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Antiparasitic activities of two sesquiterpenic lactones isolated from *Acanthospermum hispidum* D.C.

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ABSTRACT

Ethnopharmacological relevance: Aerial parts of *Acanthospermum hispidum* D.C. are often used by traditional healers in Benin for various diseases and especially for malaria.
Aim of the study: To identify active compounds from extracts of *Acanthospermum hispidum* D.CV. leaves previously shown to possess antimalarial properties and analyse *in vivo* activity and toxicity of crude extracts.
Materials and methods: Compounds were isolated from aerial part of *Acanthospermum hispidum* D.C. and structurally elucidated using extensive spectroscopic analysis. Antiplasmodial activity was evaluated *in vitro* against a chloroquine-sensitive strain of *Plasmodium falciparum* (3D7) using the measurement of the plasmodial lactate dehydrogenase activity and *in vivo* against *Plasmodium berghei berghei* by the 4-day suppressive test. Selectivity of extract and purified compounds on *Plasmodium* parasites were evaluated by using MTT test on J774 macrophage like murine cells and WI38 human normal fibroblasts and also against two other parasites: *Trypanosoma brucei brucei* and *Leishmania mexicana mexicana*. Acute and sub-acute toxicities of a crude extract were evaluated on mice.
Results: Two known sesquiterpenic lactones were isolated: **1** (15-acetoxy-8β-[(2-methylbutyryloxy)]-14-oxo-4,5-cis-acanthospermolide) and **2** (9α-acetoxy-15-hydroxy-8β-(2-methylbutyryloxy)-14-oxo-4,5-trans-acanthospermolide). **1** and **2** showed *in vitro* antiplasmodial activity against the chloroquine-sensitive strain (3D7) with IC_{50} of 2.9 ± 0.5 and 2.23 ± 0.09 μM respectively. Only **2** showed a high selectivity index (SI: 18.4) on *Plasmodium* compared to cytotoxicity against human fibroblasts cell line (WI38). **1** and **2** also showed interesting antiparasitic activities *in vitro* against *Trypanosoma brucei brucei* (IC_{50} of 2.45 ± 0.49 and 6.36 ± 1.42 μM respectively) and *Leishmania mexicana mexicana* (IC_{50} of 0.94 ± 0.05 and 2.54 ± 0.19 μM respectively). Furthermore, crude acidic water extract and fractions containing one of the two isolated compounds displayed a weak *in vivo* antimalarial activity against *Plasmodium berghei berghei* with a long half-life causing a delayed effect. *In vivo* acute (2000 mg/kg) and sub-acute (1000 mg/kg) toxicity tests on the crude acidic water extract did not show toxicity.
Conclusion: Crude acidic water extract, fractions and pure isolated compounds from *Acanthospermum hispidum* showed promising *in vitro* antiplasmodial activity. Despite our study did not show *in vivo* acute and subacute toxicities of the crude acidic water extract, its weak *in vivo* antimalarial activity and the *in vitro* cytotoxicity of pure compounds and enriched extracts containing **1** and **2** indicate that the aerial parts of *Acanthospermum hispidum* should be used with caution for malaria treatments.

1. Introduction

Malaria still remains a major public health problem in the world. Five hundred million people are exposed to this disease, with an annual death rate that the World Health Organisation (WHO/World Health Statistic, 2011) estimates to more than 800,000 people in

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2009. In addition the medical treatments face some difficulties. They are related in one hand to the appearance of *Plasmodium* strains exhibiting resistance to classical antimalarial drugs and on the other hand to the relatively expensive cost of the currently effective drugs (artemisinin derivatives – ACT) for low income populations.

The study of plants used traditionally as antimalarials is then attractive because of the possibility to find new drugs and also because of the strong adhesion of local people, for economic and also cultural reasons. This prompted our team to carry out studies on plants used in Benin by traditional healers against malaria (Bero et al., 2009). In this study, *Acanthospermum hispidum* (Asteraceae) was selected for the antimalarial activity of its dichloromethane extract *in vitro* against chloroquine-sensitive (3D7) and chloroquine-resistant (W2) strains. Aerial parts of this plant are often used in folk medicine for various purposes: vomiting, cephalgias, headaches, abdominal pains, convulsions, cough, eruptive fever, snake bites, jaundice, epilepsy, constipation, blennorrhoea, diarrhoeas, hepato-biliary disorders and malaria (Kerharo and Adam, 1974; Adjanohoun et al., 1989). Traditional healers often use a water decoction prepared with lemon juice (DNPS/MSP, 1991).

