, 5% CO_2 to induce transformation into axenic amastigote forms (Sereno and Lemesre, 1997). Axenic amastigotes were incubated at an average density of 4.10^6 cells/mL in sterile 96-well plates with various concentrations of compounds dissolved in DMSO (final concentration 1% v/v), in duplicate. Appropriate controls treated by DMSO and amphotericin B, miltefosine and pentamidine were added to each set of experiments. After a 72 h incubation period at 37 °C, 5% CO_2, the effects of the tested compounds were evaluated by estimation of the luciferase activity of the axenic amastigotes. Eighty microlitre of each well were transferred to white 96-well plates, Steady Glow reagent (Promega) was added according to the manufacturer's instructions, and the plates incubated for 2 min. The luminescence was measured in a Microbeta Luminescence Counter (PerkinElmer). Inhibitory concentration 50% (IC_{50}) was defined as the concentration of drug required to inhibit 50% of the metabolic activity of *L. infantum* amastigotes compared to the control.

2.6.1.3. Antileishmanial activity on intramacrophagic amastigotes. The effects of the compounds tested on the growth of *L. infantum* intracellular amastigotes were assessed in the following way. One hundred microlitre of J774A.1 cells were seeded in 96-well plates at an average density of 2.10^5 cells/mL and incubated for 24 h at 37 °C under 5% CO_2. *L. Infantum* promastigotes were centrifuged at 900*g* for 10 min and the supernatant replaced by the same volume of RPMI 1640, 10% FCS, pH 5.4 and incubated for 24 h at 27 °C. Then, half the macrophage medium was removed, J774A.1 cells were then infected by 100 μl of acidified promastigotes at an average density of 2.10^6 cells/mL (10:1 ratio) and plates incubated for 24 h at 37 °C. Half of the infected macrophage medium was removed and medium containing various concentrations of test compounds was added in duplicate. Appropriate controls treated with or without solvent (DMSO), and various concentrations of amphotericin B were added to each set of experiments. After 120 h incubation at 37 °C and 5% CO_2, 100 μl of well supernatant was removed and Steady Glow reagent (Promega) was added according to the manufacter's instructions. The plates were incubated for 3 min. Hundred microlitre of each well were transferred to white 96-well plates and the luminescence was measured in a Microbeta Luminescence Counter (PerkinElmer). Inhibitory concentration 50% (IC_{50}) was defined as the concentration of drug required to inhibit by 50% the metabolic activity of *L. Infantum* intracellular amastigotes compared to the control. IC_{50} were calculated by non-linear regression analysis processed on dose–response curves, using Table Curve 2D V5 software. IC_{50} values represent the means calculated from three independent experiments.

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2.6.2. Cytotoxicity evaluation on macrophages and VERO cell lines

The evaluation of cytotoxicity by MTT assay on the J774A.1 cell line (mouse macrophage cell line, Sigma-Aldrich) and VERO cells (monkey epithelial cell line, Sigma-Aldrich) was done according to Mosmann (1983) with slight modifications. Briefly, cells (5.10^4 cells/mL) in 100 μL of complete medium, [RPMI 1640 supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) for J774A.1 cell line and MEM with 10% foetal calf serum (FCS), 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), NEAA 1X for VERO cell line] were seeded into each well of 96-well plates and incubated at 37 °C, 5% CO_2. After 24 h incubation, 100 μL of medium with various product concentrations and appropriate controls (DMSO and doxorubicin) were added and the plates were incubated for 72 h at 37 °C, 5% CO_2. Each plate well was then microscope-examined to detect possible precipitate formation before the medium was pipetted out of the wells. One hundred microlitre of MTT solution (0.5 mg/mL in RPMI 1640) was then added to each well and the cells incubated 2 h at 37 °C. After this time, the MTT solution was removed and DMSO (100 μL/well) was added to dissolve the resulting formazan crystals. Plates were shaken vigorously for 5 min. Absorbance was measured at 570 nm with a microplate spectrophotometer (EON). Inhibitory concentration 50% (IC_{50}) was defined as the concentration of drug inducing 50% death of macrophages J774A.1 or VERO cells compared to the control.

2.6.3. Antiplasmodial evaluation

The antiplasmodial activity was assessed on the chloroquine-resistant *P. falciparum* strain FcM29-Cameroon, cultured continuously according to the method of Trager and Jensen (1976), in a 5% CO_2 atmosphere at 37 °C as previously described (Benoit-Vical et al., 2007). Briefly, the parasites were maintained in vitro in human red blood cells and diluted in RPMI 1640 medium, supplemented with 25 mM HEPES, 2.05 mM L-glutamine and completed with 5% human serum (French Blood Bank, EFS). The antiplasmodial activity was assessed as previously reported by Desjardins et al. (Desjardins et al., 1979) and modified as follows. Extract dilutions and compounds were tested 3 times independently, each dilution in triplicate, in 96-well plates with cultures at a parasitaemia of 1% and a haematocrit of 1%. For each test, the plates of parasite culture were incubated with products for 48 h and tritiated hypoxanthine (PerkinElmer, France) was added to the medium 24 h after the beginning of incubation (Benoit-Vical et al., 2007). The parasite culture control (with solvent only) was referred to as 100% growth. Parasite growth was estimated by [^{3}H]-hypoxanthine incorporation. Inhibitory concentration 50% (IC_{50}) was defined as the concentration of drug required to inhibit 50% of the metabolic activity of *P. falciparum* compared to the control.

