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In vitro antiprotozoal activity and cytotoxicity of extracts and fractions from the leaves, root bark and stem bark of *Isolona hexaloba*

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Abstract

Ethnopharmacological relevance: *Isolona hexaloba* (Pierre) Engl. & Diels (Annonaceae) is traditionally used in D.R. Congo against parasitic diseases including malaria.

Materials and Methods: Two crude aqueous extracts, 3 crude methanol extracts and 3 crude 80% ethanol extracts from the leaves, root bark and stem bark together with 12 subfractions from the crude 80% ethanol extracts were evaluated in vitro for their antiprotozoal activity against *Trypanosoma brucei brucei*, *T. cruzi*, *Leishmania infantum* and the chloroquine and pyrimethamine resistant K1 strain of *Plasmodium falciparum*. Their cytotoxic effects against MRC-5 cell lines were also assessed.

Results: Results indicated that the most pronounced activities against *T. b. brucei* were recorded for the crude methanol extracts of root bark ($IC_{50} = 1.97 \mu\text{g/ml}$; $SI > 32.49$) and leaves ($IC_{50} = 2.65 \mu\text{g/ml}$; $SI > 24.15$). Three samples displayed good activity against *T. cruzi*: the 80% methanol extract of leaves ($IC_{50} = 8.33 \mu\text{g/ml}$; $SI > 3.92$), its petroleum ether fraction ($IC_{50} = 8.50 \mu\text{g/ml}$; $SI = 2.52$) and the crude aqueous extract of the stem bark ($IC_{50} = 9.31 \mu\text{g/ml}$; $SI = 3.46$). The crude aqueous extract of the leaves exhibited a pronounced and selective activity against *L. infantum* ($IC_{50} = 2.00 \mu\text{g/ml}$; $SI > 32$). The crude methanol extract of leaves ($IC_{50} = 6.35 \mu\text{g/ml}$; $SI > 10.10$) and the 2 dichloromethane soluble fractions of the 80% ethanol extracts from root bark ($IC_{50} = 6.96 \mu\text{g/ml}$; $SI = 6.1$) and stem bark ($IC_{50} = 8 \mu\text{g/ml}$; $SI > 8.00$) showed good activity and selectivity against *L. infantum*. The most active samples against *Plasmodium falciparum* K1 were the leaves crude 80% ethanol extract ($0.92 \mu\text{g/ml}$) and its fractions: alkaline aqueous ($IC_{50} = 0.27 \mu\text{g/ml}$), 90% methanol ($0.90 \mu\text{g/ml}$) and dichloromethane ($1.04 \mu\text{g/ml}$), respectively, with promising selectivity indexes of $35 < SI < 237$. None of all the tested crude extracts and fractions was found to be cytotoxic against MRC-5 cell lines except the petroleum ether soluble fraction from the leaves which displayed a cytotoxic effect ($CC_{50} = 21.40 \mu\text{g/ml}$).

Conclusion: Overall, extracts of *I. hexaloba* tested here, showed good results concerning parasitic infections such as Chagas' disease, leishmaniasis, malaria and/or sleeping sickness without considerable toxicity. The 80% ethanol extracts from leaves and their fractions turned out to be of special interest as they were the most useful in the treatment of malaria.

Keywords: Antiprotozoal activity; Cytotoxicity; *Isolona hexaloba*; *Leishmania infantum*; *Plasmodium falciparum*; *Trypanosoma brucei brucei*; *Trypanosoma cruzi*.

1. Introduction

Parasitic diseases are among the most important causes of morbidity and mortality in developing countries. They can be classified in 2 groups: those caused by helminths (worms) and those caused by protozoa (unicellular organisms). Blood and tissue protozoa causing diseases such as malaria, leishmaniasis and trypanosomiasis are the most lethal. They have become a major public health concern mainly because of the development of resistance by most causative parasitic species against the classic medicines. Modern medicine has yet to find the most optimal treatments which should be cheaper, safer and more effective. Moreover, there is an urgent need to look for new lead compounds with novel mechanisms of action against these protozoa.

In developing countries, both rural and city populations are greatly dependent on medicinal plants for the treatment of different ailments including parasitic diseases. During our ethnobotanical and ethnopharmacological investigations conducted in the Nkundo area of Bolongo (Bandundu Province, Democratic Republic of Congo as presented in figure 1), in the frame of the project “Cuvette Centrale as Reservoir of Medicinal Plants in D.R. Congo” [Fruth, 2011], it was observed that the Nkundo people use the leaves, root bark and stem bark of the 15-30 m high tree *Isolona hexaloba* (Pierre) Engl. & Diels (figure 2) belonging to the Annonaceae family, alone or in mixtures with other plants, to treat various diseases including parasitic diseases such as malaria and others [Musuyu Muganza, 2006]. This plant is reported to be traditionally used as an aqueous decoction per os and/or as enema against backaches, sexual weakness, headaches, malaria, loss of appetite, pelvic pains, constipation, skin ailments etc. [Adjanohoun et al., 1988; Betti, 2002; Bouquet, 1969; Boutique, 1951; Fruth et al. 2011; Musuyu Muganza, 2006; Sandberg, 1965], and as a mouthwash against caries and toothache [Wome, 1985].

The antiprotozoal activity generally reported for plants of the Annonaceae family is correlated with the importance of this botanic family in the traditional treatment of diseases like malaria, Chagas' disease, sleeping sickness and leishmaniasis, associated with metabolites found in

various parts of the plants of this family such as alkaloids, acetogenins, polyphenols, sterols and terpenes [Ocampo & Ocampo, 2006]. Recently, increasing attention has been paid to the isolation of active compounds from *I. hexaloba*. Bisbenzylisoquinoline, oxoaporphine, proaporphine and aporphine alkaloids, several sterols, terpenes (sesquiterpenes), indolic compounds as well as many other constituents were isolated from the root and stem bark of this plant [Hocquemiller et al., 1984; Mathouet et al., 2004; Bajin ba Ndob, 2008; Sénéjoux et al., 2008]. As a contribution to the assessment of the applicability of ethnomedicinally used plants, the present study deals with the *in vitro* evaluation of the antiprotozoal activity of different crude extracts and fractions from the leaves, root bark and stem bark of *I. hexaloba* against *Trypanosoma brucei brucei*, *T. cruzi*, *Leishmania infantum* and the chloroquine and pyrimethamine-resistant K1 strain of *Plasmodium falciparum*. In order to investigate the selectivity of the antiprotozoal activities, the cytotoxic effects of the tested extracts and fractions were also assessed against MRC-5 cell lines.