This plant is also known to contain several sesquiterpenic lactones (Bohlman et al., 1979; Jakupovic et al., 1986; Cartagena et al., 2000) but our previous works (Bero et al., 2009) and those of Sanon et al. (2003) showed that extracts prepared with acidic water and fractions giving positive spots on thin layer chromatography with Dragendorff's reagent were more active than the crude extract. This prompted us to isolate and identify the two major compounds (on TLC with Dragendorff's reagent) of this extract which were also present in organic solvent fractions from the water acidic extract. To further assess the selectivity on *Plasmodium* parasites of this plant, the crude water acidic extract, fractions and purified compounds, were also tested on two other parasites: *Trypanosoma brucei brucei*, responsible for sleeping sickness and *Leishmania mexicana mexicana*, the causative agent of leishmaniosis. *In vivo* antimalarial tests were also performed on crude and enriched extracts and acute and subacute toxicities were analysed on the crude acidic water extract.

2. Materials and methods

2.1. Plant material

The aerial parts (flowers, leaves and stems) of *Acanthospermum hispidum* D.C. (Asteraceae) were collected from Danto/Porto-Novo (South East of Benin) in July 2006. A voucher specimen (number: AA 6315/HNB) was identified by Prof Akoegninou Akpovi (Abomey-Calavi University of Benin) and deposited at the National Herbarium of Abomey-Calavi University of Benin.

2.2. General experimental procedures

NMR measurements were performed on a Bruker® Avance II equipped with a cryoprobe operating at 500 MHz for ^{1}H and at 125.7 MHz for ^{13}C, using 5 mm sample tubes in $CDCl_3$ or CD_3OD solution (Euriso top®) containing TMS as the internal standard. High-resolution mass spectra were measured on a Thermo Scientific LTQ orbitrap XL mass spectrometer with ESI source in positive mode. Elemental analyses (C, H, N, S) were realized on a FlashEA 1112 series (Thermo-Interscience) and where within 0.4% of the theoretical values.

A HPLC, Hitachi Merck® with a L-6200 intelligent pump and UV L-4000 Merck® detector was used. The column was a RP18 Licrospher® (5 μm, 250 mm × 4.5 mm) and the flow rate was 1 ml/min. All solvents used were of HPLC quality (VWR). Silica gel (63 ± 200 mesh, Merck®) was used for column chromatography (CC). Fractions obtained were monitored by TLC on precoated silica gel 60 F_{254} plates (Merck®) eluted with hexane–EtOAc–MeOH (20–70–5). Interesting spots were detected under UV light at 254 nm and by positive reaction to Dragendorff's reagent.

2.3. Extraction and isolation

400 g of plant material, dried in air conditioned room (18 °C) were powdered and macerated during 24 h with water (1 l) acidified with sulphuric acid 0.5% to simulate the traditional preparation by traditional healers in Benin, who incorporate lemon juice (DNPS/MSP, 1991). After filtration, the filtrate was extracted successively with hexane (500 ml 3×) and dichloromethane (500 ml 3×). The organic phases were dried over Na_2SO_4 and evaporated to give the hexane fraction FHex (0.75 g) and the dichloromethane fraction FDCM (2.39 g).

FHex was separated over a silica gel CC eluted with the ternary solvent mixture hexane–EtOAc–acetic acid with a stepwise gradient (8–1–1)/(6–3–1)/(0–9–1) affording 70 fractions. Fractions 26–36 were combined and after evaporation under reduced pressure were chromatographed on the same column with a stepwise gradient of dichloromethane with increasing amounts of MeOH (100–0 to 93–7) as mobile phase. Thirty sub-fractions of 5 ml each were collected. Sub-fractions FHex 4–10 (73 mg) which showed one intensive orange spot on TLC with Dragendorff's reagent, were mixed, dried and separated by repeated HPLC [RP 18 (H_2O–MeOH (9–1)/MeOH): 60–100% in MeOH in 17 min] to give 2.9 mg of **1** (Rt: 17, 2 min).

The fractionation of FDCM followed the method described by Cartagena et al. (2000) used to the isolation of sesquiterpenic lactones from *Acanthospermum hispidum* with some modifications. FDCM was suspended in EtOH (130 ml) at 55 °C, diluted with H_2O (100 ml), and extracted successively with hexane (150 ml 3×) and $CHCl_3$ (150 ml 3×). The chloroform fraction evaporated at reduced pressure gave a residue (1.49 g) which was further column chromatographed over silica gel using $CHCl_3$ with increasing amounts of EtOAc (0–100%) and finally MeOH, to give 12 sub-fractions (a to l). A portion of sub-fraction FDCM-a (0.15 g of 0.76 g) which showed on TLC, several positive spots with Dragendorff, was chromatographed by HPLC [RP18 (H_2O-MeOH (9–1)/MeOH) 20–45% in MeOH in the first 17 min and then in 45–100% MeOH for the following 12 min] to give 6.4 mg of compound **2** (Rt: 26.1 min) corresponding to the most intensive orange spot.

The crude acidic water extract was prepared by maceration of 100 g of powdered aerial parts as described above. The water acidic filtrate was freeze-dried to get 8.9 g of crude acidic water extract.