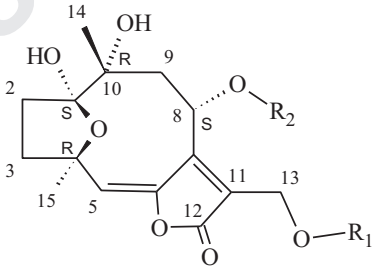
2.6.4. IC_{50}, CC_{50} and selectivity index (SI) calculation

IC_{50} on *L. infantum* and CC_{50} on macrophages and VERO cells were calculated by non-linear regression analysis processed on dose–response curves, using Table Curve 2D V5 software. IC_{50} values represent the mean value calculated from three independent experiments. Results were expressed as means followed by standard deviation. The selectivity index (SI) value allowed the comparison of the toxicity of the extracts or compounds against normal cells compared to the activity against the parasites in order to assess of their selectivity as antiparasitic. The SI on *L. infantum* was calculated as the *ratio* between the CC_{50} values against macrophages and IC_{50} values against promastigote forms. The SI on *P. falciparum* was calculated as the *ratio* between the CC_{50} values against VERO cells and IC_{50} values against *P. falciparum*. In both cases, $SI = CC_{50}/IC_{50}$.

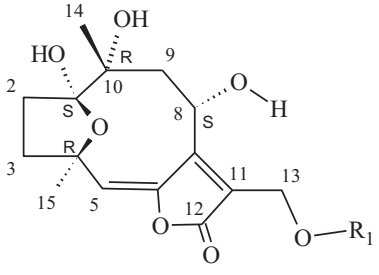
3. Results

3.1. Structural elucidation of isolated compounds by NMR analysis

Hirsutinolides **1**–**7** (Fig. 1) were clearly identified mainly on the basis of their 1D (^{1}H NMR, ^{13}C NMR) and 2D (HSQC and HMBC) NMR parameters, in $CDCl_3$. They were compared with NMR data published in the literature in the same solvent. This permitted the identification of 8-acetyl-13-ethoxypiptocarphol (**1**) ((Catalán et al., 1986), corrected in (Catalán et al., 1988)), diacetylpiptocarphol (**2**) ((Catalán et al., 1986), corrected in (Bardón et al., 1993)), piptocarphin A (**3**), piptocarphin F (**4**), piptocarphin D (**5**) ((Cowall et al., 1981), corrected in (Catalán et al., 1988)), (1S*,4R*,8S*,10R*) - 1,4-epoxy-13- ethoxy-1,8,10-trihydroxygermacra-5*E*,7 (11)-dien-6, 12-olide (**6**) (Kotowicz et al., 1998), and piptocarphol (**7**) (Bardón et al., 1993). In the course of our search for the improvement of NMR spectra of some hirsutinolides presenting enlargements of ^{1}H NMR signals (Fig. S2 Supplementary data), NMR analyses were run in CD_3OD while $CDCl_3$ and C_6D_6 were generally the solvents



	R_1	R_2
1	CH_2CH_3	$COCH_3$
2	$COCH_3$	$COCH_3$
3	$COCH_3$	$COC(CH_3)=CH_2$
4	CH_2CH_3	$COC(CH_3)=CH_2$



	R_1
5	$COCH_3$
6	CH_2CH_3
7	H

Fig. 1. Isolated compounds from *Pseudelephantopus spiralis* (Less.) Cronquist

prevailing in the literature (Bardón et al., 1993; Catalán et al., 1988; Kotowicz et al., 1998). In such conditions, it appeared that C-8 hydroxylated compounds **5**–**7** exhibited peaks distinctly split while a unique form remained in $CDCl_3$ for these three compounds (Figs. S6–S19 in Supplementary data). As far as we know, this is the first time that such a phenomenon has been described for hirsutinolides. Heteronuclear 2D NMR experiments HSQC and HMBC recorded in CD_3OD confirmed the existence of two forms of the same compound. The fact that a unique form is present in $CDCl_3$, even after a previous analysis in CD_3OD, suggests the reversibility of the reaction, and the involvement of the deuterated NMR solvents. Moreover, compounds **5**–**7** were analysed by UHPLC-MS under acidic conditions, and two main peaks displaying the same mass appeared in the chromatograms confirming the presence of two forms. It is worth noting that this phenomenon only appeared for the C-8 hydroxylated derivatives **5**–**7**. For C-8 esterified analogues **1**–**4,** only one form appeared whatever conditions suggesting the involvement of the hydroxyl function in a hydrogen bond. ^1H NMR spectra of compounds **5**–**7** recorded in $CDCl_3$ exhibited a doublet signal close to δ 6 ppm with a coupling constant of $J=12$ Hz corresponding to the non-exchangeable hydroxyl proton 8-OH coupled with H-8 close to 5.5 ppm. Therefore, the NMR signal of H-8 was a ddd ($J_{8/9a}=11$ Hz, $J_{8/9b}=2$ Hz, $J_{8/8\text{-OH}}=12$ Hz), or appeared as a doublet of doublets ($J_{8/9a}=11$ Hz, $J_{8/8\text{-OH}}=12$ Hz) given the low value of the $J_{8/9b}$ coupling constant. This large coupling constant suggests the presence of an intramolecular hydrogen bond occurring between the 8-OH and most likely the ether bridge oxygen as suggested before for a similar hirsutinolide (Yang et al., 2007). Borkosky et al. (1997) isolated a hirsutinolide structurally similar to **5** (referred as compound 7f in their publication). The NMR signal of H-8 for this compound, in $CDCl_3$, appeared as a doublet of doublets ($J_{8/9a}=2$ Hz and $J_{8/9b}=6$ Hz) indicating no intramolecular hydrogen bond. The only differences between compound **5** and the hirstunolide described by Borkosky and collaborators were the absence of a hydroxyl at C-10 and the α-orientation of the methyl at C-10. Moreover, it can be noted that a unique conformer was described for other C-8 hydroxylated hirsutinolides with an α-oriented C-10 methyl when NMR analyses were recorded in CD_3OD (Youn et al., 2012). This suggests that the presence of hydroxyl groups at C-8 and C-10 was responsible for the intramolecular bond occurring in compounds **5**–**7**. Jakupovic and colleagues reported that the quaternization of carbon 10 by an oxygenated group in hirsutinolides led to the β-orientation of the methyl group at C-10 (Jakupovic et al., 1985). From this assumption, the H-bond may occur between 8-OH and 10-OH or with the oxygen of the ether bridge because of a conformational change induced by the presence of the α-oriented hydroxyl group at C-10. Our hypothesis is therefore that this hydrogen bond is partially disrupted by CD_3OD, a polar protic solvent, and led to a mixture of two conformers.

