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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 trypansomiasis 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 >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

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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 disproportionally 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 diseases. Chagas' disease has reached global dimensions ("WHO | Epidemiology" n.d.), and leishmaniasis as well ("Leishmaniasis" n.d.). Sudan has the highest incidence of leishmaniasis in sub-Saharan countries, with 15,000–20,000 new cases per annum (Hotez and Kamath, 2009). The successful treatment of malaria is threatened by artemisinin-resistant mutants of *P. falciparum*, first reported from Southeast Asia (Ariey et al., 2014; Straimer et al., 2015; Ménard et al., 2016) and, more recently, also from Africa (Lu et al., 2017).

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 (Räz 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. In the two-concentration assay, 50 µL medium containing the corresponding samples concentration (10 or 2 µg/ml) was added to the wells of a 96 well plate. For the IC_{50} determination, 50 µl medium was added to each well and a serial sample dilution of eleven 3-fold dilution steps covering a range from 100 to 0.002 µg/ml were prepared. Then 10^5 *L. donovani* amastigotes in 50 µl medium were added to the wells, and the plate was incubated for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. After 72 h of incubation, 10 µl of resazurin solution were added to each well and the plates 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% CO2. Host cells were seeded in 96-well microtitre plates, 2×10^3 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 Hepes, 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 $[^3H]$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

TABLE 1 | Plants investigated in the present study that have a reported use as anti-infective in traditional medicine.