2.4. Parasites, cell lines and media

Plasmodium falciparum chloroquine-sensitive strain 3D7 (from Prof. Grellier of Museum d'Histoire Naturelle, Paris-France) asexual erythrocytic stages were cultivated continuously *in vitro* according to the procedure described by Trager and Jensen (1976) at 37 °C and under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The host cells were human red blood cells (A or O Rh+). The culture medium was RPMI 1640 (Gibco) containing 32 mM $NaHCO_3$, 25 mM HEPES and 2.05 mM L-glutamine. The medium was supplemented with 1.76 g/l glucose (Sigma–Aldrich), 44 mg/ml hypoxanthin (Sigma–Aldrich), 100 mg/l gentamycin (Gibco) and 10% human pooled serum (A or O Rh+). Parasites were subcultured every 3–4 days with initial conditions of 0.5% parasitaemia and 1% haematocrit.

The macrophage-like cell line, J774, derived from BALB/c murine reticulum cell sarcoma (ECACC N° 91051511 from Health Protection Agency), was cultivated *in vitro* in RPMI 1640

medium (Gibco) containing 2 mM L-glutamine supplemented with 10% heat-inactivated foetal bovine serum (Gibco) and penicillin–streptomycin (100 UI/ml–100 μg/ml).

The human non cancer fibroblast cell line, WI38 (ATCC N° CCL-75 from LGC Standards) was cultivated *in vitro* in DMEM medium (Gibco) containing 4 mM L-glutamine, 1 mM sodium pyruvate supplemented with 10% heat-inactivated foetal bovine serum (Gibco) and penicillin–streptomycin (100 UI/ml–100 μg/ml).

Trypanosoma brucei brucei strain 427 (Molteno Institute in Cambridge, UK) bloodstream forms were cultured *in vitro* in HMI9 medium containing 10% heat-inactivated foetal bovine serum (Hirumi and Hirumi, 1994).

Leishmania mexicana mexicana promastigotes MHOM/BZ/84/BEL46 (Institute of Tropical Medicine Anvers) were cultivated *in vitro* in a semi-defined medium (SDM-79) (Brun and Lun, 1994) supplemented with 15% heat-inactivated foetal bovine serum.

2.5. In vitro antiplasmodial activity

Parasite viability was measured using parasite lactate dehydrogenase (pLDH) activity according to the method described by Makler et al. (1993). The *in vitro* test was performed as described by Murebwayire et al. (2008). Chloroquine (Sigma) or artemisinin (Sigma) were used as positive controls in all experiments with an initial concentration of 100 ng/ml. First stock solutions of extracts and pure compounds were prepared in DMSO at 20 mg/ml. The solutions were further diluted in medium to give 2 mg/ml stock solutions. The highest concentration of solvent to which the parasites were exposed was 1%, which was shown to have no measurable effect on parasite viability. Extracts were tested in eight serial twofold dilutions (final concentrations: 200, 66.7, 22.2, 7.4, 2.5, 0.8, 0.3, 0.09 μg/ml) in 96-well microtitre plates. The parasitaemia and the haematocrit were 2% and 1%, respectively. All tests were performed in triplicate.

2.6. Cytotoxicity assay

The cytotoxicity of the extracts on J774 and WI38 cells was evaluated as described by Stevigny et al. (2002), using the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma)) colorimetric method based on the cleavage of the reagent by dehydrogenases in viable cells (Mosmann, 1983). Camptothecin (Sigma) was used as positive cytotoxic reference compound. Stock solutions of compounds and active fractions were prepared in DMSO at 20 mg/ml. The solutions were further diluted in medium with final concentrations of 200, 100, 50, 25, 12.5 and 6.25 μg/ml. The highest concentration of solvent to which the cells were exposed was 1%, which was shown to be non-toxic. Each extract was tested in six serial fourfold dilutions in 96-well microtitre plates. All experiments were made at least in duplicate.

2.7. In vitro test for antitrypanosomial and antileishmanial activity

The *in vitro* test was performed as described by Hoet et al. (2004). Amphotericin B (a commercial antileishmaniasis drug) and suramine (a commercial antitrypanosomal drug) were used as positive controls in all experiments with an initial concentration of 1 μg/ml. First stock solutions of crude extracts and compounds were prepared in DMSO or in ethanol/water (2:1) for water extracts at 20 mg/ml. The solutions were further diluted in medium to give 0.2 mg/ml stock solutions. Extracts and compounds were tested in eight serial threefold dilutions (final concentrations: 100, 33.3, 11.1, 3.7, 1.2, 0.4, 0.2, 0.05 μg/ml) in 96-well microtitre plates. All tests were performed in duplicate.

