Bioassay-guided isolation of active principles from Nigerian medicinal plants identifies new trypanocides with low toxicity and no cross-resistance to diamidines and arsenicals.

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Abstract

Ethnopharmacological relevance: Leaves from the plant species studied herein are traditionally used in northern Nigeria against various protozoan infections. However, none of these herbal preparations have been standardized, nor have their toxicity to mammalian cells been investigated. In search of improved and non-toxic active antiprotozoal principles that are not cross-resistant with current anti-parasitics, we here report the results of the *in vitro* screening of extracts from seven selected medicinal plant species (*Centrosema pubescens, Moringa oleifera, Tridax procumbens, Polyalthia longifolia, Newbouldia laevis, Eucalyptus maculate, Jathropha tanjorensis*), used traditionally to treat kinetoplastid infections in Nigeria, and the isolation of their bioactive principles.

Aim of the study: To investigate the efficacies of medicinal plant extracts, and of compounds isolated therefrom, against kinetoplastid parasites, assess cross-resistance to existing chemotherapy, and assay their toxicity against mammalian cells *in vitro.*

Material and methods: Plants were extracted with hexane, ethyl acetate and methanol. Active principles were isolated by bioassay-led fractionation, testing for trypanocidal activity, and identified using NMR and mass spectrometry. EC_{50} values for their activity against wild-type and multi-drug resistant *Trypanosoma brucei* were obtained using the viability indicator dye resazurin.

Results: Seven medicinal plants were evaluated for activity against selected kinetoplastid parasites. The result shows that crude extracts and isolated active compounds from *Polyalthia longifolia* and *Eucalyptus maculata*, in particular, display promising activity against drug-sensitive and multi-drug resistant *Trypanosoma brucei*. The EC_{50} value of a clerodane (16α-hydroxy-cleroda-3,13(14)-Z-dien-15,16-olide) isolated from *Polyalthia longifolia* was as low as 0.38 µg/mL, while a triterpenoid (3β,13β-dihydroxy-urs-11-en-28-oic acid) isolated from *Eucalyptus maculata* displayed an EC_{50} of 1.58 µg/mL. None of the isolated compounds displayed toxicity towards Human Embryonic Kidney cells at concentrations up to 400 µg/mL. In addition, the isolated compounds were active against *Leishmania mexicana*, as well as against *T. congolense*.

Conclusion: We have isolated a clerodane compound from *Polyalthia longifolia* that shows low toxicity, no cross-resistance with current treatments, and promising activity against both human-infective and veterinary *Trypanosoma* species.

Keywords: Antiparasite chemotherapy, ethnopharmacology, extracts, *Leishmania mexicana*, purified compounds, *Trypanosoma brucei*.

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1. Introduction

Infectious diseases that are caused by certain species of the genera *Trypanosoma* (Human African trypanosomiasis or HAT, and Chagas disease) and *Leishmania* (various forms of leishmaniasis) are amongst the neglected tropical disease, affecting many millions of people throughout the tropics. Mortality and morbidity resulting from these diseases is still very high in developing countries (Hotez and Kamath, 2009; Hotez *et al*., 2009). New and improved treatments would undoubtedly enhance the welfare of the local population and livestock (Nwodo *et al*., 2015; Giordani *et al*., 2016). While mortality due to HAT is clearly important, African Animal Trypanosomiasis (AAT or nagana) is considered the livestock disease with the

highest impact on agricultural production and animal husbandry in Africa, causing annual losses which run to billions of US dollars (Samdi *et al*., 2010). Across the tsetse belt as many as 55 million heads of cattle are at risk of infection, plus 30 million sheep and 40 million goats. It is estimated that 3 million cattle die every year from African trypanosomiasis (Samdi *et al*., 2010; Seyoum *et al*., 2013).

African trypanosomiasis and leishmaniasis as global health challenges are compounded by the lack of vaccines, making chemotherapy the only suitable alternative at the moment (La Greca and Magez, 2011). In addition, existing antiparasitic drugs are hampered by toxic side effects and the emergence of resistance (Delespaux and De Koning, 2007; Fairlamb *et al*., 2016). However, natural products have been identified as highly promising starting points for the discovery of anti-protozoan agents (e.g. Salem and Werbovetz, 2006; Omar *et al.*, 2016; Siheri *et al.*, 2016; Dike *et al*., 2016), and indeed have a long and distinguished history as essential drugs in the fight against tropical disease (e. g. Tu 2011). Indeed, over the period 1981-2002, 61% of all new chemical entities approved for infectious diseases were natural compounds or directly derived thereof (Newman *et al*., 2003).

Natural products derived from plants have played an important role in the control of diseases caused by the protozoal parasites, a classical example is malaria caused by *Plasmodium falciparum*. Current malaria treatment relies heavily on plant-derived products, including the sesquiterpene lactone artemisinin (Wells, 2011), and the alkaloid quinine (Moyo *et al*., 2016). The success stories of artemisinin, isolated from the Chinese wormwood plant (*Artemisia annua*) and of quinine, isolated from the bark of cinchona trees (*Cinchona officinalis*), both used traditionally as antimalarial therapies, justify drug discovery based on ethnopharmacological usage, as these drugs were discovered following an intensive screening of hundreds of plants traditionally used for treating malaria (Tu, 2011).These achievements notwithstanding, unfortunately, fewer efforts have been put into investigating extracts from various plants traditionally used for treating the kinetoplastid diseases like trypanosomiasis and leishmaniasis despite the undesirable side effects and marginal effectiveness of the currently available drugs.

Centrosema pubescens, Moringa oleifera, Tridax procumbens, Polyalthia longifolia, Newbouldia laevis, and *Eucalyptus maculata* thrive well in many areas of Nigeria where they grow in the wild except for *Moringa oleifera,* which is cultivated for food and for medicinal use. *Newbouldia laevis* is mostly used in northern Nigeria for boundary demarcation, while *Polyalthia longifolia* commonly called ‘‘mast tree’’ is used as an ornamental plant. These plants are highly valued and are also used for treating various infections caused by the protozoan parasites including species of *Trypanosoma, Leishmania, and Plasmodium*, especially the strains that resists existing chemotypes (Tor-Anyiin *et al*., 2003; Igoli *et al*., 2004, 2005; Alli *et al*., 2011; Abubakar *et al*., 2012; Nwodo *et al*., 2015; Bankole *et al*., 2016) . Unfortunately none of the herbal preparations from these plants have been standardized for use, nor extensively investigated for their toxicity on mammalian cells, and their activity against protozoan parasites has not been scientifically validated. Because these plants grow on many soil types, are abundant in the wild, and only their leaves are used, their utilization as herbal remedies pose no danger to biodiversity.