Plant species	Family	Vernacular name	Plant part	Traditional medicinal use
Abutilon pannosum var. *figarianum* (Webb) Verdc. (syn. *Abutilon figarianum* Webb.)	Malvaceae	Humbuk, Gargadan	Leaves	Malaria, hepatoprotective, antibacterial (Mohamed et al., 2010)
Ambrosia artemisiifolia L. (syn. *Ambrosia maritima* L.)	Asteraceae	Damsissa	Leaves	Malaria, kidney stones, renal colic, hypertension (Mahmoud et al., 1999)
Anethum graveolens L.	Apiaceae	Shabat, Dill	Fruit, seeds, oil	Colic, carminative, flatulence, and dyspepsia, joint swelling, sedative for babies, lactogenic (Jana and Shekhawat, 2010)
Annona muricata L.	Annonaceae		Leaves	Antitumor, antiparasitic (Moghadamtousi et al., 2015)
Argemone mexicana L.	Papaveraceae		Leaves	Malaria, early-stage trypansomiasis (Chibale et al., 2012)
Aristolochia bracteolata Lam.	Aristolochiaceae	Irg el Agrrab, Um Galagil	Root	Malaria, scorpion stings (Suleiman, 2015)
Azadirachta indica A.Juss.	Meliaceae	Neem	Oil	Malaria, antihelminthic (El-Tahir et al., 1999a)
Boswellia papyrifera (Caill. ex Delile) Hochst	Burseraceae	Luban	Gum	Cough, respiratory infections (Yagi et al., 2016)
Cardiospermum halicacabum L.	Sapindaceae		Leaves	Malaria, antiparasitic (Waako et al., 2005)
Combretum glutinosum Perr. ex DC.	Combretaceae	Habeil	Seeds	Fever, rheumatism (Traore et al., 2014)
Combretum hartmannianum Schweinf.	Combretaceae		Wood	Jaundice, diabetes, rheuma, wound healing, anthelminthic (Khalid et al., 2012)
Croton gratissimus var. *gratissimus* (syn. *Croton zambesicus* Müll.Arg.)	Euphorbiaceae	Um-Geleigla	Fruit	Malaria, hypertension, menstrual pain (Mohamed and Khan, 2009)
Cymbopogon citratus (DC.) Stapf	Poaceae	Lemon grass	Leaves	Kidney stones and infections, malaria (Dike et al., 2012)
Cyperus rotundus L.	Cyperaceae		Rhizome	Fever, stomach disorders, bowel irritation (Kabbashi et al., 2015)
Grewia tenax (Forssk.) Fiori	Tiliaceae	Godeim	Fruits	Malaria, iron deficiency (Gebauer et al., 2007)
Guiera senegalensis J.F.Gmel.	Combretaceae	Gubeish	Leaves	Jaundice, malaria, hyperglycemia (Suleiman, 2015)
Haplophyllum tuberculatum (Forssk.) A.Juss.	Rutaceae	Haza	Leaves	Malaria, asthma, kidney diseases, gynecological, and bowel disorders (Ahmed et al., 2010; Khalid et al., 2012)
Jatropha curcas L.	Euphorbiaceae	Habat El Muluk	Leaves	Malaria (O. Abiodun et al., 2011)
Lupinus albus subsp. *graecus* (Boiss. & Spruner) Franco & P.Silva (syn. *Lupinus termis* Forssk.	Leguminosae	Tormos	Seeds	Paste for eczema and herpes zoster (Antoun and Taha, 1981)
Moringa oleifera Lam.	Moringaceae	Shagarat al Rawag	Leaves	Antimicrobial, antipyretic, antihypertensive, antispasmodic, antiinflammatory (Ali et al., 2002; Anwar et al., 2007)
Nauclea latifolia Sm.	Rubiaceae	Karmadoda	Fruit, root bark	Malaria, abdominal disease, antimicrobial (Benoit-Vical et al., 1998; Alamin et al., 2015)
Piper cubeba L. f.	Piperaceae		Fruits	Respiratory and intestinal disorders, nephroprotective, anticancer, antimicrobial (Salehi et al., 2019)
Prosopis chilensis (Molina) Stuntz	Leguminosae	Miskeet	Leaves	Antiinflammatory, analgesic (Abodola et al., 2015)
Senna occidentalis (L.) Link (syn. *Cassia occidentalis* L.)	Leguminosae	Soreib	Aerial part	Malaria, jaundice (Suleiman, 2015)
Striga hermonthica (Delile) Benth.	Orobanchaceae	Al-buda	Stem	Malaria (Okpako and Ajaiyeoba, 2004)
Tephrosia apollinea (Delile) DC	Leguminosae	Dhawasi; Dhafra	Leaves	Antiangiogenic, antioxidant antiproliferative, anticancer (Hassan et al., 2014)
Terminalia laxiflora Engl.	Combretaceae	Darout	Bark	Fever and respiratory infections (Salih et al., 2018)
Terminalia leiocarpa (DC.) Baill. (syn. *Anogeissus leiocarpa* (DC.) Guill. & Perr.)	Combretaceae	Sahab	Bark	Cough, dysentry, giardiasis (Musa et al., n.d.)
Typha angustifolia L.	Typhaceae	Si'da	Stem	Leprosy wound bleeding, diarrhoea, anthelminthic, diuretic (Varpe et al., 2012)
Vachellia nilotica (L.) P.J.H.Hurter & Mabb. (syn. *Acacia nilotica* (L.) Delile)	Fabaceae	Sunt	Leaves	Malaria (El-Tahir et al., 1999b), respiratory infections, diarrhoea, haemorrhage (Clarkson et al., 2004)
Xanthium strumarium subsp. *brasilicum* (Vell.) O.Bolòs & Vigo (syn. *Xanthium brasilicum* Vell.)	Compositae		Leaves	Malaria (Chandel et al., 2012)
Tinospora bakis (A.Rich.) Miers	Menispermaceae	Irg alhagar	Root	Fever, diarrhoea, abdominal pain (Ahmed et al., 2010)
Ziziphus spina-christi (L.) Desf.	Rhamnaceae	Sidir	Leaves	Fever, spasmolytic, and anti- diarrhea (Khalid et al., 2012)

TABLE 2 | Plants investigated in the present study for which anti-infective properties have been examined experimentally.