2.8. Haemolysis test

The haemolytic activity of our compounds was investigated according to Fischer et al. (2003). Blood, collected in heparinized-tubes from human red blood organ, was centrifuged at 2500 rpm for 5 min. The pellet was washed three times with cold PBS pH 7.4, by centrifugation at 2500 rpm for 5 min and resuspended in the same buffer. This suspension of red blood cells was always freshly prepared and used within 24 h after collection. Stock solutions of compounds (20 mg/ml in DMSO) were diluted in PBS to obtain, after addition to the erythrocytes buffer: 200 μg/ml (the highest dose tested in antiparasitic and cytotoxic tests) with a final percentage of DMSO around 1%. The mixture was incubated for 60 min at 37 °C in a shaking water bath.

The release of haemoglobin was determined after centrifugation (2500 rpm for 5 min) by spectrophotometric analysis of the supernatant at 570 nm. Complete haemolysis was achieved using 0.2% Triton X-100 yielding the 100% control value. Less than 10% haemolysis was regarded as non-toxic effect level in our experiments. The experiments were run in triplicate and were repeated twice.

2.9. In vivo antimalarial activity

The present work was approved by the Ethical Committee for using animals at the University of Liège (no. 721). Crude extract and fractions containing **1** and **2** were assessed for *in vivo* activity in a 4-day suppressive test against *Plasmodium berghei berghei* infections in mice (Peters et al., 1999). Female NMRI mice (mean body weight 23.5 ± 2.0 g) were inoculated (2×10^7 erythrocytes parasitized with *Plasmodium berghei berghei)* intraperitoneally. The volume of inoculum was 0.1 ml. All tested mice were infected on day 0. Extracts were dissolved in 7% Tween 80 and 3% ethanol diluted 10-fold with water to provide required doses expressed in mg/kg body weight. Amount used for FHex and FDCM (200 mg/kg) followed guidelines of Fidock et al. (2004), for extracts to be tested for antiplasmodial *in vivo* activity. As the crude acidic water extract was less concentrated in **1** and **2**, we choose to test it at 500 mg/kg. The volume administered orally was 0.1 ml. Mice (five mice for each condition) were treated orally on day 0 and were given a single daily dose of extract. Two control groups of mice were used. One group was given chloroquine (10 mg/kg/body weight/d) as positive control and another group was given 7% Tween 80 and 3% ethanol diluted 10-fold in water (negative control: C−) for 4 days. A thin blood film stained with Giemsa was prepared on the 4th and 7th day for each mice and the parasitaemia was determined microscopically. The percent of parasitaemia reduction was calculated using the following equation according to Fidock et al. (2004). % reduction of parasitaemia = 100 − [(mean parasitaemia treated/mean parasitaemia negative control) × 100]. Mice stayed under observation and survivors on day 14 post-infection were considered as cured.

2.10. Acute toxicity study

This study was based on the OCDE guidelines for "acute toxic classic method essay" (OCDE, 2001). Mice were kept in animal house of "Department of traditional medicine and pharmacopeia/IRSS/CNRST" accredited by Ministry of Scientific search in Burkina Faso (n° 0-0285). Mice were maintained under standard laboratory conditions with a temperature of 21 ± 2 °C and a 12/12 dark-light cycle 2 weeks before the experiments. All animals husbandry and handling conditions were in agreement with the European Community Guidelines (1986). We proceeded to a "limit

test" which consisted in a sequential test using tree female mice per stage. They were fasted at least 12 h before oral administration of the dried acidic water extract of *Acanthospermum hispidum* at dose level 2000 mg/kg body weight and still kept fasted for 2 h before get access to water and food. The animals were closely observed for the first 4 h for any toxic symptoms and for 24 h for any mortality rate. If no mortality and no toxic symptoms appeared at the first stage, a second test was carried out to confirm the first results.

2.11. Sub-acute toxicity study

Mice were housed in the same location and kept in the same conditions as described above. Sub-acute study was based on the method described by Thanabhorn et al. (2006). Six NMRI female mice weighing 30.8 ± 3.8 g were assigned to each group. Control group received physiological water for 14 days and treated group received dried acidic water extract of *Acanthospermum hispidum* orally once daily for 14 days at the dose of 1000 mg/kg body weight. Body weight, food and water intake were monitored. All animals were sacrificed on day 15. The main organs (heart, lung, kidney, liver, stomach and spleen) were removed and weighed wet after dissection to avoid drying.

2.12. Statistical analysis

Student's *t*-test was used to test the significance of differences between results obtained for different samples, and between results for samples and controls. Statistical significance was set at $P < 0.05$.

3. Results and discussions

3.1. Extraction and isolation of the major compounds of most antiplasmodial extracts

TLC analysis of the crude water acidic extract of *Acanthospermum hispidum* showing interesting *in vitro* antiplasmodial activity showed the presence of two intensive spots positive with Dragendorff's reagent. Fractions containing these two spots (FDCM and FHex) showed higher antiplasmodial activity than the crude extract (Table 1), and were therefore subjected to a series of chromatographic separations to give compounds **1** and **2**, corresponding to these two spots. Compound **1**, by HR-MS on LTQ Orbitrap displayed a $[M+H]^+$ ion peak at *m*/*z*: 405.19098 corresponding to the elemental composition $C_{22}H_{29}O_7$ (calculated: 405.19137). Fragments at *m*/*z*: 345 $[(M\text{-}CH_3COOH)+1]^+$, 303 $[(M\text{-}C_5H_{10}O_2)+1]^+$ indicated the presence of an acetate and a saturated five-carbon atom ester.