2. Materials and methods

2.1. Plant material

Leaves, root bark and stem bark of *Isolona hexaloba* Engl. & Diels (Annonaceae) were collected between July and August 2008 at the research site Luikotale [Hohmann and Fruth, 2003] in the South-western part of the southern block of the Salonga National Park (Bandundu province, D.R. Congo). The plant was identified by B. Nlandu of the Institut National d'Etudes et de Recherches en Agronomie (INERA) and by Dr J.-P. Habari of the Department of Biology (Faculty of Sciences, University of Kinshasa). A voucher specimen PROCUV/MPI 2762 of the plant has been deposited at the herbarium of this institute. The collected plant materials were air-dried and reduced to powder (IKA[®]A11). The three powders were kept in brown covered glass bottles.

2.2. Preparation of crude extracts and fractions

2.2.1. Crude aqueous decoctions

An aqueous decoction, which is the typical traditional mode of preparation of medicinal recipes by the Nkundo people, was prepared from leaves (IHLE-1) and root bark (IHRB-1) by boiling 50 g of powder in 1.5 liter of water during 15 min. After cooling and filtration, the extracts were freeze-dried (Christ[®] ALPHA 1-4 LSC).

2.2.2. Crude methanol extracts

An amount of 50 g of leaf, root bark and stem bark powder was macerated 3 times with 500 ml MeOH for 48 h. The macerates were combined and evaporated under reduced pressure at 40-45 °C on a Rotavapor (Heidolph 94200 Bioblock) yielding the dried crude extracts IHLE-2 for leaves, IHRB-2 for root bark and IHSB-2 for stem bark, respectively.

2.2.3. 80% Ethanol extract

An amount of 50 g of leaf, root bark and stem bark powder was macerated 3 times with 500 ml 80% EtOH for 48 hours under permanent shaking. Next, each extract was filtrated and evaporated under reduced pressure at 40-45°C, yielding the corresponding dried extracts IHLE-3.1, IHRB-3.1 and IHSB-3.1 for leaves, root bark and stem bark, respectively. An amount of each dry extract (3 g) was subjected to liquid-liquid partitioning as described in Figure 3. In this fractionation scheme, each of the 3 total extracts was dissolved in distilled water, acidified with HCl 2% until pH 3 - 5 and exhaustively washed with dichloromethane (DCM). This DCM phase was evaporated to dryness. The aqueous acid phase was alkalized (pH = 9) with ammonia 10% and extracted again with DCM to afford enriched alkaloid fractions (IHLE-3.2, IHRB-3.2 and IHSB-3.2 for leaves, root bark and stem bark extracts, respectively). The aqueous phases were called alkaline aqueous fractions and denoted IHLE-3.3, IHRB-3.3 and IHSB-3.3. On the other hand, the evaporated acidic DCM phases were

dissolved in 90% MeOH and quantitatively extracted with petroleum ether: in this way the petroleum ether fractions (IHLE-3.4, IHRB-3.4 and IHSB-3.4) and the 90% MeOH fractions (IHLE-3.5, IHRB-3.5 and IHSB-3.5) were obtained. The alkaline aqueous fractions were lyophilized (Christ[®] ALPHA 1-4 LSC) and the other obtained fractions were dried under reduced pressure at 40-45 °C on a Rotavapor (Heidolph 94200 Bioblock).

2.3. Phytochemical Screening

Chemical tests were carried out on the crude aqueous, methanol and 80% ethanol extracts from leaves, root bark and stem bark of *I. hexaloba* and on the fractions of the partition of the 80% ethanol extract using standard procedures [Harborne, 1998]. The froth test (Froth Index) was used for the detection of saponins. Alkaloids were detected using Dragendorff's and Mayer's reagents resulting in the formation of an orange or yellow-white precipitate, respectively. The presence of flavonoids was determined using aluminium chloride 5% in methanol and Shinoda's reagent (HCl + magnesium turnings) giving yellow and purple colors, respectively. The reducing sugars were identified by the Fehling's reagent. Detection of anthraquinones was performed by adding ammonia solution (NH₄OH 10%) or NaOH 10% solution producing a red to red-purple color. Steroids and terpenes were screened by adding Liebermann-Burchard's reagent (acetic anhydride/ concentrated H₂SO₄) and cardiotonic glycosides by Kedde reagent, both followed by heating for 5-10 min; the appearance of purplish blue color (or other colors) is seen as positive test. Tannins were identified with FeCl₃ 5% (gallic tannins), Stiasny's reagent and Bate-Smith's reagent (BuOH/ HCl) followed by heating for 5 min resulting in the appearance of a red brown (condensed tannins). Moreover, the presence of alkaloids, flavonoids and steroids/ terpenoids was confirmed by TLC (thin layer chromatography) analysis performed on precoated silica gel 60F₂₅₄ plates (layer thickness 0.25 mm, Merck) using various mobile phases and chemical reagents for the identification of those major phytochemical groups. The following chromatographic

conditions were used: Chloroform/ methanol/ ammonia 25% (8 : 2 : 0.5) as mobile phase and Dragendorff's reagent for detection of alkaloids; n-butanol/ acetic acid/ water (4 : 1 : 5) (top layer) and ethyl acetate / acetic acid / formic acid / water (100 : 11 : 11 : 27) as mobile phases with Neu's reagent for detection of flavonoids; 2-butanon/ toluene (4 : 6) and ethyl acetate/ heptane/ methyl t-butyl ether (MTBE)/ MeOH (50 : 40 : 20 : 10) as mobile phase and Liebermann-Burchard's reagent, vanillin 1% in phosphoric acid or SbCl_3 20% in CHCl_3 for steroids and terpenoids (carotenoids). Coumarins were detected under UV (366 nm) by their blue fluorescence which becomes intense after spraying with KOH 10% (Wagner and Bladt, 1996).

