Mining Sudanese Medicinal Plants for Antiprotozoal Agents

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Neglected tropical diseases are major health hazards in developing countries. Annually, up to 30 million people are affected by either Chagas disease, African trypanosomiasis or leishmaniasis, and more than 200 million by malaria. Most of the currently available drugs have drawbacks in terms of toxicity, limited oral availability, development of resistance, or non-affordability. Tropical plants of the arid zones are a treasure chest for the discovery of bioactive secondary metabolites. This study aims to compile Sudanese medicinal plants, validate their antiprotozoal activities, and identify active molecules. We have performed a survey of medicinal plants of Sudan and selected 62 that are being used in Sudanese traditional medicine. From these, we collected materials such as leaves, stem, bark, or fruit. The plant materials were extracted in 70% ethanol and further fractionated by liquid-liquid partitioning using solvents of increasing polarity. This resulted in a library of 235 fractions. The library was tested in vitro against Plasmodium falciparum (erythrocytic stages), Trypanosoma brucei rhodesiense (bloodstream forms), Trypanosoma cruzi (intracellular amastigotes), and Leishmania donovani (axenic amastigotes). Active fractions were also tested for cytotoxicity. Of the 235 fractions, 125 showed growth inhibitory activity >80% at 10 μg/ml and further fractionated by liquid-liquid partitioning using solvents of increasing polarity. This resulted in a library of 235 fractions. The library was tested in vitro against Plasmodium falciparum (erythrocytic stages), Trypanosoma brucei rhodesiense (bloodstream forms), Trypanosoma cruzi (intracellular amastigotes), and Leishmania donovani (axenic amastigotes). Active fractions were also tested for cytotoxicity. Of the 235 fractions, 125 showed growth inhibitory activity >80% at 10 μg/ml and >50% at 2 μg/ml against at least one of the protozoan parasites. Plasmodium falciparum was the most sensitive of the parasites, followed by T. b. rhodesiense and L. donovani. Only few hits were identified for T. cruzi, and these were not selective. Contrary to expectation based on phylogeny, but in agreement with previous results, a large number of extracts displayed mutual activity against T. brucei and P. falciparum. HPLC-based activity profiling for selected active extracts was performed to identify the bioactive principles. Active compounds identified by dereplication were guieranone A from Guiera senegalensis J.F.Gmel.; pseudosemiglabrin from Tephrosia apollinea (Delile) DC; ellagic acid and quercetin from Terminalia leiocarpa (DC.) Baill.; and catechin, ethyl gallate, and epicatechin gallate from Vachellia nilotica (L.) P.J.H.Hurter & Mabb. Also the extracts of Croton gratissimus var. gratissimus and Cuscuta hyalina Roth ex Schult. exhibited promising antitrypanosomatid activity. This assessment provides a comprehensive overview of Sudanese medicinal plants and supports the notion that they are a potential source of bioactive molecules against protozoan parasites.

Keywords: HPLC activity profiling, drug discovery, Sudan, medicinal plant, Trypanosoma, Leishmania, Plasmodium
INTRODUCTION

Infections by protozoan parasites remain to be among the most devastating causes of mortality in the tropics. The trypanosomatids are a large family of flagellated protozoa, some of which cause neglected tropical diseases of high public health relevance and socio-economic impact (WHO, n.d.; Filardy et al., 2018). These are *Trypanosoma cruzi* (Chagas’ disease), *T. brucei gambiense*, and *T. b. rhodesiense* (human African trypanosomiasis or sleeping sickness), and *Leishmania* spp. (different kinds of leishmaniasis) (Stuart et al., 2008). The apicomplexan parasite *Plasmodium falciparum* is the causative agent of malaria tropica, the most dangerous form of malaria, which—despite the successes by various international bodies and philanthropic organizations—still claims an annual death toll of 435,000 (World Health Organisation, 2018). These diseases disproportionately affect the poor and vulnerable populations (WHO Expert Committee on Malaria: Twentieth Report, n.d.), calling for action to improve global well-being. A key element of the fight against protozoan neglected tropical diseases and malaria is the discovery of novel chemotherapeutic agents.

While the incidence of human African trypanosomiasis is at a historic low and a new drug, fexinidazole (Mesu et al., 2018), has recently received positive opinion by the European Medicines Agency, the prospects are slightly gloomy for other protozoal infections by protozoan parasites remain to be among the most devastating causes of mortality in the tropics. The trypanosomatids are a large family of flagellated protozoa, some of which cause neglected tropical diseases of high public health relevance and socio-economic impact (WHO, n.d.; Filardy et al., 2018). These are *Trypanosoma cruzi* (Chagas’ disease), *T. brucei gambiense*, and *T. b. rhodesiense* (human African trypanosomiasis or sleeping sickness), and *Leishmania* spp. (different kinds of leishmaniasis) (Stuart et al., 2008). The apicomplexan parasite *Plasmodium falciparum* is the causative agent of malaria tropica, the most dangerous form of malaria, which—despite the successes by various international bodies and philanthropic organizations—still claims an annual death toll of 435,000 (World Health Organisation, 2018). These diseases disproportionately affect the poor and vulnerable populations (WHO Expert Committee on Malaria: Twentieth Report, n.d.), calling for action to improve global well-being. A key element of the fight against protozoan neglected tropical diseases and malaria is the discovery of novel chemotherapeutic agents.