Compound **2** displayed a $[M+H]^+$ ion peak at *m*/*z* 421.18563 corresponding to the elemental composition $C_{22}H_{29}O_8$ (calculated 421.18627). Fragments at *m*/*z* 361 $[(M\text{-}CH_3COOH)+1]^+$ 319 $[(M\text{-}C_5H_{10}O_2)+1]^+$ also indicated the presence of an acetate and a saturated five-carbon atom ester.

^1H and ^{13}C NMR values as well as 2D experiments were in accordance with the structures given in Fig. 1 corresponding to 15-acetoxy-8β-[(2-methylbutyryloxy)]-14-oxo-4,5-cis-acanthospermolide (**1**) and 9α-acetoxy-15-hydroxy-8β-(2-methylbutyryloxy)-14-oxo-4,5-trans-acanthospermolide (**2**) previously isolated from African and American samples of *Acanthospermum hispidum* D.C. (Herz and Kalyanaraman, 1975; Bohlman et al., 1979; Cartagena et al., 2000). Furthermore, even if these two compounds showed positive spots with Dragendorff's reagent, an elemental composition analysis showed the absence of nitrogen in the two molecules and confirmed the proposed structures. It was therefore clear that **1** and **2** gave false positive reactions to Dragendorff's test (Anderson et al., 1977).

Table 1
In vitro antiplasmodial activity, cytotoxicity activity and selectivity index of extracts, fractions and compound from *Acanthospermum hispidum* D.C.

Tested substance	Cytotoxicity (IC_{50}, μg/ml) average ± SD		Antiplasmodial activity (IC_{50}, μg/ml) average ± SD	Selectivity index	Antitrypanosomal activity (IC_{50}, μg/ml) average ± SD	Selectivity index	Antileishmanial activity (IC_{50}, μg/ml) average ± SD	Selectivity index
	J774	WI38	3D7	(WI38/3D7)	Tbb	(WI38/Tbb)	Lmm	(WI38/Lmm)
Acidic water extract	>100	>100	36.9 ± 8.9	nd	>100	nd	>100	nd
FHex	7.2	nd	2.4±0.3	nd	nd	nd	nd	nd
FHex 4–10	nd	23.8 ± 0.1	9.02[a]	2.6	5.89 ± 0.1	4.04	4.7 ± 1.9	5.06
Compound **1**	1.1 (2.72[b])[a]	5.7 ± 0.2 (14.1 ± 0.5[b])	1.2 ± 0.2 (2.9 ± 0.5[b])	4.9	0.99 ± 0.2 (2.45 ± 0.49[b])	5.75	0.38 ± 0.02 (0.94 ± 0.05[b])	15
FDCM	4.5	nd	1.8 ± 0.2	nd	nd	nd	nd	nd
FDCM a	nd	38.2 ± 1.9	2.0 ± 0.8	19.1	6.03 ± 0.3	6.33	3.66 ± 2.4	10.44
Compound **2**	13.8 (32.85[b])[a]	17.3 ± 8.3 (41.19 ± 19.8[b])	0.94 ± 0.04 (2.23 ± 0.09[b])	18.5	2.67 ± 0.6 (6.36 ± 1.42[b])	6.48	1.07 ± 0.08 (2.54 ± 0.19[b])	16.21
Amphotericin B	nd	nd	nd	nd	nd	nd	0.11 ± 0.01[b]	nd
Suramine	nd	nd	nd	nd	0.11 ± 0.02[b]	nd	nd	nd
Camptothecin	0.09 ± 0.009[b]	1.2 ± 0.6[b]	nd	nd	nd	nd	nd	nd
Artemisinin	nd	nd	0.035 ± 0.003[b]	nd	nd	nd	nd	nd

SD, standard deviation; nd, not determined.
[a] Tested once.
[b] Expressed in μM.