The development of an effective chemotherapy based on a local resource will ensure new drugs that will both address the urgent need for new treatments and provide an additional economic driver for poor farming communities. In this study, we investigate the anti-kinetoplastid potential of selected herbs traditionally used for treating infections caused by trypanosomatid parasites in northern Nigeria.

2. Materials and Methods

2.1. Plant selection and collection

In Nigeria, the use of traditional herbal medicine is part of the unique tradition acceptable to the majority of the people (Sofowora, 1993; Heinrich, 2000; Adetutu *et al*., 2011). This notwithstanding, the secrecy associated with this practice has led to a paucity of scientific reporting of this knowledge in literature (Ashidi *et al*., 2010). However, the selection of traditional medicinal plants used in the present study is based on interviews with traditional healers who were willing to divulge the information. Following the method of Adetutu *et al*. (2011), ethnopharmacological methods were used to select the plants used in this research.

Briefly, the selection followed interviews with the traditional healers, using structured questionnaires to establish which plant and part of the plant is in common use against tropical fevers. Thirty-one traditional healers were approached, with eighteen volunteering to complete the survey. The information collected included local names of the plants used for treating various forms of fevers, parts of the plant commonly used, methods of preparation, and details of administration. The frequency of citation of each plant was recorded. Seven plants were consistently cited. The selected plants were purchased from the healers and their specimens were used for identification at the herbarium of Kogi State University, Nigeria. Each plant name has been checked with www.theplantlist.org and this website was last accessed on the 06/01/2017. The plants were identified as shown in Table 1:

Table 1. Botanical name and voucher number for each plant evaluated in this study.

Code	Botanical name	Voucher number
A	*Centrosema pubescens* Benth. (Family *Fabaceae*)	0446
B	*Moringa oleifera* Lam (Family *Moringaceae*)	0447
C	*Tridax procumbens* (L.) L. (Family *Compositae*)	0448
D	*Polyalthia longifolia* (Sonn.) Thwaites (Family *Annonaceae*)	0449
E	*Newbouldia laevis* (P.Beauv.) Seem. (Family *Bignoniaceae*)	0450
F	*Eucalyptus maculata* Hook. (Family *Myrtaceae*)	0451
G	*Jatropha tanjorensis* J.L.Ellis & Saroja (Family *Euphorbiaceae*)	0452

Plant collection was carried out in the months of January and February 2014 from the town of Anyigba, Kogi State, Nigeria (latitude 7º 15' - 7º 29' N and longitude 7º 11'- 7º 32' E; altitude of 410 – 430 m).

2.2. Extraction procedure

The leaves of the plants were thoroughly air dried and subsequently were ground to a fine power using a grinder before extraction.

The powdered dry leaves (20 g) were weighed into an extraction thimble (Fisher Scientific) and placed in a Soxhlet apparatus. The plant materials were extracted consecutively using solvents

of increasing polarity starting with *n*-hexane and followed by ethyl acetate and methanol (500 mL of each solvent; all obtained from Fisher Scientific (Loughborough, UK)). Each extraction stage was carried out to exhaustion. All extracts obtained were evaporated at 40 ºC; recovery of solvent, and concentration of the extracts, was carried out under vacuum using a rotary evaporator connected to a condenser. Residual solvents were further allowed to evaporate under the fume hood before samples were freeze dried in a freeze dryer. The hexane, ethyl acetate, and methanol extracts obtained were labelled as below, then stored at -20 ºC prior to analysis. The yields of the crude extracts were between 2-5 g.

Samples were labelled accordingly as:

HDK-AE; HDK-AH; HDK-AM – Ethyl acetate, Hexane, and Methanolic fractions from plant A respectively.

HDK-BE; HDK-BH; HDK-BM – Ethyl acetate, Hexane, and Methanol fractions from plant B respectively.

HDK-CE; HDK-CH; HDK-CM – Ethyl acetate, Hexane, and Methanol fractions from plant C respectively.

HDK-DE; HDK-DH; HDK-DM – Ethyl acetate, Hexane, and Methanol fractions from plant D respectively.

HDK-EE; HDK-EH; HDK-EM – Ethyl acetate, Hexane, and Methanol fractions from plant E respectively.

HDK-FE; HDK-FH; HDK-FM – Ethyl acetate, Hexane, and Methanol fractions from plant F respectively.

HDK-GE; HDK-GH; HDK-GM – Ethyl acetate, Hexane, and Methanol fractions from plant G respectively.

Bioactivity-guided fractionation was carried out according to the method described by Ene *et al* 2009, which involves testing the activity of each fraction at each stage of fractionation with the aim of further purifying each active fraction until a pure compound is obtained. As detailed below the fractionation utilizes various chromatographic techniques and purity is assessed using nmr. The yields of the pure compounds were between 10-15 mg with % purity of 80-95 % (See Table 2).

Table 2. Yield and Percentage purity of purified compounds.

Compounds	weight of purified compound (mg)	Weight of dry material (leaves) (kg)	Yield (mg/kg)	% purity
HDK20	15.21	20	0.7605	94.05
HDK40	13.75	20	0.6875	95.21
HDK52	14.22	20	0.711	94.04
HDK79	10.03	20	0.5015	80.91

2.3 Size exclusion chromatography

For non-polar fractions, Sephadex® LH-20 (GE Healthcare Life Sciences) was soaked in a solution of 5% *n*-hexane in dichloromethane or 50% dichloromethane (Sigma-Aldrich) in methanol for several hours. The slurry was then poured and packed in a glass chromatography column of appropriated size containing about 10-15 mL of solvent allowed to freely drip in order to prevent air bubbles. Samples were dissolved in a small volume of the solvent used for the mobile phase. The concentrated sample was loaded at the top of the column. Elution was with 5% *n*-hexane in dichloromethane or 50% dichloromethane in methanol. If needed, elution was continued with 100% dichloromethane or 100% methanol, respectively. For relatively polar fractions, Sephadex® was soaked in methanol and the column was then eluted with the same solvent. When column finished, Sephadex® was washed with water then methanol and kept dried for re-use.