Plant species	Part	Tested activities	IC $_{50}$ value	Active metabolite(s)	Ref
Anethum graveolens L.	Leaves	Antiplasmodial	–	Volatile oils	(Chibale et al., 2012)
Annona muricata L.	Leaves	Antileishmanial	25 µg/ml	Acetogenins	(Osorio et al., 2007)
Argemone mexicana L.	Leaves	Antiplasmodial	1.7 µg/ml	Protopine, 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., 1989a; MacKinnon et al., 1997)
Cardiospermum halicacabum 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- 4.2 µg/ml	Curcumin, demethoxycurcumin, and bis-demethoxycurcumin.	(Rasmussen et al., 2000)
Cymbopogon citratus (DC.) Stapf	Leaves	Antiplasmodial	–	Essential oils	(Tchoumbougnang 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	(Silva and Gomes, 2003)
Haplophyllum tuberculatum (Forssk.) A.Juss.	Leaves	(1) Antileishmanial (2) Trypanocidal	(1) 16.59 µg/ml and (2) 0.2 µg/ml	(1) R-(+)-limonene (2) Justicidin B	(Gertsch et al., 2003; Hemmati and Seradj, 2016; Hamdi 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	–	(Zirihi 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 tetrahydrodesoxycordifoline and 19-O-methylangustoline	(Benoit-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 (Delile) Benth.	Whole plant	Antiplasmodial	274.8 µg/ml	–	(Okpako and Ajaiyeoba, 2004)
Terminalia leiocarpa (DC.) Baill. (syn. *Anogeissus leiocarpa* (DC.) Guill. & Perr.)	Bark	Antiplasmodial	19 µg/ml	Ellagic acid, gallic acid, and gentisic acid	(Ndjonka et al., 2012)
Tinospora bakis (A.Rich.) Miers	Roots	Antiplasmodial	28.6 µg/ml	Alkaloids	(Ouattara et al., 2006)
Vachellia nilotica (L.) P.J.H.Hurter & Mabb. (syn. *Acacia nilotica* (L.) Delile)	Seed	Antiplasmodial	1.5 µg/ml	Terpenoids and tannins.	(El-Tahir et al., 1999b)
Xanthium strumarium subsp. *brasilicum* (Vell.) O.Bolòs & Vigo (syn. *Xanthium brasilicum* Vell.)	Aerial parts	Antiplasmodial, Antitrypanosomal	0.09 µg/ml *(T. brucei),* 2.95 µg/ml *(T. cruzi),* 0.16 µg/ml (*L. donovani*), and 1.71 µg/ml (*P.falciparum*)	8-Epixanthatin 1beta,5beta-epoxide	(Nour et al., 2009)
Ziziphus spina-christi (L.) Desf.	Leaves	Antileishmanial	>30 µg/ml	–	(Ali et al., 2002)

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**).

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).

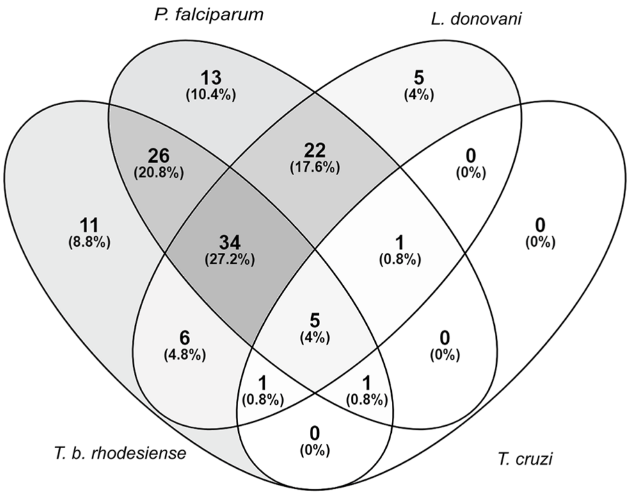

FIGURE 1 | Susceptibility of parasites against a library of Sudanese medicinal plants. Activity criteria: >80% growth inhibition at 10 µg/ml or >50% growth inhibition at 2 µg/ml against one or more of the four included parasites. Venn diagram drawn with (https://bioinfogp.cnb.csic.es/tools/venny/).

FIGURE 2 | Heat map showing two-way clustering of bioactivity of extracts, and of parasites.