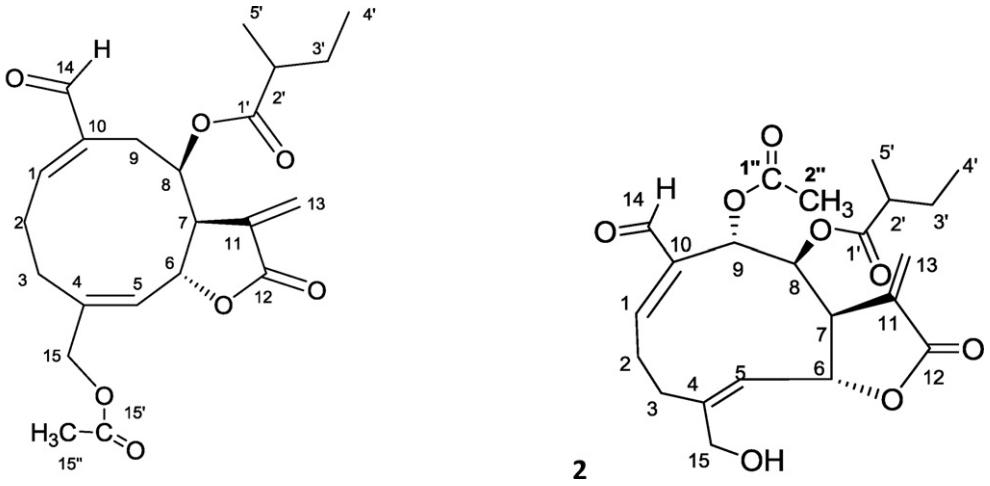

Fig. 1. Compound **1**: 15-acetoxy-8β-[(2-methylbutyryloxy)]-14-oxo-4.5-cis-acanthospermolide; compound **2**: 9α-acetoxy-15-hydroxy-8β-(2-methylbutyryloxy)-14-oxo-4.5-trans-acanthospermolide.

3.2. In vitro antiplasmodial and cytotoxic activities

Regarding the antiplasmodial activity, both compounds had similar activities but **2** had a better selectivity index (SI: 18.4). They are both cytotoxic (IC_{50} <20 μg/ml) but the effect of **1** was more significant on the murine macrophages (cancerous cells) than human fibroblasts (non-cancer cells). Among all extracts and molecules tested, FDCM-a had the best selectivity index (SI: 19.1) while preserving an interesting activity probably due to presence of **2**.

Acanthospermum hispidum was the subject of several previous studies which showed many biological activities (Araújo et al., 2008). If *in vitro* antiplasmodial activity was already described with extracts of the plant, and was shown to be higher for a specific extract defined as "alkaloid" extract (Sanon et al., 2003; Bero et al., 2009), it is the first time that sesquiterpenic lactones are clearly related to the activity of this plant. However cytotoxicity against cell lines for **1** induced the low selectivity of its antiplasmodial activity and by this way, can make it less attractive as antiplasmodial agent compared to **2**.

When we compare the *in vitro* activities of the crude extract, fractions containing **1** and **2** (FHex and FDCM) and the pure compounds (present at about 0.4% for compound **1** and 1.3% for **2**), it is clear that **1** and **2** could not account for the total activity observed. Other constituents may also have activity as for example other sesquiterpenic lactones (many of them were already described in this plant, Cartagena et al., 2000) or other compounds from other phytochemical classes. These compounds may have additive or synergistic effects with **1** or **2**.

Results on the antitrypanosomal and antileishmanial activities are also given in Table 1. While the crude acidic water extract had no interesting activity, fractions containing **1** and **2** were highly active, as well as the isolated compounds. It is worth pointing out that it is the first report of the antitrypanosomal and antileishmanial activity for these two isolated compounds. Previous studies made by Fournet et al. (1994) already tested entire plant, without success, against other strains of parasites (*Leishmania brasiliensis*, *Leishmania donovani*, *Leishmania amazonensis* and *Trypanosoma cruzi*). In addition, Hartwell and Abbott (1969) already established that many biological activities of sesquiterpenic lactones are due to the presence in their structure of α-methylene γ-lactone moiety. This moiety is very reactive with the thiol groups of vitally important components such as enzymes in various cells. The presence in **1** and **2** of this moiety could also explain the cytotoxic, antiplasmodial, antitrypanosomial and antileishmanial activities observed in this study. Nevertheless, when comparing the activities of **1** and **2**, we could observe that the presence of an acetate near this α-methylene γ-lactone moiety seems to decrease the cytotoxicity but not the antiplasmodial activity, giving a more interesting selectivity index.

Table 2
Percentage of haemolytic activity.

Tested substance (200 mg/ml)	Haemolysis (%)
Acidified aqueous extract	0.03 ± 0.01
FDCM-a	0.08 ± 0.02
FHex 4–10	0.12 ± 0.02
Triton X-100	99.72 ± 0.01

3.3. Haemolysis test

As the observed inhibition of *Plasmodium* growth could be explained by toxicity on red blood cells, we decided to observe interactions of fractions containing these two compounds as major constituents, against red blood cells to evaluate their haemolytic capacity.

None of tested extracts showed an haemolytic activity (see Table 2) indicating that the *in vitro* antiplasmodial activity of the two compounds was due to a direct action on the parasite.