3.2. Biological activities

3.2.1. Antiplasmodial, antileishmanial and cytotoxicity properties of ethanolic and aqueous extracts of *P. spiralis*

The antiplasmodial activities against *P. falciparum* of extracts obtained from the leaves of *P. spiralis* are displayed in Table 1. The aqueous extract exhibited the highest antiplasmodial activity with an IC_{50} value of 3.0 μg/mL. This corresponds to a good activity according to the standard antiplasmodial score (Willcox et al., 2011). The ethanolic extract with an IC_{50} of 21.1 μg/mL, showed a low activity. The cytoxicity was evaluated on mammalian VERO cell lines. Both extracts displayed high cytotoxicity against this healthy cell line with median cytotoxicity concentrations of 1.7 and 2.5 μg/mL for aqueous and ethanolic extracts, respectively.

Both extracts were also tested against the promastigote stage of *L. infantum* (Table 1). The aqueous extract was the more active with a good efficacy, the IC_{50} value ($IC_{50}=13.4$ μg/mL) being between 10 and 50 μg/mL (Osorio et al., 2007) while the ethanolic extract was inactive ($IC_{50}>50$ μg/mL). The cytotoxicities of extracts were also assessed on macrophages as they are the host cells of *Leishmania*. The aqueous extract was shown to be cytotoxic with a selectivity index of less than 1.

3.2.2. Biological activities of isolated compounds

The in vitro antileishmanial and antiplasmodial activities of isolated sesquiterpene lactones **2**, **3**, **5**, **6** and **7** are presented in Table 1. Compounds **1** and **4** could not be tested because of their instability.

3.2.2.1. Antiplasmodial activity and cytotoxicity of isolated compounds. The sesquiterpene lactones **2** and **3** were the most active compounds with IC_{50} values of 7.8 μM and 6.9 μM,

Table 1
Antileishmanial and antiplasmodial activities of *P. spiralis* crude extracts and isolated compounds (2, 3, 5, 6 and 7), and their cytotoxicity on macrophages and VERO cell lines. Inhibitory Concentration 50% (IC_{50})= =sample concentration inhibiting 50% of metabolic activity of parasites. Cytotoxic Concentration 50% (CC_{50})= =sample concentration providing 50% death of macrophages J774A.1 or VERO cells.

Extracts/ compounds	IC_{50} *Promastigotes L. infantum*	IC_{50} *Axenic amastigotes L. infantum*	CC_{50} Macrophages	SI[a]	IC_{50} *P. falciparum* FcM 29	CC_{50} VERO	SI[b]
H_2O extract	13.4 ± 2.6 μg/mL	–	1.0 ± 0.2 μg/mL	0.07	3.0 ± 0.6 μg/mL	1.7 ± 0.8 μg/mL	0.6
EtOH extract	> 50 μg/mL	–	ND	ND	21.1 ± 1.2 μg/mL	2.5 ± 1.0 μg/mL	0.1
2	24.1 ± 4.5 μM	4.7 ± 1.8 μM	1.4 ± 0.08 μM	0.06	7.8 ± 1.2 μM	3.7 ± 1.5 μM	0.5
3	9.5 ± 0.2 μM	2.0 ± 1.4 μM	0.9 ± 0.07 μM	0.09	6.9 ± 0.9 μM	1.8 ± 0.07 μM	0.3
5	86.3 ± 13 μM	17.0 ± 0.6 μM	5.5 ± 1.9 μM	0.06	50 μM ($n=1$)	17.6 ± 8.0 μM	0.4
6	31.5 ± 3 μM	5.4 ± 4.4 μM	3.1 ± 1.0 μM	0.10	54.9 ± 3.4 μM	75.2 ± 37 μM	1.4
7	> 100 μM	68 ± 14 μM	ND	ND	Not stable	90 ± 40 μM	ND
Amphotericin B	0.03 ± 0.01 μM	0.34 ± 0.2 μM	2.5 ± 0.2	83	–	12.2 ± 2.8 μM	–
Miltefosine	8.8 ± 2.8 μM	–	155.3 ± 15.2 μM	18	–	–	–
Pentamidine	0.5 ± 0.3 μM	–	0.53 ± 0.57 μM	1	–	–	–
Doxorubicin	–	–	0.06 ± 0.04 μM	–	–	–	–
Chloroquine	–	–	–	–	549.2 ± 68 nM	–	–
Artemisinin	–	–	–	–	13.9 ± 7.7 nM	–	–

[a] Selectivity index (SI)=CC_{50}(macrophages)/IC_{50}(promastigotes).
[b] SI=CC_{50}(VERO)/$IC_{50\pm}$(Plasmodium) ± standard deviation.