2.4 Silica gel chromatography

Open Column Chromatography was performed on silica gel 60 (mesh size 0.063-0.200 mm (Sigma-Aldrich)). The column was packed using the wet packing technique. Silica gel 60 was made into slurry using the least polar solvent of the eluting system and then poured and packed in a glass chromatography column of appropriate size. Air bubbles were eliminated by tapping the side of the column, and by allowing the tap to run while packing; excess solvent was allowed to run through and the column was left to settle. Samples were dissolved in a suitable solvent and adsorbed on a small amount of silica gel 60 (mesh size 0.063-0.200 mm), which was loaded onto the column. Elution was carried out either isocratically or using a gradient. The collected fractions were analyzed by thin layer chromatography (see supplementary figure S1 for details) and pooled according to similar chemical profiles.

2.5 Compound Identification

2.5.1 NMR spectroscopy

^{1}H and ^{13}C- and 2D NMR experiments were carried out on a JEOL (JNM LA400) 400 MHz and on a Bruker 500 or 400 MHz instrument. NMR tubes (5 mm) purchased from Wilmad-labglass were used for routine NMR experiments. Samples were dissolved in about 0.6 mL of NMR grade deuterated chloroform (Sigma-Aldrich) and taken in NMR tubes. The identification of pure compounds was first carried out by one dimensional ^{1}H and ^{13}C-NMR spectroscopy. Spectra obtained for known compounds were identified following comparison with published spectral data. Further 2D NMR experiments were carried out when necessary to accurately assign proton and carbon chemical shifts and, in some cases, to determine relative stereochemistry. Note that the NMR spectra of the main compounds identified are given as Supplementary Data (Tables S1-S4).

2.5.2 Mass spectrometry

High and low resolution electron impact mass spectra were recorded on a JEOL 505HA spectrometer using direct probe at elevated temperature (110-160℃) at 70 eV. Positive ion and negative ion mode Electrospray Ionisation (ESI) experiments were performed on a ThermoFinnigan LCQ-Deca ion trap or Orbitrap HRESI mass spectrometer (mass analyser set up at 100,000 ppm, externally calibrated at 3 ppm). According to the polarity, samples were dissolved in acetonitrile (Fisher Scientific), methanol or water (HPLC grade, produced in-house using a Millipore Milli-Q system) or in a binary mixture of these solvents to get a concentration of 100 µg/mL. Sample solution (10-20 µL) was injected along with a direct infusion of 0.1 % formic acid in acetonitrile: water (90:10) at a flow rate of 200 µL/min.

2.6. *Trypanosoma* and *Leishmania* strains

To determine the anti-kinetoplastid potential of the selected medicinal plants, the following trypanosome strains were used: (1) Wild type strain *Trypanosoma brucei* Lister 427 (WT); (2) A diamidine-resistant strain, *Tb*AT1-KO, which was obtained by knock-out of the *Tb*AT1 aminopurine transporter (Matovu et al., 2003); (3) A multi-drug resistant strain, B48, which was derived from a *Tb*AT1-KO strain after increasing exposure to pentamidine and lacks both the *Tb*AT1 transporter and the high affinity pentamidine transporter (HAPT) (Bridges et al., 2007); (4) Strain *T. brucei* R0.8, which is resistant to cAMP phosphodiesterase inhibitors, after adaptation to the PDE inhibitor Cpd A (now renamed as NPD-001) (De Koning et al., 2012; Gould et al., 2013); (5) Wild type strain *Trypanosoma congolense* (6); Wild type *Leishmania mexicana* M379 IL3000 (Al-Salabi and De Koning, 2005). All *T. b. brucei* strains were used

only as bloodstream trypomastigotes, and cultured in standard HMI-9 medium (Life Technologies), supplemented with 10 % heat inactivated Fetal Bovine Serum (FBS; Life Technologies), 14 µL β-mercaptoethanol, and 3.0 g sodium hydrogen carbonate per litre of medium (pH7.4) in vented flasks at 37 ºC in a 5% CO_2 atmosphere (Gudin *et al*., 2006). Bloodstream forms of the *T. congolense* savannah-type strain IL3000 were cultured exactly as described by Coustou et al. (Cousteau *et al*., 2010) and were kindly provided by Theo Baltz (Université Victor Segalen Bordeaux 2, Bordeaux, France). Culturing of *L. mexicana* promastigotes was performed in HOMEM with 10% FBS at 25 ºC as described (Al-Salabi *et al*., 2003).

2.7 Drug susceptibility assays

2.7.1 Drug susceptibility assay in trypanosomes

The drug susceptibilities of bloodstream form trypanosomes were determined using the resazurin (Alamar blue) assay (Gould *et al*., 2008). This involves adjusting the cell density to the desired concentration of 2×10^5 cells/mL in HMi-9/FBS. 100 µl of this suspension was added to each wells of a 96-well culture plate, pre-prepared with a double dilution of the test compounds in 100 µL of the same medium. Typically, the first well contained 400 µg/mL of test compound and dilutions would be over 23 wells (i.e. 2 rows of the plate), with the last well of row 2 receiving 100 µL of medium as drug-free control. The cells were incubated with the test compounds for a period of 48 h at 37 ºC (for *T. brucei*) or 34 ºC (for *T. congolense*), followed by the addition of 20 µL of a 500 µM resazurin sodium salt (Sigma) solution in PBS (pH 7.4), and a further 24 h incubation. Two standard drugs, pentamidine and diminazene aceturate (both obtained from Sigma) were used as control at each independent replicate determination (n>3). Fluorescence was measured using a FLUOstar Optima (BMG Labtech, Durham, NC, USA) at wavelengths of 544 nm for excitation, 590 for emission. EC_{50} values were calculated by non-linear regression using an equation for a sigmoidal dose-response curve with variable slope (Prism 5.0, GraphPad Software Inc., San Diego, CA, USA).

2.7.2 Drug susceptibility assay of *L. mexicana*

The drug susceptibility assay for promastigotes of *L. mexicana* was performed the same way as for trypanosomes, with minor modifications. Cells were grown in HOMEM medium (pH 7.4) with 10% FBS, and the final cell density in each well was adjusted to 1×10^6 cells/mL. The

plates were incubated at 25 ºC + 5% CO_2 for 72 hours followed by the addition of 20 µl of the 500 µM resazurin sodium salt/PBS to every well, and a further incubation period of 48 hours prior to reading the fluorescence.