3.4. In vivo antiplasmodial activity

To analyse the potentiality of this plant as antimalarial, the *in vivo* antimalarial activity of the plant crude acidic water extract (500 mg/kg) and fractions containing **1** and **2** (200 mg/kg each), as we did not have enough pure compounds for the test, was analysed. Neither extract nor fractions tested *in vivo* showed at day 4, a significant *in vivo* antiplasmodial activity compared to the negative control (water) (% Inhibition <25%) (Fig. 2). Furthermore, in the FDCM group we observed 60% mice death on day 4 whereas no death was recorded in the negative control group indicating toxicity for FDCM (Fig. 3). So we decided to carry out the experiment using FDCM-a, containing more **2** than FDCM and which was given at a reduced dose (50 mg/kg). At this dose, all mice were alive at day 4 (Fig. 3). Between days 4 and 7, a significant reduction of parasitaemia was observed with all fractions except FDCM (Fig. 2). Both the crude acidic water extract and the FHex fraction showed a similar percentage of inhibition, while the FDCM-a was more effective. Nevertheless, mice survival was weak during the experiment; 60% for FHex and acidic water extract and 40% for FDCM-a at day 6,

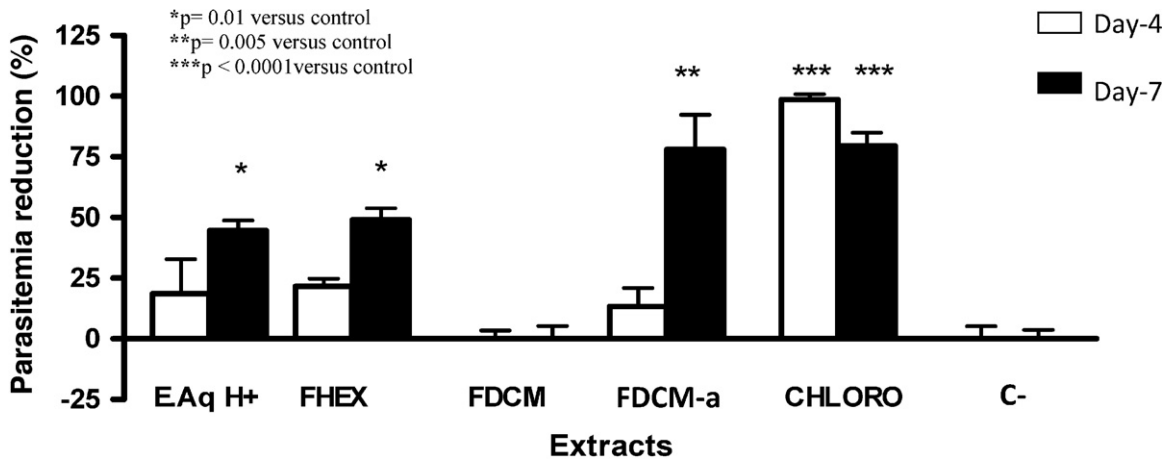

Fig. 2. *In vivo* antiplasmodial activities of extract (500 mg/kg), fractions (200 mg/kg) and sub-fractions (50 mg/kg) of *Acanthospermum hispidum*.

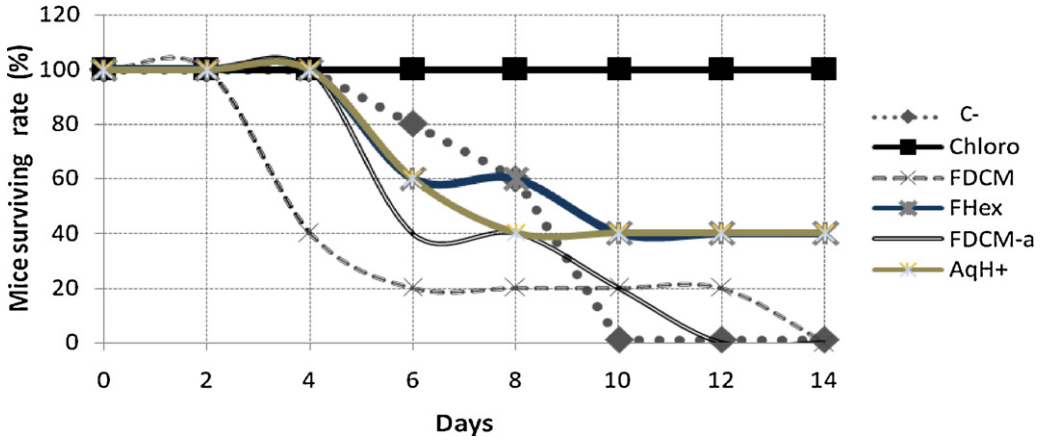

Fig. 3. Survival rate during the *in vivo* antimalarial test.

while it was of 80% in the negative control mice (Fig. 3). This survival still decreased with time especially for FDCM-a and FDCM, for which no survivors were found at the end of the experiment, as for the negative control mice (Fig. 3).