2.4. Biological evaluation

Extracts and fractions were tested against *Trypanosoma cruzi*, *Trypanosoma brucei brucei*, *Leishmania infantum*, *Plasmodium falciparum* (chloroquine and pyrimethamine-resistant K1 strain) and for cytotoxicity on MRC-5 (human lung fibroblasts) cells as reported before [Mesia et al., 2008; Musuyu Muganza et al., 2012]. Melarsoprol (for *T. brucei brucei*), benznidazol (for *T. cruzi*), miltefosine (for *L. infantum*), chloroquine diphosphate (for *P. falciparum*) and tamoxifen (cytotoxicity) were used as reference drugs. The following criteria were adopted for the evaluation of the results: $\text{IC}_{50} < 5 \mu\text{g/ml}$: pronounced activity; $5 < \text{IC}_{50} < 10 \mu\text{g/ml}$: good activity; $10 < \text{IC}_{50} < 20 \mu\text{g/ml}$: moderate activity; $20 < \text{IC}_{50} < 40 \mu\text{g/ml}$: low activity; $\text{IC}_{50} > 40 \mu\text{g/ml}$: inactive.

2.5. Toxicity in vivo An acute oral toxicity study was performed as per Mesia et al. (2010) according to OCDE guidelines (1987). Twenty four mice from the Institut National de Recherches Biomédicales (INRB) in Kinshasa were acclimatized to laboratory conditions and randomly divided in 5 test-groups and a control group receiving the vehicle (DMSO 2% in water).

Extracts and fractions having exhibited pronounced in vitro antiprotozoal activities $IC_{50} \leq 1$ $\mu\text{g/ml}$ (IHLE-3.1, IHLE-3.2, IHLE-3.3, IHLE-3.5 & IHRB-3.2) were used to test the acute toxicity in NMRI mice (22-26 g body weight) of either sex. The animals were kept fasting for overnight providing only water before administration of plant samples. Four doses (500, 1000, 1500 and 2000 mg/kg) of each sample were orally administered, as single doses, to different mice groups (n=4 each). The mice were allowed for food again 30 minutes after the gavage. On the first day, the mice were observed for 4 h after drug administration, and from the second until the 7th day, the observations were done daily during 1 h. Physiological and behavioural parameters such as grooming, hyperactivity, lethargy, righting reflex, weight, respiratory rate, convulsion and mortality were recorded.

3. Results

A total of 20 samples were tested: 2 crude aqueous extracts, 3 crude methanol extracts and 3 crude 80% ethanol extracts from the leaves, root bark and stem bark together with 12 subfractions from the crude 80% ethanol extracts. The antiprotozoam IC_{50} values, the CC_{50} values (cytotoxicity) and the selectivity index values are presented in Table 2.

The most active and most selective samples against *T.b.brucei* were the 2 methanol crude extracts IHRB-2 from root bark ($IC_{50} = 1.97$ $\mu\text{g/ml}$; $SI > 32.49$) and IHLE-2 from leaves ($IC_{50} = 2.65$ $\mu\text{g/ml}$; $SI > 24.15$).]. Nine other samples exhibited a good activity with IC_{50} values ranging from 7.19 to 9.22 $\mu\text{g/ml}$, with the crude methanol extract of stem bark (IHSB-2) displaying the best selectivity ($SI > 8.90$).

The 80% crude ethanol extract from leaves IHLE-3.1 showed good activity against *T. cruzi* with an IC_{50} value of 8.33 $\mu\text{g/ml}$ ($SI = 3.92$). Its petroleum ether fraction (IHLE-3.4) exhibited also good activity with an IC_{50} value of 8.50 $\mu\text{g/ml}$ ($SI = 2.52$). Only these 2

samples displayed a good activity against this parasite. The crude aqueous extract had previously been reported to have a good activity against *T. cruzi* ($IC_{50} = 9.31 \mu\text{g/ml}$) with a relative weak selectivity as well ($SI = 3.46$) [Musuyu Muganza et al., 2012].

The crude aqueous extract of leaves (IHLE-1) displayed a pronounced activity against *L. infantum* ($IC_{50} = 2 \mu\text{g/ml}$) and its action was appreciably selective ($SI > 32$). Three other samples exhibited a good activity: the crude methanol extract from leaves (IHLE-2) ($IC_{50} = 6.35 \mu\text{g/ml}$; $SI > 10.10$) and the alkaloid fractions of the 80% ethanol extracts from the root bark (IHRB-3.2) ($IC_{50} = 6.96 \mu\text{g/ml}$; $SI = 6.1$) and from the stem bark (IHSB-3.2) ($IC_{50} = 8 \mu\text{g/ml}$; $SI > 8$).

From the 20 samples tested, 16 exhibited a good to pronounced activity against *P. falciparum* K1. The alkaline aqueous fraction of the 80% ethanol crude extract from leaves (IHLE-3.3) which is rich in hydrophilic substances such as polyphenols, displayed the most pronounced antiplasmodial activity with an IC_{50} value of $0.27 \mu\text{g/ml}$ and had the highest selectivity ($SI > 237.04$). Ten other samples exhibited a pronounced activity against Pf-K1 and among these, 3 samples with comparable IC_{50} values and appreciable selectivity were prepared from the leaves: IHLE-3.5 ($IC_{50} = 0.90 \mu\text{g/ml}$; $SI > 71.11$), IHLE-3.1 ($IC_{50} = 0.92 \mu\text{g/ml}$; $SI = 35.53$) and IHLE-3.2 ($IC_{50} = 1.04 \mu\text{g/ml}$; $SI > 61.54$); furthermore, as for IHLE-3.2, alkaloids are believed to be involved in the pronounced activity displayed by 2 other dichloromethane fractions from the 80% ethanol crude extract of the root bark (IHRB-3.2) ($IC_{50} = 2.01 \mu\text{g/ml}$; $SI = 21.13$) and the stem bark (IHSB-3.2) ($IC_{50} = 2.23 \mu\text{g/ml}$; $SI > 28.70$). Two crude extracts from the stem bark, namely the 80% ethanol (IHSB-3.1) ($IC_{50} = 2.27 \mu\text{g/ml}$; $SI > 28.19$) and the methanol extract (IHSB-3.2) ($IC_{50} = 2.54 \mu\text{g/ml}$; $SI > 25.20$) exhibited a comparable activity. The activity and selectivity of the 90% methanol fraction of the stem bark (IHSB-3.5) ($IC_{50} = 4.44 \mu\text{g/ml}$; $SI > 14.41$) was very close to those observed for the 80% ethanol