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Plants are still considered as important sources for the discovery of novel bioactive molecules. Plants secondary metabolism represents a huge and unique reservoir of chemical diversity, which may serve as a source of new drugs, either directly or after optimization by medicinal chemistry. Independent chemoinformatic analyses have consistently shown that natural products often exhibit unique features, a high degree of structural diversity, and drug- or lead-like structural properties (Feher and Schmidt, 2003; Schmidt et al., 2012; Pascolutti et al., 2015).

A retrospective analysis showed that approximately 50% of drugs approved within the last 30 years are derived, directly or indirectly, from natural products, whereby plant derived compounds played an important role (Newman and Cragg, 2016).

Sudan’s biodiversity coupled with a deeply rooted ethnobotanical heritage is an untapped reservoir for the discovery of new bioactive natural products. Here we performed a survey of plants from Sudan that are used in traditional medicine, with a focus on malaria and neglected tropical diseases caused by protozoa. On the basis of this survey a library of plant extracts was assembled and screened against trypanosomatid parasites and *P. falciparum*. Active compounds in the most promising extracts were tracked with the aid of an activity-driven approach.

MATERIALS AND METHODS

Preparation of a Library of Plant Extracts

A total of 62 plants reputed as antiparasitic in traditional medicine in Sudan were solicited from the repository of the Faculty of Pharmacy, University of Science & Technology. The plants belonged to 35 different families, of which the Combretaceae, Leguminosae, Verbenaceae, Lamiaceae, and Compositae were the most frequent. Where available, different parts of a given plant species were included in the study.

The taxonomic identity was confirmed by the Medicinal and Aromatic Plants Research Institute, Sudan. Voucher specimens (USTH 01-USTH 62) have been deposited at the Herbarium of the faculty of Pharmacy, University of Science and Technology, Omdurman, Sudan.

Dried plant material was milled to coarse powder in a hammer mill. 100-500 g of powdered material was extracted for 24 h with 500 ml of 70% ethanol in a magnetic rod stirrer. Extracts were filtered through Whatman no. 1 filter paper and concentrated by solvent removal in a rotary vacuum evaporator. Crude extracts were suspended in water and partitioned consecutively with petroleum ether, chloroform, ethyl acetate, and n-butanol. Crude extracts and their respective fractions were allowed to dry at room temperature, weighed, and reconstituted in DMSO (10 mg/ml) to serve as stock solutions for antiparasitic testing. This resulted in a library of 235 samples.

HPLC Analyses and Microfractionation

HPLC analyses were performed on a Shimadzu HPLC system equipped with photo diode array detector (PDA) (SPD-M20A, Shimadzu), evaporative light scattering detector (ELSD) (3300, Alltech), and an electrospray ionization mass spectrometer (ESIMS) (LCMS-8030, Shimadzu). LabSolutions software was used for data acquisition and processing. The separation was performed on a C18 SunFire column (3.0 × 150 mm; 3.5 μm; Waters).

Microfractionation of the active samples was carried out by analytical RP-HPLC on an LC-MS 8030 system (Shimadzu) connected with an FC204 fraction collector (Gilson). For each fraction, a solution of 10 mg/ml was prepared in DMSO. A total of three injections were performed: 2 × 35 μl with only UV detection (254 nm) for collection (0.7 mg of fraction in total) and 1 × 35 μl with UV-ELSD-ESIMS detection without collection.

The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The gradient was 5% to 100% B in 30 min, followed by washing with 100% B for 10 min. The flow rate was 0.4 ml/min. Fractions of 1 min each were collected from minute 1 to minute 40, resulting in 40 microfractions in total. Microfractions of two successive injections of a given sample were collected into the corresponding wells of a 96-deepwell plate. Plates were then
dried in a Genevac EZ-2 evaporator (Potterat and Hamburger, 2013; Potterat and Hamburger, 2014).