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respectively. Compounds **5** and **6** were inactive against the parasite ($IC_{50} > 50$ μM) and **7** could not be tested against malaria because of its rapid degradation. In parallel, it appeared that **2** and **3** were strongly cytotoxic towards VERO cells with CC_{50} values of 3.7 and 1.8 μM, respectively. Although compound **5** was less cytotoxic ($CC_{50} = 17.6$ μM) than **2** or **3**, its antiplasmodial activity was still not selective. Compounds **6** ($CC_{50} = 75.2$ μM) and **7** ($CC_{50} = 90$ μM) exhibited no cytotoxicity.

3.2.2.2. Antileishmanial activity and cytotoxicity on macrophages of isolated compounds. Compounds **2**, **3**, **5**, **6** and **7** were tested against the promastigote and axenic amastigote stages of *L. infantum*. Generally, all the tested compounds are more active against the amastigote stage than the promastigote form of the parasite. Hirsutinolide **3** displayed the strongest activity against the promastigotes and axenic amastigotes of *L. infantum* with IC_{50} values of 9.5 and 2.0 μM respectively. Note that the IC_{50} of 9.5 μM against promastigotes is close to that of the reference miltefosine (8.8 μM). Concerning the activities of the other compounds against the two stages of the parasite, they can be ranked by decreasing the order of efficiency: **2** > **6** > **5** > **7**, **7** being totally inactive against *L. infantum*. Concerning their cytotoxicity towards macrophages, the most interesting compound, **3,** appeared much more cytotoxic than miltefosine with CC_{50} values of 0.9 and 155.3 μM, respectively. The other products **2**, **3**, **5** and **6** also exhibited strong cytotoxicities with CC_{50} between 1.4 and 5.5 μM. Compounds **2**, **3**, **5** and **6** were also tested on intramacrophagic amastigotes of *L. infantum,* and all compounds were found to be inactive at concentrations lower than their CC_{50} on macrophages.

4. Discussion

4.1. Structural identification

One of the main problems encountered during the identification of the compounds isolated in this work concerned the C-8 hydroxylated hirsutinolides **5**–**7** that exhibited two conformers when NMR analyses were recorded in CD_3OD. The hard process of NMR structural elucidation of hirsutinolides has long baffled researchers who have faced it. Indeed, the complex structural core of these germacrane-type sesquiterpene lactones and the presence of four chiral carbons have generated various representations in the plan of their formula in the literature. Consequently, this led to confusions about their stereochemistry (Catalán et al., 1988), finally resolved by the rigorous application of the conventional representation rules published by Rogers et al. (1972) for germacranolide sesquiterpenes. Moreover, due to the conformational flexibility of the 10-membered ring, the NMR spectra of hirsutinolides exhibited peak enlargement implying poorly resolved signals, attributed to the existence of several conformers in equilibrium. Analyses often have to be recorded at high temperature in C_6D_6 to limit the broadening of peaks, even so several of the signals remained broad leading to difficulties in structural elucidation (Bardón et al., 1993, 1992). This along with the high number of quaternary carbons in the structure precludes drawing inferences about stereochemistry from NOE (Catalán et al., 1988). The stereochemical determination of hirsutinolides was thus strewed with a number of missassignments over the years regarding the chiral positions at C-8 (Catalán et al., 1988) and C-10 (Cowall et al., 1981; Herz and Kulanthaivel, 1983; Jakupovic et al., 1985). Those errors were partially resolved thanks to X-ray analysis made possible for 8α-angeloyloxyhirsutinolide 13-O-acetate (Catalán et al., 1988; Jakupovic et al., 1985). However, some uncertainties still remain for various molecules of the family (Bardón et al., 1988; Pollora et al., 2000). In this work, the configuration was established by hypothesizing and comparing the chemical shifts and the coupling constants of the compounds. Knowing that two conformers coexist in CD_3OD solutions of C-8 hydroxylated hirsutinolides facilitates the understanding of complex NMR and HPLC data, making their isolation easier and avoiding errors in their identification. Isolated compound **7** has been described before as an isomeric mixture of 8α and 8β diastereoisomers (Issa et al., 2006). The $8S^*$ configuration appears to be generally accepted now concerning all hirsutinolides, i.e. with H-8 in the β position (Catalán et al., 1988; Jakupovic et al., 1985). There have been no reports as far as we know about an $8R^*$ configuration of such compounds in the literature (except early reports that have since been revised (Bohlmann et al., 1979, 1978; Catalán et al., 1986; Cowall et al., 1981)). In this context, it is fully justified to have doubts about the stereochemistry described for α-H-8 piptocarphol by Issa et al. (2006) (referred to as compound 4 in their publication). In their article, the NMR data of the two isomers were not actually published but it can be suggested that there are in fact two conformers, as some of the NMR analyses seemed to be carried out in CD_3OD. This kind of phenomenon might also occur for other germacranolides exhibiting an ether bridge spatially close to a hydroxyl function.