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

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

Plant	Part	Fraction	Cytotoxicity [µg/ml]	
			IC_{50}	IC_{90}
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 (Caill. ex Delile) Hochst	Gum	Petroleum ether	31.6	83.5
Commiphora myrrha (T.Nees) Engl.	Gum	Methanol	5.5	9.9
Croton gratissimus var. *gratissimus* (syn. *Croton zambesicus* Müll.Arg.)	Fruits	Chloroform	32.5	81.8
Cuscuta hyalina Roth ex Schult.	Stem	Chloroform	19.6	30.2
Cymbopogon citratus (DC.) Stapf	Leaves	Ethyl acetate	53.8	N/A[a]
Cyperus rotundus L.	Rhizome	Ethyl acetate	64.3	N/A[a]
Guiera senegalensis J.F.Gmel.	Leaves	Ethyl acetate	16.0	67.8
Haplophyllum tuberculatum (Forssk.) A.Juss.	Root	Chloroform	6.3	10.3
Moringa oleifera Lam.	Leaves	Ethyl acetate	89.6	N/A[a]
Prosopis chilensis (Molina) Stuntz	Leaves	Chloroform	5.9	9.8
Struthanthus concinnus Mart.	Branches	Ethyl acetate	44.6	86.1
Tephrosia apollinea (Delile) DC	Leaves	Chloroform	15.5	51.8
Vachellia nilotica (L.) P.J.H.Hurter & Mabb. (syn. *Acacia nilotica* (L.) Delile	Leaves	Ethyl acetate	21.5	83.0
Xanthium strumarium subsp. *brasilicum* (Vell.) O.Bolòs & Vigo (syn. *Xanthium brasilicum* Vell.)	Leaves	Petroleum ether	13.3	28.8

[a]*N/A, not achievable.*

testing against amastigote *T. cruzi*. Concentration-response curves allowed the calculation of both 50% and 90% inhibitory concentrations (IC_{50} and IC_{90}; **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* Muell. Arg. (Euphorbiaceae), processed fruits of *Nauclea latifolia* Sm. *(*Rubiaceae), leaves of *Lippia lacunosa* Mart. & Schauer (Verbenaceae), and *Xanthium strumarium* subsp. *brasilicum* (Vell.) O.Bolòs & Vigo (syn. *Xanthium brasilicum* Vell.) (Compositae), and the mango *Mangifera indica* L. fruit peels (Anacardiaceae). In addition, the water fraction of processed fruits of *Nauclea latifolia*. showed significant inhibition of *T. b. rhodesiense* at the two tested concentrations.

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.(Convolvulaceae) 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 IC_{50} of 53.8 µg/ml (**Table 3**).

HPLC-Based Activity Profiling

The ethyl acetate fraction of *Ziziphus spina-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 (IC_{50} = 16 µg/ml; **Table 3**).

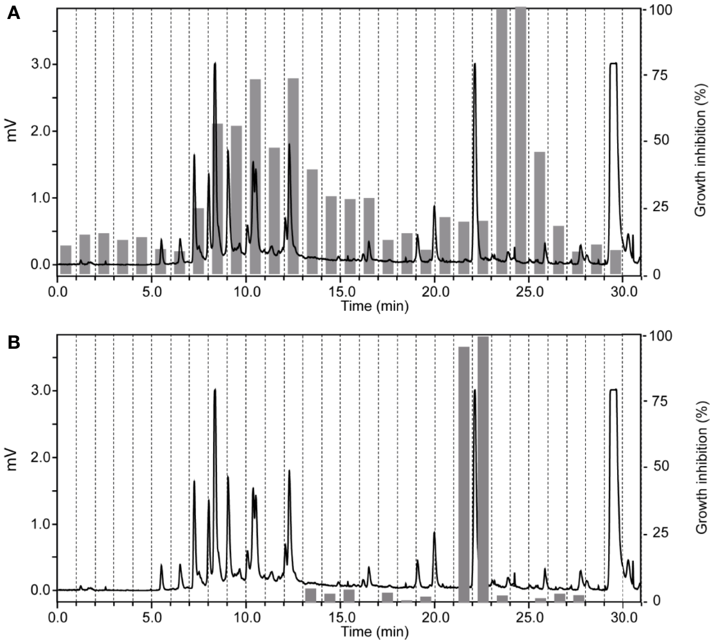

FIGURE 3 | HPLC-based activity profiling of an ethyl acetate fraction from leaves of *Ziziphus spina-christi* (L.) Desf. The ELSD chromatogram of the fraction separation on an analytical RP-HPLC column is shown. Activity of the 1-min micro-fractions is indicated for trypanocidal **(A)** and antileishmanial activity **(B)**, expressed as % of growth inhibition.