This evolution of the activity shown in Figs. 2 and 3 could suggest that fractions tested possess long half-life and only have a delayed effect, but also that one of them (FDCM) is not devoid of toxicity. After 14 days, only two extracts (crude acidic water and FHex) decreased the number of deaths as compared to untreated mice. Higher doses could have been used but as some toxicity (higher death rate than untreated controls at day 6) was already observed at 500 mg/kg, we did not used higher doses.

3.5. Acute toxicity study

Given the (cyto)toxicity observed, the toxicity of the crude acidic water extract, close to the form used in traditional medicine was evaluated.

In the acute toxicity study, the acidified aqueous extract of *Acanthospermum hispidum* given per os at the dose of 2000 mg/kg body weight, did not show any lethality and no particular toxic symptoms were noted.

At the end of the oral acute toxicity study, the LD_{50} of this aqueous extract was extrapolated as higher than 2 g/kg body weight. According to the Organisation for Economic Cooperation and Development (OCDE, 2001) guidelines for acute toxicity, an LD_{50} dose of 2 g/kg and above is categorized as unclassified and hence the drug was considered to be safe. Hussain et al. (1990) arrived at the same conclusions for the acute toxicity study using methanolic extract of the aerial part of *Acanthospermum hispidum*. Araújo et al. (1989) also confirmed the non-acute toxicity of a root extract of *Acanthospermum hispidum* given intraperitoneally (LD_{50} = 2 g/kg body weight).

3.6. Sub-acute toxicity study

In the subacute toxicity, at 1000 mg/kg of acidic water extract, no death was observed at the end of the treatment period and no significant effect was observed on body weight.

No particular signs were found with regard to clinical symptoms. The weight of organs (heart, lung, liver, kidneys, and spleen) was unaltered in the treated group compared to the control group (Fig. 4). Macroscopic observation of those organs did not reveal any enlarging, congestion, and necrosis lesions. Only the presence of a light fat film all around stomach of one mice of the treated group was noticed.

As no signs of toxicity were observed in acute and subacute studies with water acidic extract at a high dose, the discrepancy observed with *in vivo* antimalarial tests could be explained by a higher sensitivity of infected mice which had infected blood cells and inflammations.

A previous study showed toxic effect to mice of shoots and seeds of *Acanthospermum hispidum* (Ali and Adam, 1978) mixed at different doses (1–50% of diet) with a mice classical diet. They observed lesions in the kidneys, liver, heart, lungs, spleen, intestines, biliary vesicle, and after a long period administration (within 9–172 days)

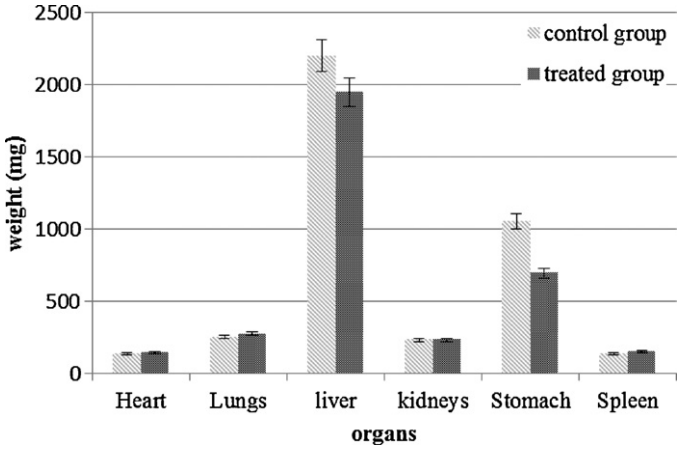

Fig. 4. Organs weights comparison after subacute test with 1000 mg/kg/day of acidic water extract during 14 days.

death. That is why, FDA (Food and Drug Administration) classified *Acanthospermum hispidum* as "restricted" plant (Araújo et al., 2008) but it was another part of the plant and doses were higher than those used in our work.

4. Conclusions

Our work on the aerial part of *Acanthospermum hispidum* allowed us to isolate two antiparasitic sesquiterpenic lactones showing a high activity on *Plasmodium falciparum*, *Leishmania mexicana mexicana* and *Trypanosoma brucei brucei*, but also some cytotoxicity. While **1** showed a low selectivity index, **2** seemed more interesting (SI = 18.4 on *Plasmodium*). The *in vivo* antimalarial tests performed with crude extract and enriched fractions showed a weak activity after a few days of treatment, but not enough to get a good clinical efficiency. Only two extracts (the acidic water extract and FHex) showed antimalarial activities, increasing the survival of mice at day 14 but seemed to increase toxicity of the infection at the beginning of the treatment. Acute and sub-acute toxicity tests realized on the crude acidic water extract at higher dose than those tested in the antimalarial *in vivo* study, did not indicate any toxicity of the extract, but nevertheless, because of *in vitro* cytotoxicity of isolated compounds and their enriched fractions, this plant should be used with caution for malaria treatment.

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