2.8 Cytotoxicity assay using Human Embryonic Kidney (HEK) Cells 293T

Toxicity of drugs to mammalian cells was tested using Human Embryonic Kidney (HEK) Cells. Briefly, HEK Cells were grown in a standard culture containing 500 mL Dulbecco's Modified Eagle's Medium (DMEM) (Sigma), 50 mL new born Calf Serum (NBCS) (Gibco), 5 mL Penicillin/Streptomycin (Gibco) and 5 mL L-Glutamax (200 mM, Gibco). The cells were incubated at 37 ºC + 5% CO_2 ratio in vented flasks and were passaged when they reached 80-85% confluence. For the assay, harvested cells were adjusted to a density of 3×10^5 cells/mL and 100 µL of this suspension was added to each well of a 96-well plate, each well containing 3×10^4 cells. The plate was incubated at 37 ºC + 5% CO_2 for 24 hours to allow the cells to adhere to the bottom of the plate. Drug stocks were prepared by doubling dilution in DMEM medium in a separate 96-well plate; phenyl arsine oxide (PAO (Sigma)) was used as positive control. At the end of the 24 hours incubation period, 100 µL/well of the plate that contains the drug dilution series was transferred to the opposite well on the plate that contained the HEK cells. The plate was then incubated at 37 ºC + 5% CO_2 for an additional 30 h followed by the addition of 10 µL of sodium resazurin solution and a further incubation at 37 ºC + 5% CO_2 for 24 hours prior to reading the fluorescence as above. EC50 values were determined from Sigmoid curves (Prism 5.0) and for each compound the Selectivity Index (SI) was calculated as EC_{50}(HEK)/ EC_{50}(test organism).

3. Results

3.1 Antitrypanocidal activity and cross resistance studies of extracts using wild type and multi-drug resistant strains of *T. brucei.*

A total of 21 dried extracts from seven plants were screened against protozoan parasites. Extracts were selected for further purification according to a slightly modified version of the protocol of Dua *et al*. 2011. The trypanocidal activities of the primary extracts were tested at a starting concentration of 400 µg/mL, as described by Dua *et al*. (2011). None of the methanol fractions showed measureable activity, probably because all the active ingredients had already

been extracted by the earlier hexane and ethyl acetate washes. Another notable observation was that none of the extracts showed reduced activity against the multi-drug resistant strain B48, and are therefore not cross-resistant with first-line trypanosomiasis drugs such as pentamidine, melarsoprol, diminazene and Cymelarsan. The one exception was the hexane extract of plant A, which was in fact significantly more active against B48 ($P<0.001$; Fig. 1).

The trypanocidal activities of the hexane (H) and ethyl acetate (E) extracts with EC_{50} values <100 μM are depicted in Figure 1, and a full list is given in Table 3. Of particular interest were those with an EC_{50} value below 15 μg/mL, being the hexane extract of plant D (HDK-DH, 2.4 ± 0.1 μg/mL), and the ethyl acetate extracts of plants E (HDK-EE. 4.2 ± 0.7 μg/mL) and F (HDK-FE, 12.3 ± 0.3 μg/mL); these were selected for further purification.

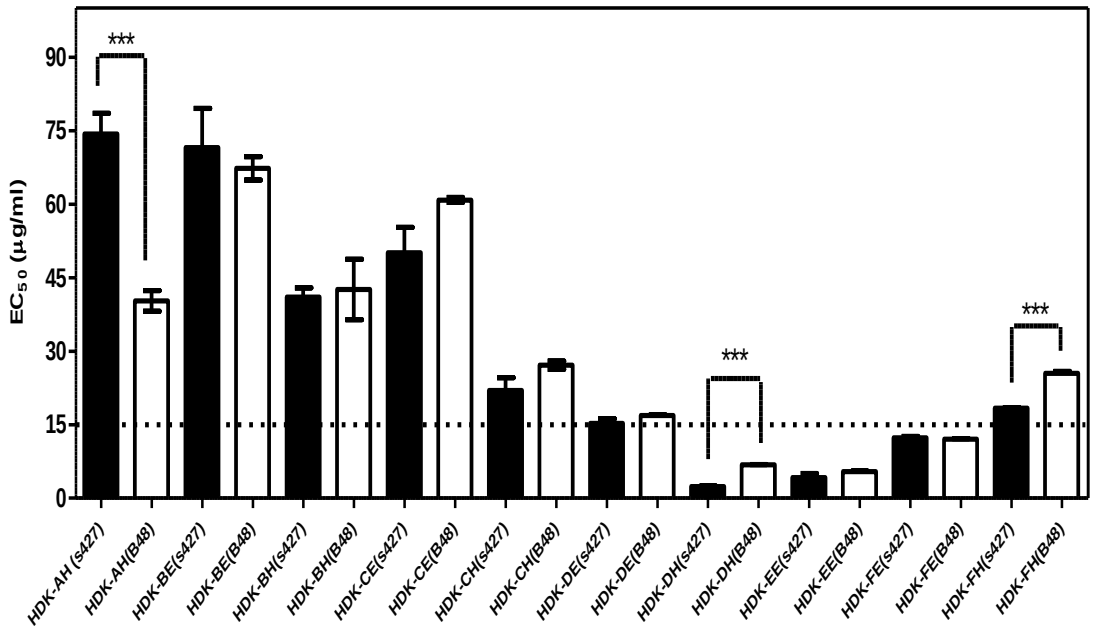


Figure 1. Drug sensitivity assays with medicinal plant extracts against T. brucei s427WT and B48 cell lines. The figure shows all the EC50 values from hexane (H), ethyl acetate (E) and methanol (M) extracts that could be obtained using the protocol described in the Methods section. When not shown the EC50 value was >400 μg/mL. The hexane fraction of Centrosema pubescens (HDK-AH) was more active against multi-drug resistant strain B48 than against wild type s427; no other significant differences ($p<0.05$) between the two strains were observed. Bars represent the average EC_{50} values and SEM of at least three independent

*determinations. ***, P<0.001* by Student's unpaired t-test. The dotted line identifies the fractions with EC_{50} values ≤15 µg/mL.

3.2 *In vitro* selectivity of extracts: therapeutic index relative to Human Embryonic Kidney cells

All 21 crude extracts from the seven medicinal plants were tested on Human Embryonic Kidney (HEK) 293-T cells in vitro, using a modified, and more sensitive, resazurin assay, involving a total incubation period of 78 hours. The result showed that at high doses extracts HDK-CH, HDK-DH, HDK-EE, and HDK-FH displayed a cytostatic, rather than cytotoxic, activity against HEK cells (EC_{50} <100 µM), whereas other extracts displayed less toxicity (Table 3). Indeed, no EC_{50} value could be obtained for 11 out of 21 extracts, using a top concentration of 400 µg/mL. The selectivity index (SI), defined as the in vitro EC_{50} value of the extract against the host cell divided by the EC_{50} value against the target cell, reached >20 but could not be determined where either EC_{50} value could not be obtained due to either low efficacy or low toxicity.