**Activity Testing Against Trypanosoma brucei rhodesiense**

*In vitro* activity was tested against bloodstream-form *T. b. rhodesiense* STIB 900, which had been obtained in 1982 from a Tanzanian patient and adapted to axenic culture (Baltz et al., 1985). The culture medium was MEM supplemented with 25 mM HEPES, 1 g/L additional glucose, 1% MEM nonessential amino acids, 0.2 mM 2-mercaptoethanol, 1 mM Na-pyruvate, and 15% heat inactivated horse serum. In the two-concentration assay, 50 µl medium containing the corresponding samples concentration (10 µg/ml or 2 µg/ml) was added to the wells of a 96 well plate. For the IC_{50} determination, a 50-µl medium was added to each well, and a serial sample dilution of 11 threefold dilution steps covering a range from 100 to 0.002 µg/ml were prepared. Then 10^{4} *T. b. rhodesiense* in 50 µl medium was added to the wells, and the plate was incubated for 72 h at 37°C in a humidified atmosphere of 5% CO_{2}. A 10-µl resazurin solution (12.5 mg resazurin dissolved in 100 ml distilled water) was added to each well and incubated for a further 2 to 4 h (Ráz et al., 1997). Plate reading was performed in a Spectramax Gemini XS microplate fluorometer (Molecular Devices Corporation) using an excitation wavelength of 536 nm and emission wavelength of 588 nm. Melarsoprol was used as reference drug. Final in-test DMSO concentration did not exceed 1%. All assays were performed in two independent replicates at least.

**Activity Testing Against Leishmania donovani**

*L. donovani* amastigotes strain MHOM/ET/67/L82 were grown in axenic culture in SM medium at pH 5.4 with 10% heat-inactivated fetal bovine serum, at 37°C in a humidified atmosphere of 5% CO_{2}. A 10-µl resazurin solution (12.5 mg resazurin dissolved in 100 ml distilled water) was added to each well and incubated for another 2 h (Mikus and Steverding, 2000). Plate reading was performed as described for *T. brucei*. Miltefosine was used as reference drug. Final in-test DMSO concentration did not exceed 1%. All assays were performed in two independent replicates at least.

**Activity Testing Against Trypanosoma cruzi**

All tests were performed with the *T. cruzi* Tulahuen strain C2C4, which expresses the β-galactosidase (*LacZ*) gene (Buckner et al., 1996). L6 rat skeletal myoblasts served as host cells. Cultures were maintained in RPMI 1640 medium supplemented with 10% FBS and 1.7 µM L-glutamine at 37°C in a humidified atmosphere of 5% CO_{2}. Host cells were seeded in 96-well microtitre plates, 2 × 10^{4} per well in 100-µl medium. After 24 h, 50 µl of a suspension of 1 × 10^{5}/ml trypomastigote *T. cruzi* were added. The medium was replaced at day 4, test samples were added, and the plates incubated for further 4 d. Finally, 50 µl of 2.5× CPRG/Nonidet solution was added to all wells. A color reaction was visible within 2-6 h, which was quantified in an absorbance reader at 540 nm (Spectramax). Benznidazole was used as reference drug. Final in-test DMSO concentration did not exceed 1%. All assays were performed in two independent replicates at least.

**Activity Testing Against Plasmodium falciparum**

*In vitro* antimalarial activity was tested against the erythrocytic stages of *P. falciparum* NF54, originally isolated from a patient at Schiphol airport. The parasites were grown in human erythrocytes in RPMI 1640 supplemented with 0.5% ALBUMAX II, 25 mM Heps, 25 mM NaHCO_{3} (pH 7.3), 0.36 mM hypoxanthine, and 100 U/ml neomycin and kept in an atmosphere of 3% O_{2}, 4% CO_{2}, and 93% N_{2} in humidified modular chambers at 37°C. In the two-concentration assay, 100 µl medium containing the corresponding samples concentration (final sample concentration of 10 or 2 µg/ml) was added to the wells of a 96-well plate. For the IC_{50} determination, a 50-µl medium was added to each well and a serial sample dilution of 11 threefold dilution steps covering a final range from 100 to 0.002 µg/ml were prepared. Then 100-µl parasite (erythrocytes at 1.25% final hematocrit and 0.3% final parasitemia) was added. After 48 h of incubation with test compounds, 0.25 µCi of [^{3}H]hypoxanthine was added per well, and the plates were incubated for an additional 24 h. Cells were harvested onto glass-fiber filters, and radioactivity was counted using a Betaplate liquid scintillation counter. Artemisinin was used as reference drug. Final in-test DMSO concentration did not exceed 1%. All assays were performed in two independent replicates at least.

**Clustering According to Antiprotozoal Activity**

Two-way clustering was performed on the bioactivity data measured at 2 µg/ml (*Supplementary Table S1*). Percent inhibition was converted to decimals, and the maximum was set to 1. For sake of clarity, we included only one fraction per plant, i.e. the one which had exhibited the highest activity against any of the four protozoan parasites. Hierarchical clustering was performed with the Eisen lab programs *Cluster* and *Treeview* (Eisen et al., 1998) using Euclidean distance and average linkage.

**Cytotoxicity Testing**

L6 rat skeletal myoblast cells were seeded in 96-well microtiter plates at 2 × 10^{4} cells/ml in RPMI 1640 medium supplemented with 10% FBS and 1.7 µM L-glutamine. The cells were allowed to
attach overnight, then test compounds were added. After 72 h of incubation, 10 µl of resazurin solution (see above) was added, and the plates were incubated for an additional 2 h. Plates were read in a fluorescence scanner at 536 nm excitation and 588 nm emission wavelength. Podophyllotoxin was used as reference. All assays were performed in two independent replicates at least.