4.2. Biological analysis

The results showed that the traditional preparation (aqueous extract) of *P. spiralis,* exhibited a good antiplasmodial activity against *P. falciparum* whereas the ethanolic extract was only weekly active. This result validates the traditional use of this herb in the treatment of malaria or associated symptoms in South America. Concerning the antileishmanial activity against *L. infantum,* while the ethanolic extract was inactive, the aqueous extract again presented a good activity. From the seven hirsutinolide-type sesquiterpene lactones isolated from *P. spiralis*, five were tested for their antiprotozoal activities. Compounds **2** and **3** were the most active molecules against both parasites but also the most toxic on both VERO cells and macrophages. Furthermore, they had no effect on intramacrophagic amastigotes at concentrations non-toxic for the macrophage. Numerous sesquiterpene lactones are known to be bioactive as they interact through Michael type additions with various nucleophilic biological targets (Ghantous et al., 2010; Kupchan et al., 1971; Merfort, 2011; Schmidt, 2006). This confers them a large spectrum of biological activities, including antiplasmodial and antileishmanial activities (Lavault et al., 2005; Tiuman et al., 2005; Toyang et al., 2013) also reported for hirsutinolides (Chea et al., 2006; Odonne et al., 2011; Pillay et al., 2007). It has been suggested by Pillay et al. (2007) that the antiplasmodial activity of hirsutinolides is due to their 2(5H) furanone moiety. More generally, Schmidt et al. (2009) demonstrated that the α,β-unsaturated carbonyl moiety was the key pharmacophore, not only for antiprotozoal activity, but also for the cytotoxic properties of sesquiterpene lactones (Schmidt, 2006). Thereby, it is not surprising that compound **3**, the only compound tested here displaying a methacrylate ester in its structure, was also the most active molecule. Hirsutinolides **5**–**7**, with a free OH on C-8, were found to be the least active compounds suggesting that the etherification of this hydroxyl group is important for activity and cytotoxicity. Two of the isolated hirsutinolides (**1** and **2)** have already been isolated before from the traditional antileishmanial herb *P. spicatus*, and tested for their in vitro antileishmanial activities against axenic amastigotes of *Leishmania amazonensis*, one of the cutaneous Leishmaniasis species. Both were reported to be strongly active with IC_{50} values of 0.37 μM (**1**) and 0.20 μM (**2**), close to the activity displayed by Amphotericin B ($IC_{50} = = 0.41$ μM). Hirsutinolide **1** was not tested by our team but the activity of compound **2** against amastigotes of *L. infantum* ($IC_{50} = 4.7$ μM) appeared to be more than

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20 times less than against amastigotes of *L. amazonensis*. This could be explained by inter-species *Leishmania* variability regarding to the efficacy of different drugs (Croft et al., 2006; Morais-Teixeira et al., 2011). Cytotoxicity towards host cells is a very important criterion for assessing the selectivity of the pharmacological activities observed. Given the strong activity against *L. amazonensis* displayed by **2** and its absence of cytotoxicity on various tumoral mammalian cell lines reported in the literature (Buskuhl et al., 2010), Odonne et al. reported it to be a good antileishmanial agent. However, considering the poor selectivity of **2** with respect to macrophages, and its lack of activity on intramacrophagic amastigotes in the present work, the antileishmanial potential of this compound would appear to require reassessment.

Despite the good antiprotozoal activity displayed by the aqueous extract of *P. spiralis* and isolated compounds **2** and **3**, probably responsible for the extract's efficacy, their cytotoxicity underlined in this study appeared to be critical. Furthermore, aqueous extracts and all the individual compounds tested were found to be cytotoxic to macrophages. As bioactive compounds, hirsutinolides have already been pointed out as potent inhibitors of various tumoral cell lines. Buskuhl et al. (2010) even warned about the potential genotoxicity of compounds **1** and **2**. In the light of these results and in order to assess the real safety of *P. spiralis* preparations in folk medicine, it appeared advisable to carry out further toxicological evaluations, particularly in vivo assays. A suitable method for the determination of the most cytotoxic compounds, **2** and **3**, in the traditional preparation is also required as they represent 0.3% (w/w) of the aqueous extract. Moreover, owing to the lack of selectivity of both aqueous extract and individual hirsutinolides against *L. infantum*, an assessment of the safety of the traditional antileishmanial remedy obtained from *P. spicatus* seems to be necessary as previously proposed by Odonne et al. (2011).

5. Conclusion

The *in-vitro* antiplasmodial activity of aqueous extract and of its purified main compounds **2** and **3** from *P. spiralis* may underlie the use of this plant in South American folk medicine to treat malaria. So far, no work has been reported as to the phytochemical composition of this traditional remedy and *P. spiralis* extracts had not been tested in vitro for potential antiplasmodial and antileishmanial activities. In addition, because of the toxicity of the active components of the aqueous extract, diacetylpiptocarphol (**2**) and piptocarphin A (**3**), against healthy cell lines and macrophages, further studies are needed to assess the safety of the traditional preparation in *in-vivo* animal models. It also appears advisable to develop an analytical method for the quantification of diacetylpiptocarphol (**2**) and piptocarphin A (**3**) in the traditional *P. spiralis* preparation.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2015.05.014.

References

Bardón, A., Catalán, C.A.N., Gutiérrez, A.B., Herz, W., 1988. Piptocarphol esters and other constituents from *Vernonia cognata*. Phytochemistry 27, 2989–2990 http://dx.doi.org/10.1016/0031-9422(88)80706-4.

Bardón, A., Kamiya, N.I., De Léon, C.A. De Ponce, Catalán, C.A.N., Díaz, J.G., Herz, W., 1992. Glaucolides and related sesquiterpene lactones from *Vernonia nudiflora* and *Chrysolaena propinqua*. Phytochemistry 31, 609–613. http://dx.doi.org/10.1016/0031-9422(92)90045-R.

