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

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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), diacetyl-piptocarpoph (2), piptocarphins A (3), F (4) and D (5), (15,4R,8S,10R)-1,4-epoxy-13-ethoxy-1,8,10-trihydroxygermacra-5E,7(11)-dien-6,12-olide (6), and piptocarpoph (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 IC50 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 (CC50 < 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 CD3OD 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.

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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
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 Leishmaniasis 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; Wilcox 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., 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 Pasto mula, Singh et al., 2014). Moreover, it was con...
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 H2O–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–H2O 1:1 and subjected to SPE C18 cartridge (10 g) under vacuum (P = 5 mm Hg) then eluted with increasing amounts of MeOH in H2O (50–100%). This afforded 13 fractions (A–M). Fraction A (113 mg) was chromatographed on SPE C18 cartridge (10 g) with H2O 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 H2O, 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 (Rt = 7.8 min) and 4.7 mg of 1 (Rt = 9.9 min). Semi-preparative HPLC (MeCN-0.1% HCOOH in H2O, 11:9, 3 mL/min) performed on pooled fractions B (6.8 mg) and C (21.7 mg) gave 3 (7.2 mg, Rt = 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 H2O, 3:2, 3 mL/min) to yield 4 (2.8 mg, Rt = 12.5 min).

Procedure B: isolation of 2 and 3: The entire crude aqueous extract (270 g) was suspended in H2O (200 mL) and extracted by CH2Cl2 (6 × 200 mL). Evaporation of the CH2Cl2 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 H2O 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 CH2Cl2 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 CH2Cl2 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. L. infantum promastigotes in log phase were centrifuged at 900g 10 min, cell medium was then replaced by MAAs-20 medium and maintained at 37 °C, 5% CO2 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 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% (IC50) 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 900g 10 min, cell medium was then replaced by MAAs-20 medium and maintained at 37 °C, 5% CO2 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 less than 0.5% v/v), in triplicate. 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% CO2, 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% (IC50) was defined as the concentration of drug required to inhibit by 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 2.10^6 cells/mL and incubated for 24 h at 37 °C under 5% CO2. L. Infantum promastigotes were centrifuged at 900g 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 (1:1 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% CO2, 100 μl of well supernatant was removed and Steady Glow reagent (Promega) was added according to the manufacturer'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% (IC50) was defined as the concentration of drug required to inhibit by 50% of the metabolic activity of L. infantum intramacrophagic amastigotes compared to the control. IC50 were calculated by non-linear regression analysis processed on dose–response curves, using Table Curve 2D VS software. IC50 values represent the means calculated from three independent experiments.
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% CO₂ 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 5% human serum (French Blood Bank, EFS). The medium 24 h after the beginning of incubation (Benoit-Vical et al., and tritiated hypoxanthine (PerkinElmer, France) was added to the plates of parasite culture and incubated with products for 48 h. Extract dilutions and compounds were tested 3 times independently. 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 [3H]-hypoxanthine incorporation. Inhibitory concentration 50% (IC₅₀) 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. IC₅₀, CC₅₀ and selectivity index (SI) calculation

IC₅₀ on L. infantum and CC₅₀ on macrophages and VERO cells were calculated by non-linear regression analysis processed on dose–response curves, using Table Curve 2D V5 software. IC₅₀ 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 their selectivity as antiparasitic. The SI on L. infantum was calculated as the ratio between the CC₅₀ values against macrophages and IC₅₀ values against promastigote forms. The SI on P. falciparum was calculated as the ratio between the CC₅₀ values against VERO cells and IC₅₀ values against P. falciparum. In both cases, SI = CC₅₀/IC₅₀.

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 (¹H NMR, ¹³C NMR) and 2D (HSQC and HMBC) NMR parameters, in CDCl₃. They were compared with NMR data published in the literature in the same solvent. This permitted the identification of 8-acetyl-13-ethoxypiptocarpoph (1) (Catalán et al., 1986), corrected in (Catalán et al., 1988), diacetylpiptocarpoph (2) (Catalán et al., 1986), corrected in (Bardón et al., 1993), piptocarpoph A (3), piptocarpoph F (4), piptocarpoph D (5) (Cowall et al., 1981), corrected in (Catalán et al., 1988), (15S,4R,8S,10R)-1,4-epoxy-13-ethoxy-1,8,10-trihydroxygermacra-5,7(E)-dien-6,12-olide (6) (Kotowicz et al., 1998), and piptocarpoph (7) (Bardón et al., 1993). In the course of our search for the improvement of NMR spectra of some hirsutinolides presenting enlargements of ¹H NMR signals (Fig. S2 Supplementary data), NMR analyses were run in CD₃OD while CDCl₃ and C₆D₆ were generally the solvents.

![Fig. 1. Isolated compounds from Pseudelephantopus spiralis (Less.) Cronquist](image-url)
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 CDCl3 for these three compounds (Figs. S6–S10 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 CD3OD confirmed the existence of two forms of the same compound. The fact that a unique form is present in CDCl3, even after a previous analysis in CD3OD, 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 CDCl3 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 (J8/9a = 11 Hz, J8/9b = 2 Hz, J8/9b = 8–OH = 12 Hz), or appeared as a doublet of doublets (J8/9a = 11 Hz, J8/9b = 12 Hz) given the low value of the J8/9b 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 7fi in their publication). The NMR signal of H-8 for this compound, in CDCl3, appeared as a doublet of doublets (J8/9a = 2 Hz and J8/9b = 12 Hz) indicating no intramolecular hydrogen bond. The only differences between compound 5 and the hirsutinolide 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 CD3OD (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 CD3OD, 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 IC50 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 IC50 of 21.1 μg/mL, showed a low activity. The cytotoxicity 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 IC50 value (IC50 = 13.4 μg/mL) being between 10 and 50 μg/mL (Osorio et al., 2007) while the ethanolic extract was inactive (IC50 > 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

In the 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 IC50 values of 7.8 μM and 6.9 μM,
respectively. Compounds 5 and 6 were inactive against the parasite (IC₅₀ > 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 IC₅₀ values of 3.7 and 1.8 µM, respectively. Although compound 5 was less cytotoxic (CC₅₀ = 17.6 µM) than 2 or 3, its antiplasmodial activity was still not selective. Compounds 6 (CC₅₀ = 75.2 µM) and 7 (CC₅₀ = 90 µM) exhibited no cytotoxicity.

3.2.2. Anti-leishmanial 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 IC₅₀ values of 9.5 and 2.0 µM respectively. Note that the IC₅₀ 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 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 CC₅₀ values of 0.9 and 155.3 µM, respectively. The other products 2, 3, 5 and 6 also exhibited strong cytotoxicities with CC₅₀ 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 CC₅₀ 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 CD₃OD. 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 germacraneide 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 CD₃OD 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 misassignments 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-angeloyloxylhirsutinolide 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 CD₃OD 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 (Böhlmann 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 piptocarpoph 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 CD₃OD. This kind of phenomenon might also occur for other germacraneides 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 confirms them a large spectrum of biological activities, including antiprostamidial 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 antiprostamidial activity of hirsutinolides is due to their 2(5H) furanone moiety. More generally, Schmidt et al. (2009) demonstrated that the αβ-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 IC₅₀ values of 0.37 µM (1) and 0.20 µM (2), close to the activity displayed by Amphotericin B (IC₅₀ = 0.41 µM). Hirsutinolide 1 was not tested by our team but the activity of compound 2 against axenic amastigotes of L. infantum (IC₅₀ = 4.7 µM) appeared to be more than...
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 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.

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