extract from the root bark (IHRB-3.1) ($IC_{50} = 4.48 \mu\text{g/ml}$; $SI > 14.29$); the activity of the petroleum ether fraction from leaves (IHLE-3.4, $IC_{50} = 4.88 \mu\text{g/ml}$) is also close to that of the 2 previous samples but IHLE-3.4 showed the highest cytotoxicity from all tested samples ($CC_{50} = 21.4 \mu\text{g/ml}$) and therefore its selectivity index is rather low ($SI = 4.39$). The 5 remaining samples showed a good activity with $5 < IC_{50} < 10 \mu\text{g/ml}$.

Concerning acute toxicity in NMRI mice, it was tested up to a concentration of 2.0 g/ kg. But even at this high level of dosage, the tested plant samples did not produce any signs of toxicity and LD_{50} values were estimated to be higher than 2000 mg/ kg.

4. Discussion

Among the Nkundo people, medicinal preparations from *I. hexaloba* are preferably made by decoction [Musuyu Muganza, 2006]. Looking at the activity of crude aqueous extracts from this plant, it is noteworthy that the root bark aqueous extract (IHRB-1) showed a good activity against *P. falciparum* K1 ($IC_{50} = 8.39 \mu\text{g/ml}$; $SI > 7.63$) and *T.b. brucei* ($IC_{50} = 7.41 \mu\text{g/ml}$; $SI > 8.64$), while the crude aqueous extract from leaves (IHLE-1) displayed a low activity against *P. falciparum* K1 ($20 < IC_{50} < 40 \mu\text{g/ml}$), a good activity against *T.b. brucei* ($IC_{50} = 9.22 \mu\text{g/ml}$; $SI > 6.94$) and a pronounced activity against *L. infantum* ($IC_{50} = 2.0 \mu\text{g/ml}$; $SI > 32$). In our previous screening, it had already been found that the crude aqueous extract from the stem bark of this plant displayed a pronounced activity against *T.b. brucei* ($IC_{50} = 1.95 \mu\text{g/ml}$; $SI = 16.52$) [Musuyu Muganza et al., 2012].

The diversity of phytoconstituents with antiprotozoal activities has been documented in many reviews covering substances from different phytochemical groups [Xu and Pieters, 2013; Rocha et al., 2005; Hoet et al., 2004]. From a phytochemical point of view, alkaloids (bisbenzylisoquinolines, oxoaporphines, proaporphines or aporphines), several sterols, along with terpenes and other indolic compounds were isolated from the root and stem bark of *I.*

hexaloba [Bajin ba Ndob, 2008; Sénéjoux et al., 2008; Mathouet et al., 2004; Hocquemiller et al., 1984]. Our phytochemical screening was conducted on the aqueous, methanol and 80% ethanol crude extracts from leaves, stem bark and root bark of *I. hexaloba* and on the fractions from the partition of the 80% ethanol extracts. The presence of steroids and/or terpenes, carotenoids, alkaloids, reducing sugars and phenolic compounds (flavonoids, tannins), as presented in Table 1, was certified and these constituents are believed to account for the observed biological activities. Similar to Cabalion et al. (1980), our phytochemical screening revealed the presence of alkaloids in leaves, root bark and stem bark and all 3 DCM fractions enriched in alkaloids displayed a pronounced activity against *P. falciparum* K1 with the leaves sample being the most active and, according to the TLC profiles, the phytochemically richest of the 3 DCM fractions. Cycleanine, norcycleanine and isochondodendrine, which are bisbenzylisoquinoline (BBIQ) alkaloids, have been isolated from the stem bark and root bark of *I. hexaloba* [Hocquemiller et al., 1984] and 2 other BBIQ alkaloids (curine and guattegaumerine) were obtained from the roots [Laouirem et al., 2008]; 3 oxoaporphine alkaloids (atherospermidine, liriodenine and lysicamine), along with 3 sterols (campesterol, β -sitosterol and stigmasterol), caryophyllene oxide, 3 fatty acids (linoleic acid, palmitic acid and stearic acid) and 2 indolic compounds (5-formyl-indole and 5-(3-oxo-but-1-enyl)-indole) were isolated from a chloromethylenic extract of defatted stem bark of *I. hexaloba* [Sénéjoux et al., 2008] and cazolobine, a sesquiterpene derivative, was isolated from the roots of the same plant [Mathouet et al., 2004].

To the best of our knowledge most of these compounds have not yet been reported from the leaves of this plant. Moreover, it is well known that many BBIQs with different chemical structures isolated from other plant species had been shown to possess antiprotozoal potential against different strains of *P. falciparum* [Murebwayire et al., 2008; Longanga Otshudi et al., 2005; Mambu et al., 2000; Angerhofer et al., 1999; Marshall et al., 1994], *Entamoeba histolytica* [Marshall et al., 1994], *Trypanosoma brucei* [Murebwayire et al., 2008; Camacho