Table 3. EC_{50} value, Resistance Factor (RF) and Selectivity Index (SI) of crude ethyl acetate (E), Hexane (H), and methanol (M) extracts from seven Nigerian medicinal plants.

Fractions	***T.b.* s427 WT (µg/mL)**	**T.b. B48 (µg/mL)**	**RF**	**HEK (293T) cells (µg/mL)**	**SI**
HDK-AE	92.2 ± 2.1	ND	ND	>500	>5.4
HDK-AH	74.3 ± 4.2	40.3 ± 2.1	0.54[3]	386 ± 24	5.2
HDK-AM	233 ± 11	ND	ND	>500	>2
HDK-BE	71.5 ± 8.0	67.3 ± 2.4	0.94	492 ± 37	6.9
HDK-BH	41.0 ± 1.9	42.6 ± 6.2	1.04	>500	>12
HDK-BM	>250	ND	ND	>500	≥2
HDK-CE	50.1 ± 5.2	60.8 ± 0.5	1.22	198 ± 12	4.0
HDK-CH	22.0 ± 2.6	27.1 ± 0.8	1.23	80.0 ± 17	3.6
HDK-CM	222 ± 5	ND	ND	>500	>2
HDK-DE	15.2 ± 1.0	16.9 ± 0.2	1.11	114 ± 3	7.5
HDK-DH	2.4 ± 0.1	6.8 ± 0.1	2.9[3]	50.9 ± 2.2	21.6
HDK-DM	129 ± 18	ND	ND	>500	>3.9
HDK-EE	4.2 ± 0.7	5.4 ± 0.1	1.28	56.2 ± 1.9	13.3

HDK-EH	98.5 ± 1.1	ND	ND	>500	>5
HDK-EM	150 ± 10	ND	ND	>500	>3.3
HDK-FE	12.3 ± 0.3	12.0 ± 0.1	0.98	246 ± 17	20.1
HDK-FH	18.4 ± 0.1	25.4 ± 0.5	1.38[3]	86.9 ± 9.9	4.7
HDK-FM	141 ± 16	ND	ND	>500	>2.8
HDK-GE	>500	ND	ND	378 ± 17	<1
HDK-GH	>500	ND	ND	>500	ND
HDK-GM	>500	ND	ND	>500	ND
Pentamidine	3.4 ± 0.5[1]	1040 ± 180[1]	305	ND	ND
PAO[2]	ND	ND	ND	118 ± 6[1]	ND

Results were expressed as average EC_{50} of at least three independent determinations. Extracts HDK-CH, HDK-DH, HDK-EE, and HDK-FH were cytostatic rather than cytotoxic at high doses on HEK cells. Values are average of EC_{50} values of 4 independent determinations, expressed in µg/mL. [1]EC50 values for pentamidine and PAO are given in nM. [2]PAO, phenylarsine oxide. [3]P<0.001, Student's unpaired t-test t-test, comparing WT and B48. ND, not determined.

3.3 Anti-Leishmanial activity of the primary plant extracts

In order to determine the *in vitro* anti-leishmania activity of extracts from the selected plants, the extracts were tested on *Leishmanial mexicana* (M379) promastigotes (Fig. 2). The result revealed that hexane extracts from *Tridax procumbens* and *Polyalthia longifolia* (HDK-CH and HDK-DH) gave the best activity against *L. mexicana in vitro* (EC_{50} values of 16.8 and 17.2 µg/mL respectively), while hexane extracts from *Moringa oleifera* (HDK-BH), *Newbouldia laevis (HDK-EH), Eucalyptus maculata* (HDK-FH), and ethyl acetate fractions of *Tridax procumbens* (HDK-CH) and *Polyalthia longifolia* (HDK-DH) all displayed moderate anti-leishmanial activity *in vitro* (EC_{50} 50-70 µg/mL). Extracts not included in figure 2 were inactive against *L. mexicana* under our experimental condition. There is only moderate correlation between the antileishmanial and antitrypanosomal activities of these extracts, but, importantly, extract HDK-DH displayed the strongest activity against both of the parasites.

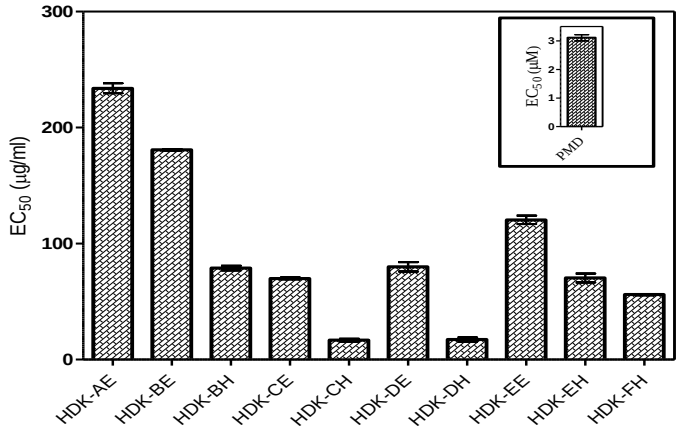


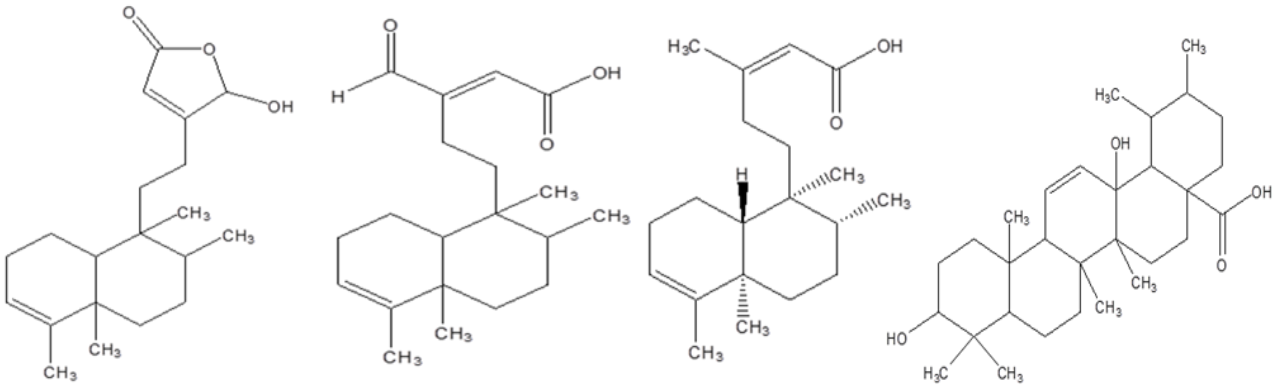
Figure 2. Effect of Nigerian plant extracts on L. mexicana promastigotes. The data shown are the average EC_{50} ± SEM of three separate experiments. Inset*: pentamidine (PMD), a standard drug, was used as internal control.*