### RESULTS

**Review of Medicinal Plants From Sudan**

Ethnopharmacological literature review based on scholarly databases (PubMed, Medline, SciFinder) and other supporting documents revealed that 34 of the 62 plants had been recorded...
for use against leishmaniasis, trypanosomiasis or malaria, including the symptoms related to any of these diseases (Table 1). Several of the plants had also been investigated pharmacologically and had exhibited anti-infective activity (Table 2).

### Table 2: Plants investigated in the present study for which anti-infective properties have been examined experimentally.

| Plant species                  | Part               | Tested activities | IC₅₀ value | Active metabolite(s)                  | Ref                        |
|--------------------------------|--------------------|-------------------|------------|---------------------------------------|----------------------------|
| Anethum graveolens L.          | Leaves             | Antiplasmodial    | –          | Volatile oils                         | (Chiba et al., 2012)       |
| Annona mucronata L.            | Leaves             | Antileishmanial   | 25 µg/ml   | Acetogenins                           | (Osorio et al., 2007)      |
| Argemone mexicana L.           | Leaves             | Antiplasmodial    | 1.7 µg/ml  | Proteopine, allocryptopine, and berberine | (Simoes-Pires et al., 2014) |
| Aristolochia bracteolata Lam.  | Root               | Antiplasmodial    | < 5 µg/ml  | –                                     | (El-Tahir et al., 1999b)   |
| Azadirachta indica A.Juss.     | Leaves             | Antiplasmodial    | 2.5 µg/ml  | Gedunin                               | (Khalid et al., 1988b; MacKinnon et al., 1997) |
| Cordospermum halacabum L.      | Leaves             | Antiplasmodial    | 42 µg/ml   | –                                     | (Kaushik et al., 2015)     |
| Combretum glutinosum Perr. ex DC. | Leaves         | Trypanocidal      | 26.5 µg/ml | –                                     | (Traore et al., 2014)      |
| Combretum hartmannianum Schweinf. | Bark              | Antiplasmodial    | 0.2 µg/ml  | –                                     | (Ali et al., 2002)         |
| Commiphora myrrha (Nees) Engl. | Gum resin          | Trypanocidal      | 8.1 µg/ml  | –                                     | (Okba et al., 2018)        |
| Croton Gratissimus var. Gratissimus (syn. Croton zambesicus Müll.Arg.) | Root               | Antiplasmodial    | –          | Sesquiterpenes, monoterpenes, and alkaloids | (Okokon and Nwafor, 2009) |
| Curcuma longa L.               | Rhizome            | Antiplasmodial    | 3–40.2 µg/ml | Demethoxycurcumin, and bis-demethoxycurcumin. | (Rasmussen et al., 2003) |
| Cymbopogon citratus (DC.) Stapf | Leaves             | Antiplasmodial    | –          | Essential oils                        | (Tchouboungbang et al., 2005) |
| Cyperus rotundus L.            | Whole plant        | Antiplasmodial    | –          | Terpenes, monoterpenes, and sesquiterpenes. | (Peerzada et al., 2015)    |
| Guiera senegalensis J.F.Gmel.  | Leaves and roots   | Antiplasmodial    | 4.08 µM    | Guiranone A                           | (Silver and Gomes, 2003)   |
| Haplodphylly tuberculatum (Forssk.) A.Juss. | Leaves (1) | Antileishmanial | (1) 16.59 µg/ml and (2) 0.2 µg/ml | (1) R-(+)-limonene and (2) Justicidin B | (Gertsch et al., 2003; Hemmati and Seraç, 2016; Harandi et al., 2018) |
| Jatropha curcas L.             | Seeds              | Trypanocidal      | 1.9 µg/ml (T. brucei) and 7.4 µg/ml (T. cruzi) | Phorbol esters            | (Khalid, 2012)               |
| Mangifera indica L.            | Stem bark          | Antiplasmodial    | >50 µg/ml  | –                                     | (Zirni et al., 2005)       |
| Moringa oleifera Lam.          | Leaves             | Antileishmanial   | 5.25 µM    | Niazinin                              | (Kaur et al., 2014)        |
| Nauclea latifolia Sm.          | Stem and root      | Antiplasmodial    | 0.9–3 µg/ml | Alkaloids tetrahydrodesoxycurcifoline and 19-O-methylangustoline | (Biennot-Vical et al., 1998; Boucherle et al., 2016) |
| Piper cubeba L. f.             | Fruits             | Antitrypanosomal against T. cruzi amastigotes | 87.9 µg/ml | Essential oil                          | (Esperandim et al., 2013)  |
| Senna Occidentalis (L.) Link (syn. Cassia occidentalis L.) | Leaves             | Antiplasmodial    | <3 µg/ml   | Anthraquinones, terpenes, and flavonoids. | (Tona et al., 2004)        |
| Striga hermonthica (Delle) Bentham. | Whole plant       | Antiplasmodial    | 274.8 µg/ml | –                                     | (Okpako and Ajayi, 2004)   |
| Terminalia leiocarpa (DC.) Ball. (syn. Anogeissus knoepf (DC.) Guill. & Perr.) | Bark               | Antiplasmodial    | 19 µg/ml   | Ellagic acid, gallic acid, and gentisic acid | (Ndjonka et al., 2012)     |
| Tinospora baxis (A.Rich) Miers | Roots              | Antiplasmodial    | 28.6 µg/ml | Alkaloids and tannins.                | (Ouattara et al., 2006)    |
| (syn. Acacia nilotica (L.) Delle) | Seed               | Antiplasmodial    | 1.5 µg/ml  | –                                     | (El-Tahir et al., 1999b)   |
| Xanthium strumarium subsp. brasiliicum (Vell.) O.Bolos & Vigo (syn. Xanthium brasiliicum Vell.) | Aerial parts        | Antiplasmodial, Antitrypanosomal | 0.09 µg/ml (T. brucei), 2.96 µg/ml (T. cruzi), 0.16 µg/ml (L. donovani), and 1.71 µg/ml (P. falciparum) | 8-Epoxanthatin 1beta,Sbeta-epoxide | (Nour et al., 2009) |
| Zeopbus spin-a-christi (L.) Desf. | Leaves             | Antileishmanial   | >30 µg/ml  | –                                     | (Ali et al., 2002)         |