et al., 2002], *Trypanosoma cruzi* [Fournet et al., 1997] and *Leishmania* species [Camacho et al., 2002; Fournet et al., 1988]. Some known BBIQs from *I. hexaloba* were also investigated for these antiprotozoal activities. Thus, cycleanine and isochondodendrine were previously reported to produce 94-99% inhibition of the growth of two *Leishmania* species including *L. brasiliensis brasiliensis* and *L. donovani* for cycleanin; and 77-98.6% inhibition of the growth of 3 *Leishmania* species including *L. brasiliensis brasiliensis*, *L. mexicana amazonensis* and *L. donovani* for isochondodendrine at the tested concentration of 50 µg/ml. At the lowest tested concentration of 10 µg/ml, they still produced 64.3% inhibition for cycleanin on *L. donovani* and 65.3-75.9% inhibition for isochondodendrin on the three tested *Leishmania* species, respectively [Fournet et al., 1988]. Interestingly, cycleanine and curine were reported to show high in vivo efficacy against *T. cruzi* infection of C3/He mice when administered at an oral dose of 2 mg/kg and 10 mg/kg body weight. No obvious toxicity could be noticed as assessed by appearance, weight change, blood parameters and organ histology of the treated animals. They showed mortality times (MT₅₀) of 134.16 - 135.29 and 129.37 - 210.09 days respectively depending on tested strains of *T. cruzi*. The effect of these BBIQs could be due to a blocking of the Ca²⁺ channel for the regulation of *T. cruzi* infectivity to invade host cells, or their selective immunosuppressive properties suggesting a mechanism of action of BBIQ on trypomastigote growth [Fournet et al., 1997]. Moreover, cycleanine was previously reported to exhibit a pronounced antiplasmodial activity against the W2 clone of *P. falciparum* and the chloroquine-sensitive D6 clone with IC₅₀ values of 247 and 73 nM, respectively, in the absence of cytotoxicity against KB cells (ED₅₀ > 33700 nM), resulting in SI values of > 460 and > 140 indicating its high selectivity against these parasites [Angerhofer et al., 1999]. Furthermore, this BBIQ isolated from *Epinetrum villosum* root bark (Menispermaceae) had been reported to display pronounced antiplasmodial activity against the chloroquine-resistant FcB1 strain of *P. falciparum* with an IC₅₀ value of 2.8 ± 1.6 µg/ml (45.0 nM), in the absence of cytotoxicity against HCT-116 human colon carcinoma cell lines (ATCC CCL 247),

resulting in a high selectivity index of 118 [Longanga Otshudi et al., 2005]. Mambu et al. (2000) had reported pronounced antiplasmodial activity for isochondodendrine isolated from *Isolona ghesquiereina* stem bark against *P. falciparum* cultured in human blood ($IC_{50} = 892 \pm 3$ nM), in the absence of cytotoxicity effect against L-6 cell lines, resulting in an SI of 51. In addition, this BBIQ alkaloid isolated from *E. villosum* root bark had also been found to exhibit strong antiplasmodial activity against the chloroquine-resistant FcB1 strain of *P. falciparum* ($IC_{50} = 0.10 \pm 0.01$ μ g/ml or 0.26 nM), with an SI of 175. Although cycleanine N-oxide, a derivate of cycleanine has not yet been isolated from *I. hexaloba*, this compound isolated from *E. villosum* had been previously reported to exhibit antiplasmodial activity against the chloroquine-resistant FcB1 clone of *P. falciparum* ($IC_{50} = 8.6 \pm 2.2$ μ g/ml or 13.48 μ M), in the absence of appreciable cytotoxicity on HCT-116 human colon carcinoma cell-lines ($CC_{50} = 41.8 \pm 4.15$ μ g/ml) [Longanga Otshudi et al., 2005].

These previously reported results clearly show that BBIQs seem to be at least in part responsible of the antiprotozoal potential of *Isolona hexaloba* since the alkaloid fractions were among the most active ones. Another class of alkaloids (oxoaporphines) has been isolated from *I. hexaloba* with compounds like atherospermidine, liriodenine and lysicamine [Sénéjoux et al., 2008]. Atherospermidine and liriodenine have also been isolated from leaves of *Annona mucosa* but only liriodenine was tested for its antileishmanial activity on promastigote forms ($IC_{50} = 1.43 \pm 0.58$ μ g/ml, SI = 13.36 towards *L. amazonensis* TH-8 and $IC_{50} = 55.92 \pm 3.55$ μ g/ml, SI = 0.34 towards *L. braziliensis* M2903) along with other active samples. Liriodenine was found to be the most cytotoxic ($LC_{50} = 19.11 \pm 1.06$ μ g/ml) of all tested samples against the mice peritoneal macrophages (de Lima et al., 2012); the same 2 alkaloids were isolated from *Annona foetida* too alongside with another oxoaporphine (O-methylmoschatoline) and one pyrimidine- β -carboline-one (annomontine); all the isolated alkaloids (excepted atherospermidine) were tested for trypanocidal activity (*T. cruzi*). O-

methylmoschatoline was found to be the most active against both epimastigote ($IC_{50} = 92.0 \pm 18.4 \mu\text{g/ml}$) and trypomastigote ($IC_{50} = 3.8 \pm 1.8 \mu\text{g/ml}$) forms of *T. cruzi*. Liriodenine displayed this trypanocidal activity with respective IC_{50} values of $177.0 \pm 10.6 \mu\text{g/ml}$ and $4.0 \pm 0.2 \mu\text{g/ml}$. The trypanocidal activity against trypomastigote forms of *T. cruzi* was even stronger than that of the positive control (crystal violet, $IC_{50} = 12.8 \pm 0.9 \mu\text{g/ml}$) [Costa et al., 2011]. This antiprotozoal profile was also found by Février et al. (1999) who noted that liriodenine from *Rollinia emarginata* lysed all the tested *Leishmania* strains (*L. braziliensis* 2903, *L. amazonensis* PH-8 and *L. donovani* HS-70) at a concentration of $5 \mu\text{g/ml}$ and that this oxoaporphine exhibiting a significant trypanocidal action in vitro by reducing the number of parasites in infected murine blood by 53%. Waechter et al. (1999) worked on antileishmanial and antitrypanosomal activities of extracts and compounds isolated from the stem bark of *Unonopsis buchtienii* found that lysicamine was devoid of trypanocidal activity in vitro against *Trypanosoma brucei brucei* (CMP strains) while liriodenine showed a weak activity ($IC_{100} = 50 \mu\text{g/ml}$) and that liriodenine displayed the highest leishmanicidal activity against *Leishmania major* and *L. donovani* with IC_{100} value of $3.12 \mu\text{g/ml}$.