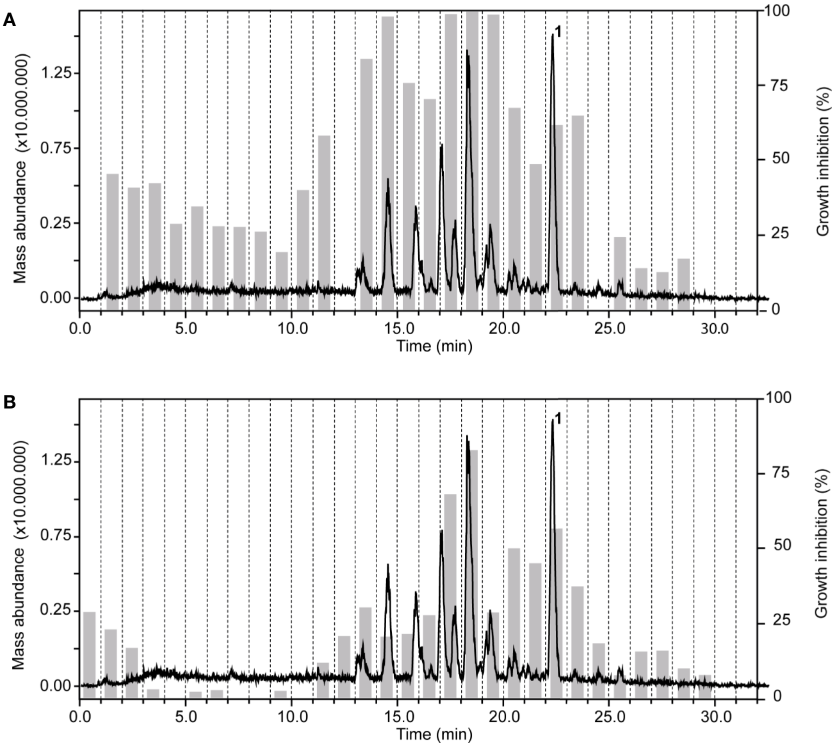

FIGURE 4 | HPLC-ESIMS (base peak chromatogram) and activity profile of a chloroform fraction from leaves of *Guiera senegalensis* J.F.Gmel. Similar time window for trypanocidal **(A)** and antiplasmodial activity **(B)** was found. Peak **1** refers to guieranone A.

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 $[M+H]^+$ ion at *m/z* 316 in the MS, and λ_{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 $[M+H]^+$ ion at *m/z* 393 in the ESIMS, and λ_{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 μg/ml, and moderate cytotoxicity (IC_{50} of 21.5 μg/ml against L6; **Table 3**). In the HPLC activity profile (**Figure 6**) peaks with $[M+H]^+$ ions at *m/z* 291.0 and *m/z* 442.9 in the ESIMS, and with λ_{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-z (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 μg/ml or >50% growth inhibition at 2 μg/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