Bardón, A., Montanaro, S., Catalán, C.A.N., Diaz, J.G., Herz, W., 1993. Piptocarphols and other constituents of *Chrysolaena verbascifolia* and *Lessingianthus rubricaulis*. Phytochemistry 34, 253–259.

Benoit-Vical, F., Lelièvre, J., Berry, A., Deymier, C., Dechy-Cabaret, O., Cazelles, J., Loup, C., Robert, A., Magnaval, J.-F., Meunier, B., 2007. Trioxaquines are new antimalarial agents active on all erythrocytic forms, including gametocytes. Antimicrob. Agents Chemother. 51, 1463–1472. http://dx.doi.org/10.1128/AAC.00967-06.

Blair Trujillo, S., Madrigal, B., 2005. Plantas antimalaricas de Tumaco: Costa Pacifica colombiana. Universidad de Antioquia, Medellín.

Bohlmann, F., Brindöpke, G., Rastogi, R.C., 1978. A new type of germacranolide from *Vernonia* species. Phytochemistry 17, 475–482. http://dx.doi.org/10.1016/S0031-9422(00)89342-5.

Bohlmann, F., Mahanta, P.K., Dutta, L.N., 1979. Weitere hirsutinolide aus *Vernonia*-arten. Phytochemistry 18, 289–291. http://dx.doi.org/10.1016/0031-9422(79)80073-4.

Borkosky, S., Bardón, A., Catalán, C.A.N., Díaz, J.G., Herz, W., 1997. Glaucolides, hirsutinolides and other sesquiterpene lactones from *Vernonanthura pinguis*. Phytochemistry 44, 465–470. http://dx.doi.org/10.1016/S0031-9422(96)00481-5.

Buskuhl, H., de Oliveira, F.L., Blind, L.Z., de Freitas, R.A., Barison, A., Campos, F.R., Corilo, Y.E., Eberlin, M.N., Caramori, G.F., Biavatti, M.W., 2010. Sesquiterpene lactones from *Vernonia scorpioides* and their *in vitro* cytotoxicity. Phytochemistry 71, 1539–1544. http://dx.doi.org/10.1016/j.phytochem.2010.06.007.

Bussmann, R.W., Sharon, D., 2006. Traditional medicinal plant use in Loja province, Southern Ecuador. J. Ethnobiol. Ethnomed. 2, 44. http://dx.doi.org/10.1186/1746-4269-2-44.

Canizales, S.A., Celemín, J.S., Mora-Delgado, J., 2010. Diversidad y uso de arvenses en pasturas de fincas ganaderas del Alto Magdalena (Tolima, Colombia). Zootec. Trop. 28, 427–437.

Catalán, C.A.N., De Iglesias, D.I.A., Kavka, J., Sosa, V.E., Herz, W., 1988. Glaucolides and related sesquiterpene lactones from *Vernonia chamaedrys*. Phytochemistry 27, 197–202. http://dx.doi.org/10.1016/0031-9422(88)80613-7.

Catalán, C.A.N., De Iglesias, D.I.A., Kavka, J., Sosa, V.E., Herz, W., 1986. Sesquiterpene lactones and other constituents of *Vernonia mollissima* and *Vernonia squamulosa*. J. Nat. Prod. 49, 351–353.

Chea, A., Hout, S., Long, C., Marcourt, L., Faure, R., Azas, N., Elias, R., 2006. Antimalarial activity of sesquiterpene lactones from *Vernonia cinerea*. Chem. Pharm. Bull. 54, 1437–1439.

Cowall, P., Cassady, J.M., Chang, C.-J., Kozlowski, J.F., 1981. Isolation and structure determination of piptocarphins A-F, cytotoxic germacranolide lactones from *Piptocarpha chontalensis*. J. Org. Chem. 46, 1108–1114. http://dx.doi.org/10.1021/jo00319a014.

Cragg, G.M., Newman, D.J., 2013. Natural products: a continuing source of novel drug leads. Biochim. Biophys. Acta - Gen. Subj. 1830, 3670–3695. http://dx.doi.org/10.1016/j.bbagen.2013.02.008.

Croft, S.L., Sundar, S., Fairlamb, A.H., 2006. Drug resistance in leishmaniasis. Clin. Microbiol. Rev. 19, 111–126. http://dx.doi.org/10.1128/CMR.19.1.111-126.2006.

Desjardins, R.E., Canfield, C.J., Haynes, J.D., Chulay, J.D., 1979. Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. Antimicrob. Agents Chemother. 16, 710–718. http://dx.doi.org/10.1128/AAC.16.6.710.

Domingues Passero, L.F., Laurenti, M.D., Santos-Gomes, G., Soares Campos, B.L., Sartorelli, P., Lago, G., Henrique, J., 2014. Plants used in traditional medicine: extracts and secondary metabolites exhibiting antileishmanial activity. Curr. Clin. Pharmacol. 9, 187–204.

Fournet, A., Munoz, V., 2002. Natural products as trypanocidal, antileishmanial and antimalarial drugs. Curr. Top. Med. Chem. 2, 1215–1237. http://dx.doi.org/10.2174/1568026023393011.

Ghantous, A., Gali-Muhtasib, H., Vuorela, H., Saliba, N.A., Darwiche, N., 2010. What made sesquiterpene lactones reach cancer clinical trials? Drug Discov. Today 15, 668–678. http://dx.doi.org/10.1016/j.drudis.2010.06.002.