### Testing for Antiparasitic Activity

The original extracts and all fractions obtained by partitioning were tested at two concentrations, 2 and 10 µg/ml, against the following panel of protozoan parasites: *T. b. rhodesiense* bloodstream form, *T. cruzi* intracellular amastigote form grown...
in rat L6 cells, *L. donovani* axenic amastigote form grown at low pH, and *P. falciparum* erythrocytic stage grown in human erythrocytes. Percent inhibition was calculated in comparison to untreated controls. All tests were carried out in independent duplicates. The results are compiled in Supplementary Table S1.

Extracts that exhibited >80% growth inhibition at 10 µg/ml, or >50% growth inhibition at 2 µg/ml against at least one of the tested parasites was considered active. Of the 235 extracts in our library, 125 (53%) fulfilled these activity criteria. A total of 34 (27%) of the active extracts exhibited activity against *T. b. rhodesiense*, *L. donovani*, and *P. falciparum* collectively. Regarding parasite species-selective inhibition, *P. falciparum* appeared to be the most susceptible parasite, followed by *T. b. rhodesiense* and *L. donovani*. Among the tested parasites *T. cruzi* was the least susceptible towards the plant extracts (Figure 1).

### Two-Way Clustering of the Bioactivity Data

We used the screening results obtained with 2 µg/ml for two-way clustering, i.e. clustering the plants according to their bioactivity, and clustering the parasites according to their susceptibility (Figure 2). Per plant only one fraction from the partitioning was included, i.e. the one which had displayed the highest activity against any of the four parasites. This approach clearly confirmed the notion that *T. b. rhodesiense* and *P. falciparum*, despite their large phylogenetic distance, have a similar susceptibility profile. It also highlighted *T. cruzi* as the least susceptible of the four tested parasites (Figure 2). There was no clear separation between the medicinal plants with reported anti-infective use (printed in red in Figure 2) and the rest. Regarding antiplasmodial activity, the plants that had a reported use against malaria (n=17; Table 1) were slightly more active against *P. falciparum in vitro*, both at 2 µg/ml (mean inhibition of 43% vs. 39%) and at 10 µg/ml (mean inhibition of 89% vs. 75%). However, these differences were not statistically significant (p=0.70, two-tailed Mann-Whitney test).

### Testing for Cytotoxicity

Extracts with antiparasitic activity were also tested for cytotoxicity. This was done against rat L6 skeletal myoblast cells, the same cell line that had been used as host cells for...
testing against amastigote *T. cruzi*. Concentration-response curves allowed the calculation of both 50% and 90% inhibitory concentrations (IC50 and IC90) (Table 3). The cytotoxicity data of the tested fractions cannot directly be compared to their antiparasitic activity because the antiparasitic and cytotoxic activity of a given fraction can be due to different molecules. Nevertheless, the aim was to identify non-toxic fractions for the following HPLC-based activity profiling and identification of active compounds.