3.4 Bioactivity-guided isolation of the active constituents from extracts

Between 150 - 200 fractions were collected from the column for each extract selected for fractionation, and the isolation of active compound was guided by the activity of each fraction. Progress of purification was monitored with NMR and mass spectrometric techniques.

After column chromatography the HDK-DH crude extracts yielded a fraction that was very active against trypanosomes. This was followed by further purification on a Sephadex column, which gave a pure and active compound, analyzed by ^{1}H and ^{13}C-NMR. Nuclear Overhauser Experiments, HMBC, HQSC, and COSY were then carried out to determine the exact structure and relative stereoisomer. Mass spectrometry was also performed to confirm the structure and its purity. The result was a clerodane-type diterpenoid with an *alpha,beta*-unsaturated lactone ring in the side chain identified as clerodane (16-α-hyroxy-cleroda-3-13(-14)-Z-dien-15,16-olide; HDK-20).

In addition, polyalthialdioc acid (HDK-79) and Kolavenic acid (HDK-52) were also isolated pure from other fractions of the crude hexane extract of plant D, *Polyalthia longifolia* (Figure 3).



HDK-20 HDK- 79 HDK-52 HDK-40

Figure 3. Anti-kinetoplastid compounds isolated from Polyalthia longifolia (HDK - 20, 79 and 52) and Eucalyptus maculata (HDK-40). HDK-20 is 16-α-hyroxy-cleroda-3-13(-14)z-dien-15,16-olide; HDK-79 is kolavenic acid; HDK-52 is polyalthiadioc acid; and HDK-40 is 3β,13β-dihydroxy-urs-11-en-28-oic acid.

Fractionation of extract HDK-FE yielded a series of compounds, including triterpenes, aromatics (xanthones), cinnamate/chalcone types, aliphatics, and triterpenoids. Some of these were found in combination in the fractions, or with other impurities. However, the active fraction on trypanosomes was, after chemical analyses, identified as an ursane type triterpenoid ($C_{30}H_{48}O_4$; HDK-40) with a molecular weight of 472.36 g/mol. Signals for seven skeletal methyl groups, of which two were doublets and five were singlets, were contained in the ^{1}H-NMR spectrum. The presence of 30 carbon atoms in its ^{13}C-NMR, coupled with the signal from the ^{1}H-NMR spectrum was suggestive of the fact that this compound was an ursane-type triterpenoid. Furthermore, analyses of the ^{1}H-NMR spectrum of this compound revealed two olefinic protons and a secondary hydroxyl group whose splitting pattern and chemical shift were characteristic of a 3β-equatorial hydroxy in the nucleus of a typical ursane-type triterpenoid.

In addition, two active compounds were identified from the crude extract of HDK-EE. The NMR and mass spectrometry analyses revealed them to be pheophytin A and B (HDK-28 and HDK-23 respectively) with molecular formula $C_{55}H_{74}O_5N_4$ and $C_{55}H_{72}N_4O_6$ and molecular masses of 870 and 885.18 g/mol respectively.

3.5 Trypanocidal activity of the purified active compounds

The isolated natural compounds were tested on wild-type s427 trypanosomes as well as a panel of s427-derived cell lines adapted to high levels of resistance of current and experimental

classes of trypanocides. B48 is highly resistant to virtually all diamidines and melaminophenyl arsenicals; AQP2/3-KO lacks the AQP2/AQP3 genetic locus and is moderately resistant to pentamidine and melarsoprol; TbAT1-KO is somewhat resistant to pentamidine and highly resistant to diminazene; R0.8 is highly resistant to inhibitors of cAMP phosphodiesterases TbrPDEB1 and B2 (Gould *et al*., 2013), an important class of new therapeutic leads against trypanosomiasis (Shakur et al., 2011; Gould et al., 2011). The results are summarized in Table 4.

Compound HDK-20 displayed the highest activity against s427-WT trypanosomes, with an EC_{50} value of 0.38 ± 0.05 µg/mL, corresponding to approximately 1 µM. HDK-23, HDK-40 and HDK-79 also displayed activity below 5 µg/mL, but the activity of HDK-52 was rather disappointing at 12.3 µg/mL. HDK20, HDK-79 and HDK-52 are closely related compounds from the same plant, *Polyalthia longifolia*, and most likely metabolites of each other. Clearly, the sidechain appears to have a large impact on the trypanocidal activity with the closed β-lactone highly effective; this gives a potential starting point for derivatization towards even higher anti-parasitic activity.

Testing of all purified compounds on our panel of drug resistant trypanosome lines identified only minor variations in sensitivity, which although statistically significant in some cases, were always well below even a 2-fold difference from EC_{50} value against the wild-type control (Table 4), in contrast to very high levels of resistance to the control drugs. As the resistance mechanisms for these drug classes are now well understood both in standard laboratory strains and in field isolates (Bridges et al., 2007; Delespaux and De Koning, 2007; Munday et al., 2015) we can conclude with confidence that the natural compounds reported here are not cross-resistant with key trypanocide classes such as the diamidines, melaminophenyl arsenicals and PDE inhibitors. Indeed, HDK-52 was significantly more active against all the resistant strains of *T. brucei* than the wild type.

3.6 Cytotoxicity of the purified compounds

The purified compounds were tested for their effects on human embryonic kidney (HEK) cells, using the same protocol as used for the crude extracts, so as to determine whether the antiprotozoal activity is the result of general toxicity or is more specifically antiprotozoal. The toxicity assays showed that the natural compounds that have an effect on *T. brucei* have almost no inhibitory activity on HEK cell growth and viability. Selectivity indices (SI, EC_{50} of HEK

cells / EC_{50} of parasites) for trypanosomes are not shown as none of the compounds tested were toxic to HEK cells at the highest concentration tested (200 µg/mL) (Fig. 4). However, this means that SI values range between >8 for HDK-28 and >526 for HDK-20. We conclude that the purification increased not only the activity of the plant extracts but also dramatically improved the selectivity of the product.