Herz, W., Kulanthaivel, P., 1983. Piptocarphol esters from *Piptocarpha opaca*. Phytochemistry 22, 1286–1287. http://dx.doi.org/10.1016/0031-9422(83)80245-3.

Internal report: base de datos de las plantas medicinales 2010. IIAP. 2014 [www document]. Scribd. URL. ⟨http://www.scribd.com/doc/219478120/Base-de-Datos-de-Las-Plantas-Medicinales-2010-IIAP⟩ (accessed 4.24.14).

Issa, H.H., Chang, S.M., Yang, Y.L., Chang, F.R., Wu, Y.C., 2006. New sesquiterpene lactones from the aerial parts of *Pseudoelephantopus spicatus*. Chem. Pharm. Bull. 54, 1599–1601.

Jakupovic, J., Schmeda-hirschmann, G., Schuster, A., Zdero, C., Bohlmann, F., King, R.M., Robinson, H., Pickardt, J., 1985. Hirsutinolides, glaucolides and sesquiterpene lactone from *Vernonia* species. Phytochemistry 25, 145–158. http://dx.doi.org/10.1016/S0031-9422(00)94520-5.

Kotowicz, C., Bardón, A., Catalán, C.A.N., Cerda-García-Rojas, C.M., Joseph-Nathan, P., 1998. Glaucolides and hirsutinolides from *Vernonanthura squamulosa*. Phytochemistry 47, 425–428.

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Kupchan, S.M., Eakin, M.A., Thomas, A.M., 1971. Tumor inhibitors. 69. Structure–cytotoxicity relationships among the sesquiterpene lactones. J. Med. Chem. 14, 1147–1152.

Kvist, L.P., Christensen, S.B., Rasmussen, H.B., Mejia, K., Gonzalez, A., 2006. Identification and evaluation of Peruvian plants used to treat malaria and leishmaniasis. J. Ethnopharmacol. 106, 390–402. http://dx.doi.org/10.1016/j.jep.2006.01.020.

Lavault, M., Landreau, A., Larcher, G., Bouchara, J.-P., Pagniez, F., Le Pape, P., Richomme, P., 2005. Antileishmanial and antifungal activities of xanthanolides isolated from *Xanthium macrocarpum*. Fitoterapia 76, 363–366. http://dx.doi.org/10.1016/j.fitote.2005.03.019.

Macbride, J.F., 1936. Flora of Peru. Field Museum of Natural History, Chicago, U.S.A..

Merfort, I., 2011. Perspectives on sesquiterpene lactones in inflammation and cancer. Curr. Drug Targets 12, 1560–1573.

Morais-Teixeira, E., de, Damasceno, Q.S., Galuppo, M.K., Romanha, A.J., Rabello, A., 2011. The *in vitro* leishmanicidal activity of hexadecylphosphocholine (miltefosine) against four medically relevant *Leishmania* species of Brazil. Mem. Inst. Oswaldo Cruz 106, 475–478. http://dx.doi.org/10.1590/S0074-02762011000400015.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Ochoa, J.G., Andrade, G.I., 2003. The introduced flora to Machu Picchu sanctuary: an inventory and management priorities for biodiversity conservation. Ecol. Boliv. 38, 141.

Odonne, G., Herbette, G., Eparvier, V., Bourdy, G., Rojas, R., Sauvain, M., Stien, D., 2011. Antileishmanial sesquiterpene lactones from *Pseudelephantopus spicatus*, a traditional remedy from the Chayahuita Amerindians (Peru). Part III. J. Ethnopharmacol. 137, 875–879. http://dx.doi.org/10.1016/j.jep.2011.07.008.

Odonne, G., Valadeau, C., Alban-Castillo, J., Stien, D., Sauvain, M., Bourdy, G., 2013. Medical ethnobotany of the Chayahuita of the Paranapura basin (Peruvian Amazon). J. Ethnopharmacol. 146, 127–153. http://dx.doi.org/10.1016/j.jep.2012.12.014.

Osorio, E., Arango, G.J., Jiménez, N., Alzate, F., Ruiz, G., Gutiérrez, D., Paco, M.A., Giménez, A., Robledo, S., 2007. Antiprotozoal and cytotoxic activities *in vitro* of colombian Annonaceae. J. Ethnopharmacol. 111, 630–635. http://dx.doi.org/10.1016/j.jep.2007.01.015.

Pillay, P., Vleggaar, R., Maharaj, V.J., Smith, P.J., Lategan, C.A., Chouteau, F., Chibale, K., 2007. Antiplasmodial hirsutinolides from *Vernonia staehelinoides* and their utilization towards a simplified pharmacophore. Phytochemistry 68, 1200–-1205. http://dx.doi.org/10.1016/j.phytochem.2007.02.019.

Pollora, G.C., Bardón, A., Catalán, C.A., Gedris, T.E., Herz, W., 2000. Sesquiterpene lactones from *Chrysolaena platensis*. Biochem. Syst. Ecol. 28, 707–711.

Q3 Pruski, J.F., 2011. Asteraceae, Flora Mesoam.. Missouri Botanical Garden Press, Missouri, pp. 608–625.

Q4 Rogers, D., Moss, G.P., Neidle, S., 1972. Proposed conventions for describing germacranolide sesquiterpenes. J. Chem. Soc. Chem. Commun., 142. http://dx.doi.org/10.1039/c39720000142.

Romero, G.A.S., Boelaert, M., 2010. Control of visceral leishmaniasis in Latin America —a systematic review. PLoS Negl. Trop. Dis. 4, e584. http://dx.doi.org/10.1371/journal.pntd.0000584.