### Extracts With Selective Anti-Trypanosomatid Activity

The most potent and selective activity against *T. b. rhodesiense* was exhibited by the chloroform fraction of the leaves of *Terminalia catappa* L. (Combretaceae), which showed 98% inhibition at 10 µg/ml and 80% inhibition at 2 µg/ml. Five of the ethyl acetate fractions showed growth inhibition > 85% at 10 µg/ml: fruits of *Croton gratissimus var. gratissimus* (syn. *Croton zambesicus* Mull. Arg.), fruits of *Commiphora myrrha* (Nees) Engl., *Cuscuta hyalinum* Roth ex Schult., *Cymbopogon citratus* DC. (Poaceae) exhibited selective activity above 80% inhibition at 10 µg/ml. However, the ethyl acetate fraction, in particular, exhibited cytotoxicity on L6 cells with an IC50 of 53.8 µg/ml (Table 3).

Only five percent of the library extracts were preferentially active against *L. donovani*. These were mostly lipophilic, e.g., the chloroform fraction of *Ambrosia artemisiifolia* L. (syn. *Ambrosia maritima* L.) (Asteraceae) leaves and the petroleum ether fractions of *Piper cubeba* L. f. (Piperaceae) fruits, *Portulaca oleracea* L. (Portulacaceae) aerial parts, and *Typha angustifolia* L. (Typhaceae) stem. *Trypanosoma cruzi* was the least sensitive among the tested parasites. Only the crude extract of *Annona muricata* L. (Annonaceae) leaves and the methanolic fraction of *Commiphora myrrha* (Nees) Engl. (Burseraceae) oil and resin inhibited the growth of intracellular *T. cruzi* more than 50% at 2 µg/ml. However, these activities were not specific for *T. cruzi* (Figure 1, Supplementary Table S1).

### Extracts of Selective Antiplasmodial Activity

Thirteen fractions showed >80% growth inhibition of *P. falciparum* at 10 µg/ml, but none showed >50% growth inhibition at 2 µg/ml. Among the most active ones were the chloroform fraction of *Cuscuta hyalina* Roth ex Schult. (Convulvulaceae) stem and the ethyl acetate fractions of the leaves of *Abutilon pannosum* var. *figarianum* (Webb) Verdc. (syn. *Abutilon figarianum* Webb) (Malvaceae), *Annona muricata*, *Tephrosia apollinea* (Delile) DC (Leguminosae), and *Cardiospermum halicacabum* L. Moreover, both the chloroform and the ethyl acetate fractions of the leaves of *Cymbopogon citratus* (DC.) Stapf (Poaceae) exhibited selective antiplasmodial activity above 80% inhibition at 10 µg/ml. However, the ethyl acetate fraction, in particular, exhibited cytotoxicity on L6 cells with an IC50 of 53.8 µg/ml (Table 3).

### HPLC-Based Activity Profiling

The ethyl acetate fraction of *Ziziphus spinosa-christi* (L.) Desf. (Rhamnaceae) leaves had shown >80% growth inhibition at 10 µg/ml, and >50% inhibition at 2 µg/ml across all parasites (Supplementary Table S1). HPLC-based activity profiling revealed that the time-windows of antiparasitic activity against *L. donovani* on the one side, and against *T. b. rhodesiense* and *P. falciparum* on the other side, were different. The antitrypanosomal and antiplasmodial activity was associated with more polar, earlier eluting compounds, while the antileishmanial activity was located in the more lipophilic and later eluting compounds (Figure 3).

In the chloroform fraction of *Guiera senegalensis* J.F.Gmel. (Combretaceae) leaves the two time windows of activity against *T. b. rhodesiense* and *P. falciparum* were identical (Figure 4), likely indicating molecules of dual activity. However, the chloroform fraction also had a relatively high cytotoxicity (IC50 = 16 µg/ml; Table 3).

### Table 3 | Cytotoxicity of antiprotozoal extracts as determined against rat L6 skeletal myoblast cells in vitro.

| Plant | Part | Fraction | Cytotoxicity [µg/ml] | IC50 | IC90 |
|-------|------|----------|-----------------------|------|------|
| Ambrosia artemisiifolia L. (syn. Ambrosia maritima (L.)) | Leaves | Ethyl acetate | 38.1 | 85.8 |
| Annona muricata L. | Leaves | Chloroform | 20.3 | 71.3 |
| Argemone mexicana L. | Leaves | Ethyl acetate | 58.5 | 91.9 |
| Boswellia papyrifera (Call. ex Delle) Hochst | Gum | Petroleum ether | 31.8 | 83.5 |
| Commiphora myrrha (T.Nees) Engl. | Gum | Methanol | 5.5 | 9.9 |
| Cuscuta hyalinum Roth ex Schult. | Fruits | Chloroform | 32.5 | 81.8 |
| Cymbopogon citratus (DC.) Stapf | Stems | Chloroform | 19.6 | 30.2 |
| Cyperus rotundus L. | Leaves | Ethyl acetate | 53.8 | N/A* |
| Guiera senegalensis J.F.Gmel. | Leaves | Ethyl acetate | 16.0 | 67.8 |
| Haplophyllum tuberculatum (Forssk.) A.Juss. | Root | Chloroform | 6.3 | 10.3 |
| Morina oleifera Lam. | Leaves | Ethyl acetate | 89.6 | N/A* |
| Prosopis chilensis var. | Leaves | Chloroform | 5.9 | 9.8 |
| Struthanthus concinnus Mart. | Branches | Ethyl acetate | 44.6 | 86.1 |
| Vachellia nilotica (L.) P.J.H.Hurter & Mabb. (syn. Acacia nilotica (L.) Delile) | Leaves | Chloroform | 15.5 | 51.8 |
| Xanthium strumarium subsp. brasilicum (Veil.) O.Bolòs & Vigo (syn. Xanthium brasilicum Veil.) | Leaves | Ethyl acetate | 21.5 | 83.0 |
| | | Petroleum ether | 13.3 | 28.8 |