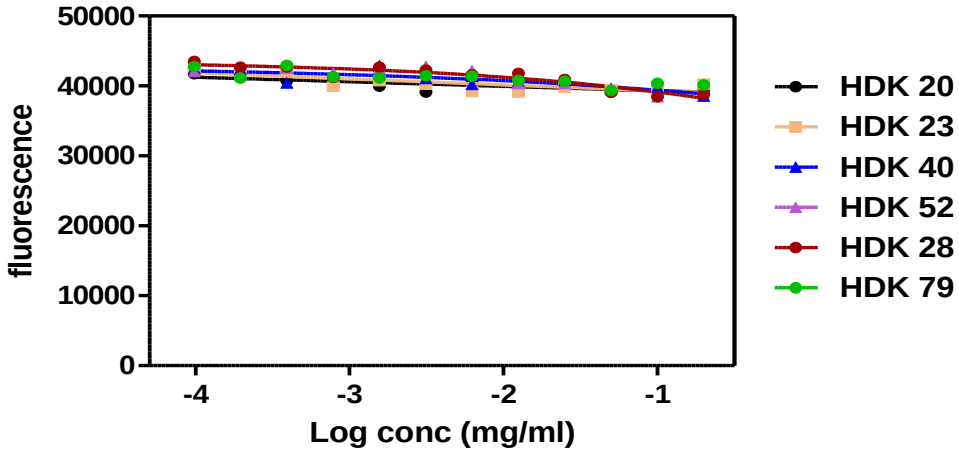


Fig. 4. Cytotoxicity assay of natural compounds on HEK 293-T cells, with 24 h cyto-adherence, 30 h incubation with drugs, and 24 h with sodium resazurin (alamar blue).None of the isolated compounds displayed sigmoidal curves, showing that they were not toxic to mammalian cells at concentrations >200 mg/mL. The experiment shown was representative of three independent repeats with identical outcomes.

3.7 Activity of Purified compounds against *Trypanosoma congolense* and *Leishmania mexicana*.

The purified compounds were next tested against *T. congolense* in order to determine their prospects of being developed and used as veterinary drugs to treat animal African trypanosomiasis (AAT or nagana), a condition that causes billions of dollars in economic losses in sub-Sharan Africa (Giordani et al., 2016). Most of the compounds were somewhat less potent against *T. congolense* than against *T. brucei* under the assay conditions, but displayed a similar order of potency: HDK-20 >> HDK-40 > HDK-52 = HDK-79. In addition, the compounds were tested against *L. mexicana* promastigotes. As for the other two protozoan species, HDK-20 was the most potent of the compounds tested, and the order against *L. mexicana* was HDK-20 >> HDK-52 > HDK-40 > HDK-79. Figure 5 displays the EC_{50} values against all three pathogens

side-by-side. The level of anti-parasite activity for HDK-20 makes this a promising lead compound against kinetoplastid pathogens.

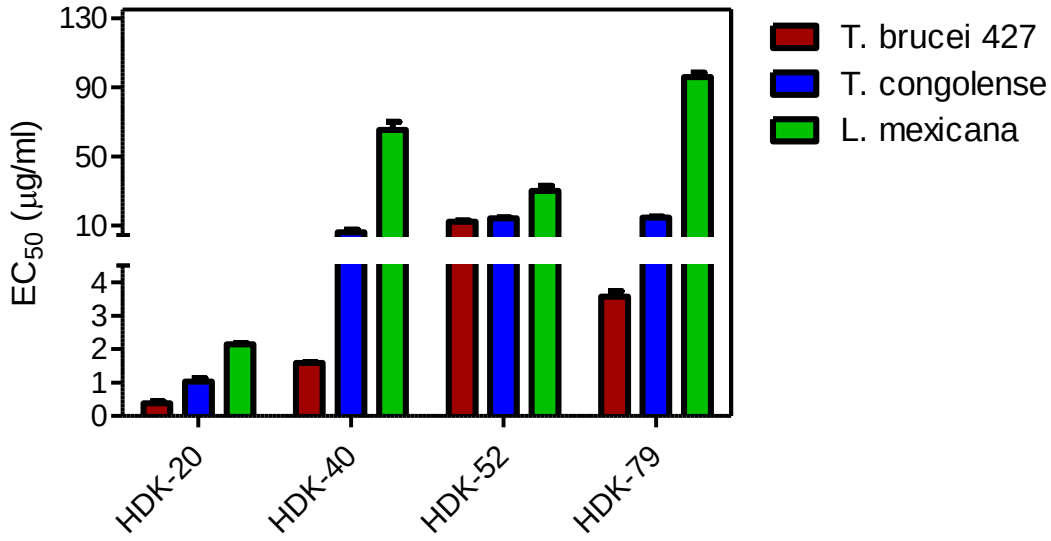


Figure 5. Effects of purified natural compounds against three pathogenic protozoa: bloodstream forms of T. brucei s427 and T. congolense strain IL3000, and promastigotes of L. mexicana strain M379. EC_{50} values shown are the average and SEM of 3 - 4 independent determinations.

4. Discussion

There is an increased interest in the antiprotozoal screening of plant extracts related to traditional therapy (Ndjakou Lenta *et al.*, 2007; Osorio *et al*., 2007), apparently due to their very low toxicity, the stability of natural compounds, and their reported reduced prospects for resistance or cross resistance to existing drugs (Nour *et al*., 2009). We report here on the antiprotozoal activity of the extracts of seven Nigerian medicinal plants.

Several of the crude ethyl acetate and hexane extracts displayed promising activity, whereas the methanol extracts were markedly less active. The low activity of the methanol extracts may simply be the result of the plants material already having been extracted with hexane and ethyl acetate, or genuinely indicate that the less polar solutes from these plants have the best anti-protozoal activity. The polar compounds extracted by methanol generally display less cellular penetration than hydrophobic ones (Orsi and Essex, 2010), and this may contribute to the lack of in vitro anti-parasite activity.

The extracts of *C. pubescens, M. oleifera, T. procumbens* and *J. tanjorensis* (plants A, B, C and G, respectively) did not display any *significant in vitro* anti-trypanosomal activity under our experimental design. However, these plants may contain compounds with other anti-protozoal or antibacterial activities, for which we did not test in the current study. Furthermore, the plants may in fact contain anti-trypanosomal compounds, but perhaps only in low amounts at the time of gathering the material. It must also be remembered that in the traditional usage the plant preparations are taken orally and the active principle may be activated in vivo.