Nevertheless, other constituents such as steroids and terpenes may possess antiprotozoal properties as well even if sterols (such as β -sitosterol and stigmasterol) were found inactive against these tested *Leishmania* strains in Waechter et al. (1999). In our study, the 90% methanol soluble fractions rich in steroids and terpenes as presented in Table 1, were found to display good to pronounced antiprotozoal activity with IC_{50} values ranging from 0.9 to $8.5 \mu\text{g/ml}$ towards *P. falciparum* K1 and *T.b.brucei* in particular. Polyphenols have also been cited to be active against protozoa. Ellagic acid for instance was found to be active against *Plasmodium falciparum*, in vitro as well as in vivo, as reported by Banzoui et al. (2002) and Soh et al. (2009).

In another study, 132 flavonoids from different subclasses (flavone, flavonol, flavanone, isoflavone and chalcone) were evaluated for their in vitro antitrypanosomal (*T.b. rhodesiense*) activity. The activities of 7, 8-hydroxyflavone and quercetagenin were found to be the most pronounced and the most selective with respective IC_{50} (0.16 & 0.8 μ M) and SI (1019 & 571) values [Raez, 1998].

In the present study, the polyphenol-rich fraction (IHLE-3.3) from leaves, which also probably contains polar alkaloids according to the phytochemical screening, displayed the greatest activity and the most specific action against *P. falciparum* K1 as indicated in Table 2. Toxicity testing was carried out for the most antiprotozoally active samples mainly for those exhibiting IC_{50} values ≤ 1.0 μ g/ml in order to situate their tolerances limits. According to the obtained results, the tested plant samples did not produce any mortality or toxicity signs and LD_{50} values were estimated higher than 2000 mg/kg in NMRI mice.

5. Conclusion

The results of the present study provide further data on the antiprotozoal activity of the aqueous, methanol and 80% ethanol crude extracts from leaves, root bark and stem bark of *I. hexaloba* as well as the respective different fractions of the latter extract. In particular, the preeminence of the antiplasmodial activity of the 80% ethanol extract from the leaves and most of its respective fractions in comparison to those observed for samples from the stem bark and the root bark was established. Although the stem bark is the most used plant part in traditional medicine as reported by Musuyu Muganza (2006), it turns out that the leaves are the part which would be most advisable because of their renewability and above all, given that their samples displayed the most important activity with the best selectivity indices especially against *P. falciparum*. Globally, the obtained results tend to validate/ corroborate the traditional use of this plant species against parasites especially as the most antiprotozoally active tested plant samples can seemingly be considered devoid of toxicity in mice. Therefore,

the most active fractions have been selected for extensive further studies aiming to isolate and identify the active constituents. Moreover, complementary in vivo investigations will be considered in order to fully evaluate the expressed antiprotozoal potential and the subchronic/ long term toxicity profiles of the most active fractions and/or their constituents.

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Conflicts of Interest

The authors declare no conflict of interests regarding the publication of this paper.

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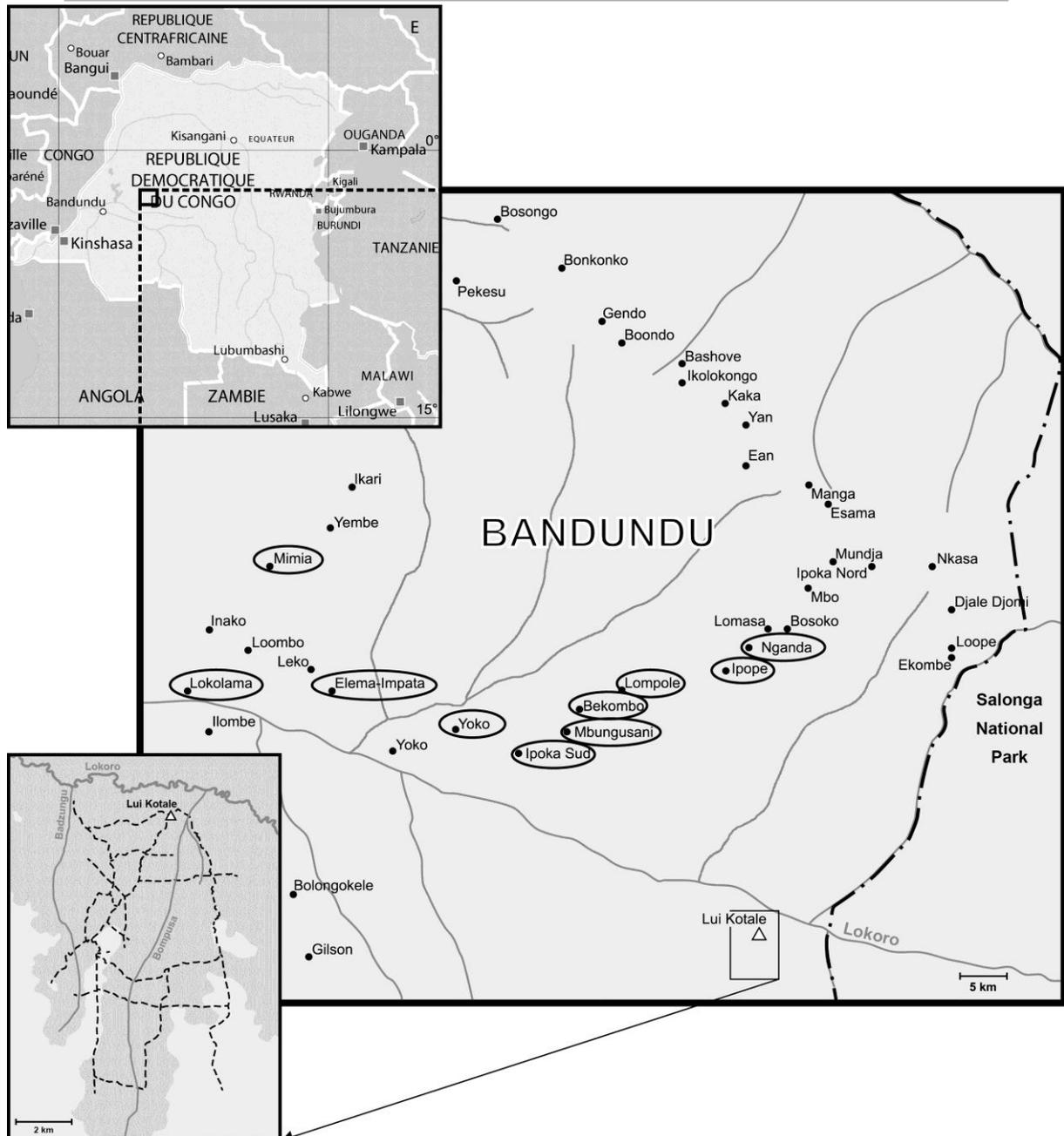


Fig. 1: Map of the D.R. of Congo with focus on the area of ethnobotanical investigations and plant sample collection (Lui Kotale study site). Villages that contributed to the data set are circled (from Fruth et al. 2011).