*N/A* not achievable.
FIGURE 3 | HPLC-based activity profiling of an ethyl acetate fraction from leaves of Ziziphus spinosa-christi (L.) Desf. The ELSD chromatogram of the fraction separation on an analytical RP-HPLC column is shown. Activity of the 1-min micro-fractions is indicated for trypanocidal (A) and antileishmanial activity (B), expressed as % of growth inhibition.

FIGURE 4 | HPLC-ESIMS (base peak chromatogram) and activity profile of a chloroform fraction from leaves of Guiera senegalensis J.F.Gmel. Similar time window for trypanocidal (A) and antimalarial activity (B) was found. Peak 1 refers to guieranone A.
Dereplication of Active Principles

HPLC-based activity profiling, in combination with on-line spectroscopic data (MS and UV) and comparison with natural products databases was used to dereplicate known active compounds. The antiplasmodial activity of Guiera senegalensis J.F.Gmel was in accordance with previous reports. In the window of activity a HPLC peak was detected which exhibited a \([\text{M+H}]^+\) ion at \(m/z\) 316 in the MS, and \(\lambda_{\text{max}}\) 241 and 276 nm in the UV spectrum. This peak was assigned to guieranone A (MW 316.35 g/mol), a compound previously reported from this species (Silva and Gomes, 2003). The chloroform fraction of Tephrosia apollinea (Delile) DC. leaves was active against three parasites (Supplementary Table S1), as well as cytotoxic in L6 cells (Table 3). In the window of activity a HPLC peak exhibiting a \([\text{M+H}]^+\) ion at \(m/z\) 393 in the ESIMS, and \(\lambda_{\text{max}}\) 256 and 310 nm in the UV spectrum corresponded to pseudosemiglabrin, a major secondary metabolite in this plant (Waterman and Khalid, 1980), of known antioxidant and anti-inflammatory activity.

The ethyl acetate fraction of the leaves, roots, and seeds of Terminalia leiocarpa (DC.) Baill. (syn. Anogeissus leiocarpa (DC.) Guill. & Perr.) (Combretaceae) exhibited promising inhibitory activity against T. b. rhodesiense and P. falciparum (Figure 5). In the active time window HPLC peaks with MS and UV data indicative for ellagic acid and quercetin were seen, and their identity was confirmed by co-injection of authentic samples. The two compounds have been previously reported from T. leiocarpa (Ndjonka et al., 2012; Oboh et al., 2017). Ellagic acid has been previously shown to possess antiplasmodial activity (Banzouzi et al., 2002) which has been attributed to the inhibition of beta-haematin formation in the parasite (Dell’Agli et al., 2003). The antiplasmodial activity of quercetin (Ganesh et al., 2012) has been associated with the inhibition of a parasite protein kinase (Wiser et al., 1983). The leaf extract of Vachellia nilotica (L.) P.J.H.Hurter & Mabb. (syn. Acacia nilotica (L.) Delile) (Fabaceae) inhibited T. b. rhodesiense and P. falciparum at 2 \(\mu\)g/ml, and moderate cytotoxicity (IC\(_{50}\) of 21.5 \(\mu\)g/ml against L6 (Table 3). In the HPLC activity profile (Figure 6) peaks with \([\text{M+H}]^+\) ions at \(m/z\) 291.0 and \(m/z\) 442.9 in the ESIMS, and with \(\lambda_{\text{max}}\) 277 and 280 nm in the UV spectra were detected in the active time window. These peaks corresponded to catechin (Wulf et al., 2008) and epicatechin gallate (Salem et al., 2011), respectively. The occurrence of these compounds in V. nilotica has been reported (Khalid et al., 1989b; Dikti Vildina et al., 2017). Catechins were found to possess antiplasmodial activity by inhibiting both the ATPase and chaperone functions of the P. falciparum heat shock proteins (PfHsps) through direct binding to PfHsp70-1 and PfHsp70-2 (Zininga et al., 2017). In addition, a peak corresponding to ethyl gallate was detected in the active time window. Gallate esters are known inhibitors of trypanosome alternative oxidase, and they can increase intracellular glycerol to toxic levels resulting in trypanocidal activity (Jeacock et al., 2017). However, we cannot exclude that ethyl gallate was formed from gallic acid during ethanol extraction.