Q5 Schmidt, T.J., 2006. Structure–activity relationships of sesquiterpene lactones. In: Atta-ur Rahman (Ed.), Studies in Natural Products Chemistry, Bioactive Natural Products (Part M). Elsevier, pp. 309–392.

Schmidt, T.J., Khalid, S.A., Romanha, A.J., Alves, T.M.A., Biavatti, M.W., Brun, R., Da Costa, F.B., De Castro, S.L., Ferreira, V.F., De Lacerda, M.V.G., et al., 2012a. The potential of secondary metabolites from plants as drugs or leads against protozoan neglected diseases—Part II. Curr. Med. Chem. 19, 2176–2228.

Schmidt, T.J., Khalid, S.A., Romanha, A.J., Alves, T.M., Biavatti, M.W., Brun, R., Da Costa, F.B., de Castro, S.L., Ferreira, V.F., de Lacerda, M.V., et al., 2012b. The potential of secondary metabolites from plants as drugs or leads against protozoan neglected diseases—Part I. Curr. Med. Chem. 19, 2128–2175.

Schmidt, T.J., Nour, A.M.M., Khalid, S.A., Kaiser, M., Brun, R., 2009. Quantitative structure–antiprotozoal activityrelationships of sesquiterpene lactones. Molecules 14, 2062–2076. http://dx.doi.org/10.3390/molecules14062062.

Sereno, D., Lemesre, J.L., 1997. Axenically cultured amastigote forms as an *in vitro* model for investigation of antileishmanial agents. Antimicrob. Agents Chemother. 41, 972–976.

Singh, N., Mishra, B.B., Bajpai, S., Singh, R.K., Tiwari, V.K., 2014. Natural product based leads to fight against leishmaniasis. Bioorg. Med. Chem. 22, 18–45. http://dx.doi.org/10.1016/j.bmc.2013.11.048.

Tiuman, T.S., Ueda-Nakamura, T., Garcia Cortez, D.A., Dias Filho, B.P., Morgado-Díaz, J.A., de Souza, W., Nakamura, C.V., 2005. Antileishmanial activity of parthenolide, a sesquiterpene lactone isolated from *Tanacetum parthenium*. Antimicrob. Agents Chemother. 49, 176–182. http://dx.doi.org/10.1128/AAC.49.11.176-182.2005.

Toyang, N.J., Krause, M.A., Fairhurst, R.M., Tane, P., Bryant, J., Verpoorte, R., 2013. Antiplasmodial activity of sesquiterpene lactones and a sucrose ester from *Vernonia guineensis* Benth. (Asteraceae). J. Ethnopharmacol. 147, 618–621 http://dx.doi.org/10.1016/j.jep.2013.03.051.

Trager, W., Jensen, J.B., 1976. Human malaria parasites in continuous culture. Science 193, 673–675.

Tye, A., 2001. Invasive plant problems and requirements for weed risk assessment in the Galapagos Islands, Weed Risk Assessment. CSIRO Publishing, pp. 153–175.

Valadeau, C., Pabon, A., Deharo, E., Albán-Castillo, J., Estevez, Y., Lores, F.A., Rojas, R., Gamboa, D., Sauvain, M., Castillo, D., Bourdy, G., 2009. Medicinal plants from the Yanesha (Peru): evaluation of the leishmanicidal and antimalarial activity of selected extracts. J. Ethnopharmacol. 123, 413–422.

WHO | Leishmaniasis Fact sheet N°375, 2014. [www document]. WHO. URL. ⟨http://www.who.int/mediacentre/factsheets/fs375/en/⟩ (accessed 12.16.14).

Q6 WHO | Malaria Fact sheet N°94, 2014 [www document]. WHO. URL. ⟨http://www.who.int/mediacentre/factsheets/fs094/en/⟩ (accessed 12.16.14).

WHO Expert Committee on the Control of the Leishmaniases and Meeting, 2010. Control of the leishmaniases: report of a meeting of the WHO expert committee on the control of leishmaniases, 22–26th March 2010. World Health Organization, Geneva.

Willcox, M., Benoit-Vical, F., Fowler, D., Bourdy, G., Burford, G., Giani, S., Graziose, R., Houghton, P., Randrianarivelojosia, M., Rasoanaivo, P., 2011. Do ethnobotanical and laboratory data predict clinical safety and efficacy of anti-malarial plants? Malar. J. 10, S7. http://dx.doi.org/10.1186/1475-2875-10-S1-S7.

Willcox, M.L., Bodeker, G., 2004. Traditional herbal medicines for malaria. BMJ 329, 1156–1159. http://dx.doi.org/10.1136/bmj.329.7475.1156.

Yang, Y.-L., Chang, S.-M., Wu, C.-C., Hsieh, P.-W., Chen, S.-L., Chang, F.-R., Hung, W.-C., Issa, H.H., Wu, Y.-C., 2007. Cytotoxic sesquiterpene lactones from *Pseudoelephantopus spicatus*. J. Nat. Prod. 70, 1761–1765. http://dx.doi.org/10.1021/np070331q.

Youn, U.J., Park, E.-J., Kondratyuk, T.P., Simmons, C.J., Borris, R.P., Tanamatayarat, P., Wongwiwatthananukit, S., Toyama, O., Songsak, T., Pezzuto, J.M., Chang, L.C., 2012. Anti-inflammatory sesquiterpene lactones from the flower of *Vernonia cinerea*. Bioorg. Med. Chem. Lett. 22, 5559–5562. http://dx.doi.org/10.1016/j.bmcl.2012.07.010.