Fig. 2: Flowering branch of *Isolona hexaloba*

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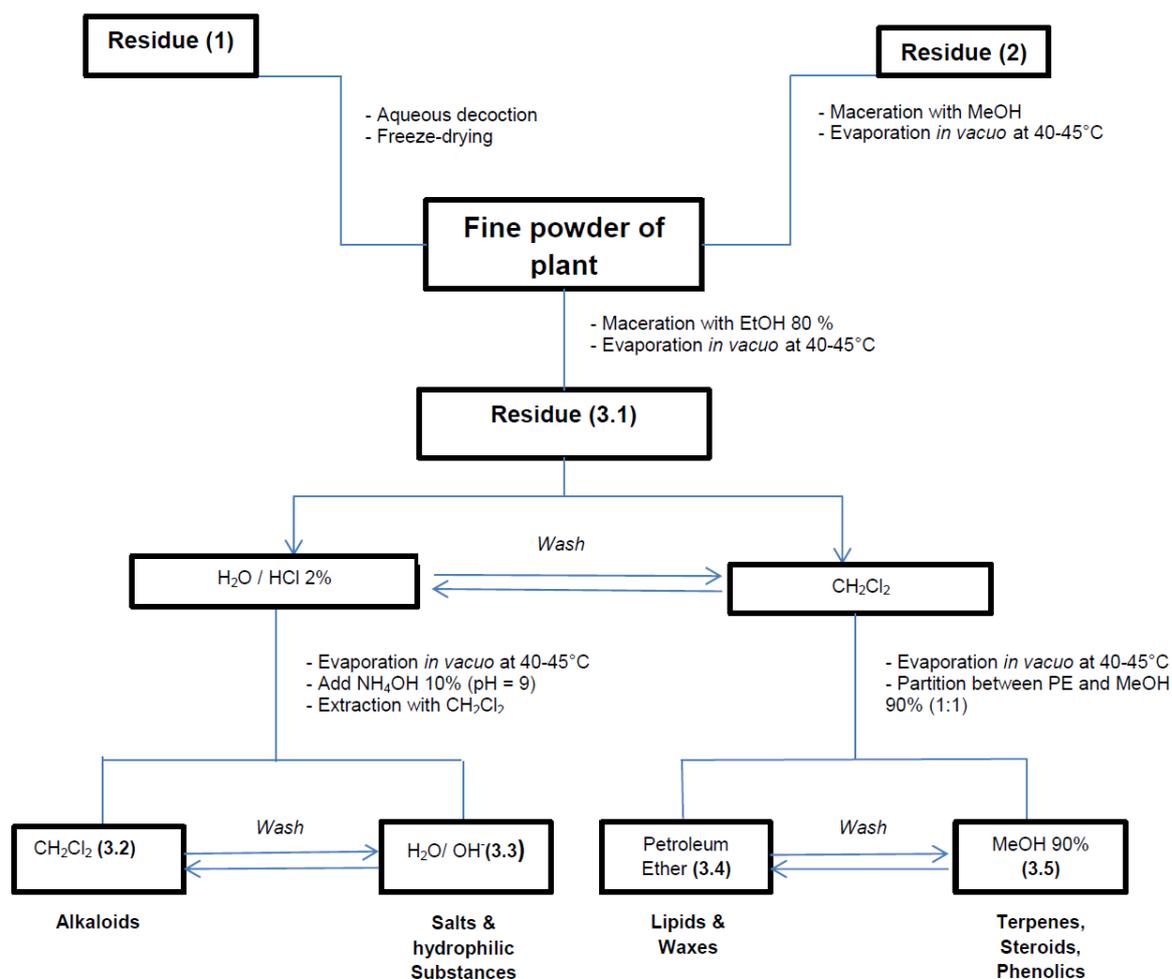


Fig. 3: Fractionation scheme of the crude extract (residue 3.1)

Table 1: Phytochemical screening results

Phytochemical groups\ Samples	IHLE-1	IHRB-1	IHSB-1	IHLE-2	IHRB-2	IHSB-2	IHLE-3.1	IHLE-3.2	IHLE-3.3	IHLE-3.4	IHLE-3.5	IHRB-3.1	IHRB-3.2	IHRB-3.3	IHRB-3.4	IHRB-3.5	IHSB-3.1	IHSB-3.2	IHSB-3.3	IHSB-3.4	IHSB-3.5	
Alkaloids/ Dragendorff Reagent	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Alkaloids/ Mayer Reagent	+	+	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Anthraquinones/ NaOH 10%	-	-	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Cardiotonic glycosides/ Kedde Reagent	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carotenoids/ SbCl ₃ 20% in CHCl ₃	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Coumarins/ KOH 10% & UV 366 nm	-	+	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Flavonoids/ AlCl ₃ 5% in MeOH Reagent	+	+	+	+	+	+	+	-	+	-	-	+	-	+	-	-	+	-	+	-	-	
Flavonoids/ Neu Reagent	+	+	+	+	+	+	+	-	+	-	-	+	-	+	-	-	+	-	+	-	-	
Flavonoids/ Shinoda Reagent	+	+	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Reducing sugars/ Fehling reagent	+	+	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Saponin/ Froth test/ Froth index	125	<100	<100	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Tannins (condensed)/ Bate-Smith reagent	+	+	+	+	+	+	nd	nd	+	nd	nd	nd	nd	+	nd	nd	nd	nd	+	nd	nd	+
Tannins/ FeCl ₃ 5%	+	+	+	+	+	+	+	-	+	-	-	+	-	+	-	-	+	-	+	-	-	-
Tannins/ Stiasny reagent	+	+	+	+	+	+	+	-	+	-	-	+	-	+	-	-	+	-	+	-	-	-
Terpenes & Steroids/ Liebermann-Burchard Reagent	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-	+	+	-	-	-	-	+
Terpenes & Steroids/ vanillin 1% in phosphoric acid	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-	+	+	-	-	-	-	+