**DISCUSSION**

A total of 62 Sudanese plants were selected on the basis of their traditional use as medicinal plants, with an emphasis on plants that had been used to treat protozoal diseases. Of these plants a library of 235 extracts was prepared and tested against four protozoan parasites: *Plasmodium falciparum* (erythrocytic stages), *Trypanosoma brucei rhodesiense* (bloodstream forms), *Trypanosoma cruzi* (intracellular amastigotes), and *Leishmania donovani* (axenic amastigotes). The methods used were standard in vitro tests for drug discovery, where the measured signals correlated with the number of parasites. Screening of the library resulted in 125 potential hits that fulfilled the chosen activity criteria, i.e. >80% growth inhibition at 10 \(\mu\)g/ml or >50% growth inhibition at 2 \(\mu\)g/ml against one or more of the four parasites. A total of 11 extracts were solely active against *T. b. rhodesiense*, 13 against *P. falciparum*, and 5 against *L. donovani*. A total of 27 extracts exhibited activity against three parasites. The percentage of extracts that displayed activity against both *T. brucei* and *P. falciparum* (21%) was considerably higher than that with activity against *T. brucei* and *L. donovani* (5%), despite the fact that trypanosomes and leishmania are taxonomically related...
trypanosomatid parasites. This somehow surprising result is in agreement with previous screening campaigns reports (Mokoka et al., 2011; Kaiser et al., 2015; Llurba Montesino et al., 2015). The lack of overlap between activity against *T. cruzi* and *L. donovani* is not unusual and has been documented previously (Witschel et al., 2012; Zulfiqar et al., 2017). They are different parasites living in different compartments, i.e. cytoplasma for *T. cruzi* but acidic environment for *Leishmania*.

Interestingly, a major part of these extracts were from plants of the family Combretaceae (*Guiera senegalensis* J.F.Gmel., *T. leiocarpa* (DC.) Baill., *Combretum glutinosum* Perr. ex DC., *Combretum indicum* (L.) DeFilipps (syn. *Quisqualis indica* L.), and *Terminalia laxiflora* Engl.). Plants of this family are known to be rich in phenolic compounds. The lowest number of hits was found for *T. cruzi*. This may be due, in part, to the fact that *T. cruzi* amastigotes (which are the clinically relevant stages for chemotherapy) cannot be grown axenically. Hence, activity can only be identified if the antiparasitic activity against *T. cruzi* is significantly higher than cytotoxicity in L6 cells used for culturing the parasite.

Our findings corroborate previously reported activities of some plants, e.g. for *Z. spina-christi* (Mubaraki et al., 2017), *G. senegalensis* (Fiot et al., 2006), *Terminalia* spp. and *X. strumarium* (Nour et al., 2009; Abiodun et al., 2012; Ndjonka et al., 2012). Antiprotozoal activities of some other plants are reported here for the first time, e.g. the antitrypanosomal activity of *Cuscuta hyalina* Roth ex Schult., *Combretum indicum* (L.) DeFilipps, and *Croton gratissimus* var. *gratissimus*. HPLC activity profiling, in combination with on-line spectroscopy, enabled a rapid identification of some of the active compounds by dereplication (*Figure 7*), i.e. guieranone A (1) from *G. senegalensis*, pseudosemiglabrin (2) from *T. apollinea*, ellagic acid (3), and quercetin (4) from *T. leiocarpa*, and catechin (5), ethyl gallate (6), and epicatechin gallate (7) from *V. nilotica*. HPLC-based activity

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**FIGURE 6** | HPLC-UV trace of the ethyl acetate fraction of *Vachellia nilotica* (L.) P.J.H.Hurter & Mabb. leaves detected at 254 nm, and the % of inhibition of the 1-min micro-fractions against *Trypanosoma brucei rhodesiense* (A), *Leishmania donovani* (B), and *Plasmodium falciparum* (C). Peaks 5 to 7 refer to catechin, ethyl gallate, and epicatechin gallate, respectively.
profiling will also be of use for the identification of antiprotozoal compounds from promising Sudanese plants such as Croton gratissimus var. gratissimus and Cuscuta hyalina Roth ex Schult., which exhibited interesting antitrypanosomatid activity. In summary, we have compiled a comprehensive library of Sudanese medicinal plants and demonstrate that they are a promising source of bioactive molecules against protozoan parasites.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: AM, PM, MK, MH, SK. Performed the experiments: AM, MK. Analyzed the data: PM, MH, SK. Wrote the paper: AM, PM, MK, MH, SK.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00865/full#supplementary-material

TABLE S1 | Screening results of protozoan activity of plant extracts at two concentrations, 10 and 2 µg/ml.
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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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