It has previously been reported that Nigerian medicinal plants contain active anti-parasite constituents (Ene *et al*., 2014; Igoli *et al*., 2011). However, the plants that we report on here have not previously been investigated for their anti-kinetoplastid activities, nor have their active compounds been identified before now. The only exception is a report that methanol extracts of *P. longifolia* leaves (our plant D) inhibit the growth of *L. donovani* promastigotes, with an IC_{50} value of 4.18 µg/mL (Pal *et al.*, 2011). In our screens, too, *P. longifolia* displayed the most promising anti-leishmanial activity, with the crude hexane extract displaying a similar IC_{50}

against *L. mexicana* promastigotes. *P. longifolia* leaf extracts have also been reported to possess activity against pathogenic fungi such as *Candida albicans*, and against gram-positive bacteria, particularly S*taphylococcus aureus*, but not against gram-negative strains (Chanda and Nair, 2010).

The second-most promising medicinal plant in our study was *E. maculata* (plant F), with both the ethyl acetate and hexane extracts displaying anti-trypanosomal EC_{50} values below 20 µg/mL; this was apparently attributable the presence of the ursane type triterpene HDK-40, isolated from the ethyl acetate extract. Several previous studies reported anti-infectious activities of *Eucalyptus* species. Extracts of *E. maculata* leaves with methanol-dichloromethane were previously shown to have activity against gram-positive bacteria (Takahashi *et al*., 2004). Extracts from *E. sideroxylon* and *E. torquata* similarly displayed activity against gram-positive bacteria and some fungi (Ashour, 2008), and the ethyl acetate fraction from *E. camaldulensis* displayed a 100% growth inhibition against the protozoan *Trichomonas vaginalis* in vitro at a concentration of 12.5 mg/mL (Hassani et al., 2013).

Extracts of *N. laevis* (plant E) have been reported to exercise anti-diabetic (ethanolic extract of leaves; Kolawole and Akanji, 2013), bactericidal (methanolic leaf extract; Usman and Osuji, 2007), analgesic and anti-inflammatory (ethanolic flower extract; Usman *et al*., 2008) activities but we are not aware of any studies regarding their anti-protozoan activities. Here, we report the anti-kinetoplastid activity of *N. laevis* and attribute its activities to the presence of pheophytin, a compound that was previously isolated from *Loniera hypoglauca* in a screen for activity against hepatitis C virus, and was shown to inhibit its NC3 serine protease (Wang *et al*., 2009). We found both pheophytin A and B to exhibit a good level of anti-trypanosomal activity, but pheophytin B was approximately 15-fold more potent than pheophytin A.

Our initial NMR data revealed the presence of some common classes of active compounds in these plants under study, including terpeniods, aromatics, and pheophytins (Salem and Werbovetz, 2006; Wink, 2012). We attribute the remarkable anti-kinetoplastid activity of *Polyalthia longifolia* extracts and their isolated compounds observed in this study to the presence of clerodane diterpenes, of which the closed lactone form, HDK-20, was more active against the kinetoplastids than the aldehyde or carboxylic acid moieties on the scaffold. HDK-20 and HDK-79 have previously been described in extracts of *P. longifolia*, with antifeedant

(Phadnis *et al*., 1988) and anti-inflammatory properties (inhibition of NO production by macrophages; Wu *et al*., 2014).

Wink (2008) reported that lipophilic secondary metabolites like the terpenoids have detergent-like properties that can disrupt biological membranes, alter their fluidity and inhibit the function of membrane proteins, including those involved in cellular signaling and transport. Although we observed no major differences in activity against our cell lines with altered drug transport (B48, AQP2/3-KO, AT1-KO) or cAMP signaling (R0.8), HDK-52 consistently displayed a significantly higher activity against the transporter mutants than against the wild-type, a situation that was reversed for HDK-79, and both were somewhat less effective against the cAMP-desensitized clone R0.8 as well. However, we do not propose that chemical disruption of the lipid bilayer can be the main mode of action of these compounds, as this would fail to explain the considerable selectivity *vis-à-vis* the *Leishmania mexicana* or human cells.

The active principles isolated in this work could be potentially developed to treat either human or animal African trypanosomiasis (HAT and AAT, respectively). Whereas HAT, commonly known as sleeping sickness, is a devastating, fatal disease, its disease burden is steadily declining (Simarro *et al*., 2015) while AAT continues to cause huge economic losses, and places serious limitations on agriculture in sub-Saharan Africa – not just for cattle rearing (beef, dairy) but also for draught power for ploughs, carts, etc. The main AAT pathogens are *Trypanosoma congolense* and *Trypanosoma vivax* (Giordani et al., 2016), and we therefore also tested some of the active principles on *in vitro* cultures of *T. congolense*. Interestingly, the same order of potency for these compounds applied for both *T. brucei* and *T. congolense*, with HDK-20 again being by far the most active agent. Together with the similarly potent activities against multi-drug resistant trypanosome lines, this appears to indicate that the clerodane diterpenes may have general utility against African trypanosomes. This is important, as African livestock may be infected by any of several *Trypanosoma* species and any drug must be able to act on all of them, as identification of (sub)-species for individual animals is currently impracticable. Although the bio-assay fractionation was guided by activity against *T. brucei*, the extracts and their isolated compounds were also tested on *L. mexicana*, as the plants are considered to have broad activity against protozoan infections. The anti-*Leishmania* efficacy of some of these plants (hexane extracts of *T. procumbans* and *P. longifolia*) and their purified compounds (HDK-20), affirms

their continuous use in folkloric medicine, and we propose that at least some of the plants are good prospects for new drugs against kinetoplastid diseases.

5. Conclusion

Our results have shown that the medicinal plants used in this research are reservoirs of active principles against trypanosomes; these natural compounds are nontoxic to mammalian cells *in vitro* and with no prospects for cross-resistance with existing drugs like pentamidine, melarsoprol, cymelarsan and diminazene aceturate. The active principles identified could serve as lead compounds towards the identification of more efficient anti-trypanosome drugs. We conclude that the Nigerian flora is indeed an appropriate starting point in the search for new and more efficient anti-kinetoplastid molecules, and that our results validate the traditional usage of at least some of these plants for the treatment of tropical fevers.

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