Table 2: Antiprotozoal and cytotoxic activity of different crude extracts and fractions from *Isolona hexaloba*

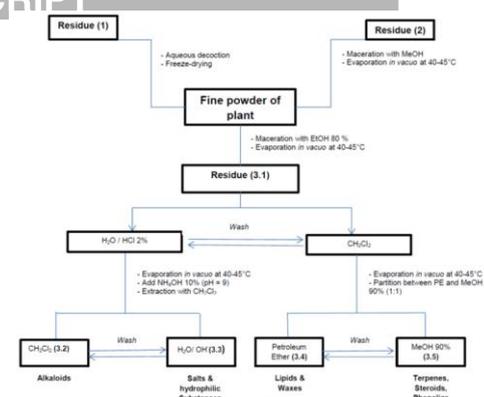
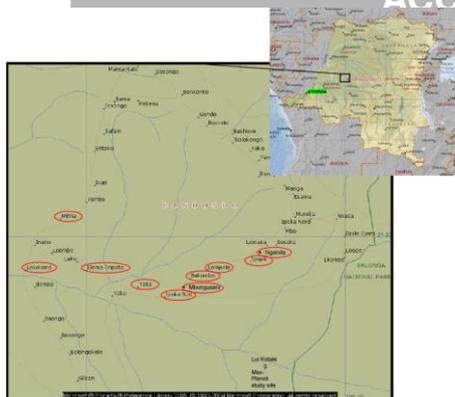
Tested Samples	Antiprotozoal IC ₅₀ (µg/ml) activity				Cytotoxicity CC ₅₀ (µg/ml)	MRC-5 Selectivity Index			
	T.b.b	T. c.	L. inf.	Pf-K1		MRC-5/ Tbb	MRC-5/ Tc	MRC-5/ L. inf.	MRC-5/ Pf-K1
IHLE-1	9.22	30.05	2.00	23.59	>64.00	>6.94	>2.13	>32	>2.71
IHRB-1	7.41	33.07	32.46	8.39	>64.00	>8.64	>1.94	>1.97	>7.63
IHLE-2	2.65	33.71	6.35	6.03	>64.00	>24.15	>1.90	>10.10	>10.61
IHRB-2	1.97	21.30	10.08	8.18	>64.00	>32.49	>3.00	>6.35	>7.82
IHSB-2	7.19	15.06	24.05	2.54	>64.00	>8.90	>4.25	>2.66	>25.20
IHLE-3.1	10.21	8.33	32.46	0.92	32.69	3.20	3.92	1.00	35.53
IHLE-3.2	34.27	32.79	34.56	1.04	>64.00	>1.87	>1.95	>1.85	>61.54
IHLE-3.3	>64.00	>64.00	>64.00	0.27	>64.00	nd	nd	nd	>237.04
IHLE-3.4	8.17	8.50	11.31	4.88	21.40	2.62	2.52	1.89	4.39
IHLE-3.5	8.43	16.54	50.80	0.90	>64.00	>7.59	>3.87	>1.26	>71.11
IHRB-3.1	8.57	34.56	20.32	4.48	>64.00	>7.47	>1.85	>3.15	>14.29
IHRB-3.2	7.23	10.34	6.96	2.01	42.47	5.87	4.11	6.10	21.13
IHRB-3.3	>64.00	>64.00	>64.00	>64.00	>64.00	nd	nd	nd	nd
IHRB-3.4	8.17	21.71	27.27	9.92	34.56	4.23	1.59	1.27	3.48
IHRB-3.5	8.30	20.71	26.91	5.59	>64.00	>7.71	>3.09	>2.38	>11.45
IHSB-3.1	40.82	34.27	21.11	2.27	>64.00	>1.57	>1.87	>3.03	>28.19
IHSB-3.2	33.71	17.55	8.00	2.23	>64.00	>1.90	>3.65	>8.00	>28.70
IHSB-3.3	>64.00	>64.00	>64.00	>64.00	>64.00	nd	nd	nd	nd
IHSB-3.4	>64.00	>64.00	>64.00	>64.00	>64.00	nd	nd	nd	nd
IHSB-3.5	31.74	45.02	32.46	4.44	>64.00	>2.02	>1.42	>1.97	>14.41
Benznidazol	nd	3.19	nd	nd	nd	nd	nd	nd	nd
Chloroquine	nd	nd	nd	0.34	nd	nd	nd	nd	nd
Suramine	0.05	nd	nd	nd	nd	nd	nd	nd	nd
Miltefosine	nd	nd	3.17	nd	nd	nd	nd	nd	nd
Tamoxifen	nd	nd	nd	nd	13.7	nd	nd	nd	nd

IHLE: *Isolona hexaloba* leaves, IHRB: *Isolona hexaloba* root bark, IHSB: *Isolona hexaloba* stem bark;

3.1: 80% crude ethanol extract, 3.2: Dichloromethane fraction rich in alkaloids, 3.3: Alkaline aqueous fraction rich in salts & hydrophilic substances; 3.4: Petroleum ether fraction rich in lipids and waxes; and 3.5: 90% methanol fraction rich in steroids and terpenes from each selected plant part;

T.b.b: *Trypanosoma brucei brucei*, T.c: *Trypanosoma cruzi*, L.inf: *Leishmania infantum*, MRC-5: Human Fetal Lung Fibroblast Cells; Pf-K1: *Plasmodium falciparum* chloroquine and pyrimethamine resistant K-1 strain of *P. falciparum*, nd: not determined.

graphical abstract



Accepted manuscript