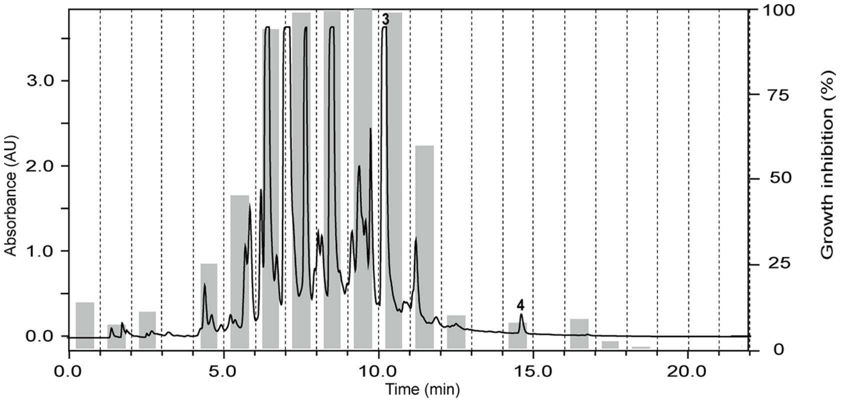

FIGURE 5 | HPLC-PDA antiplasmodial activity profiling of the ethyl acetate fraction of the leaves of *Terminalia leiocarpa* (DC.) Baill. recorded at 254 nm. Peaks **3** and **4** refer to ellagic acid and quercetin, respectively.

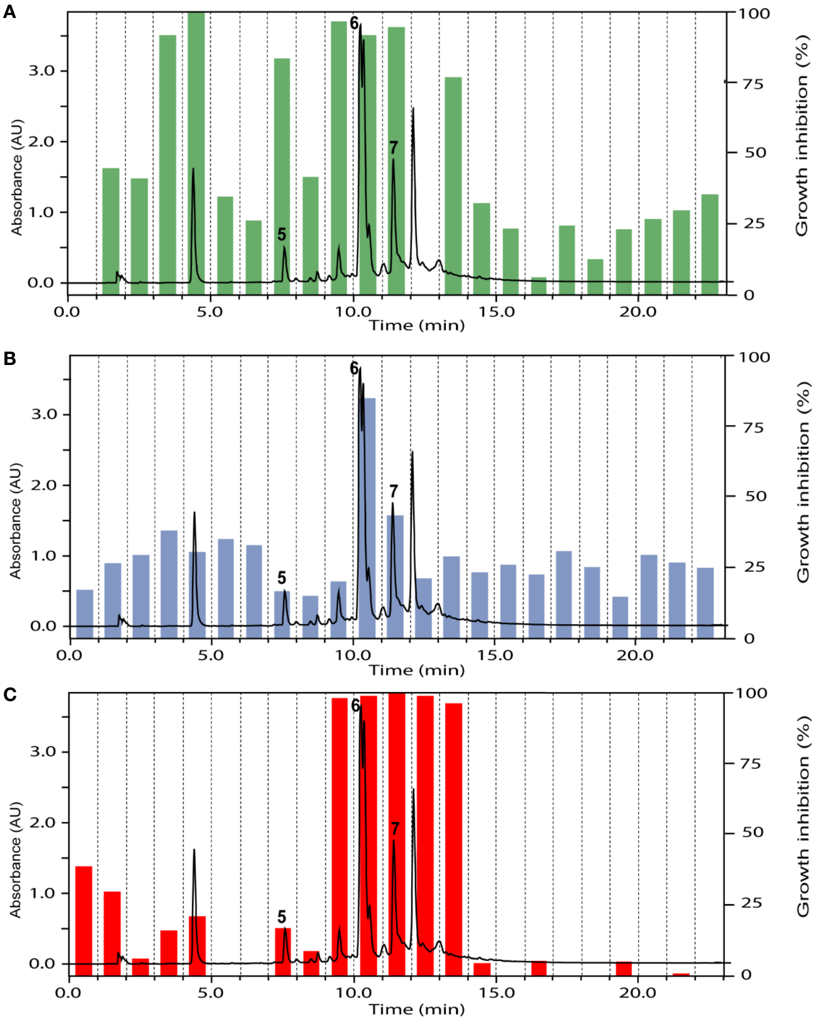

FIGURE 6 | HPLC-UV trace of the ethyl acetate fraction of *Vachellia nilotica* (L.) P.J.H.Hurter & Mabb. leaves detected at 254 nm, and the % of inhibition of the 1-min micro-fractions against *Trypanosoma brucei rhodesiense* **(A)**, *Leishmania donovani* **(B)**, and *Plasmodium falciparum* **(C)**. Peaks **5** to **7** refer to catechin, ethyl gallate, and epicatechin gallate, respectively.

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

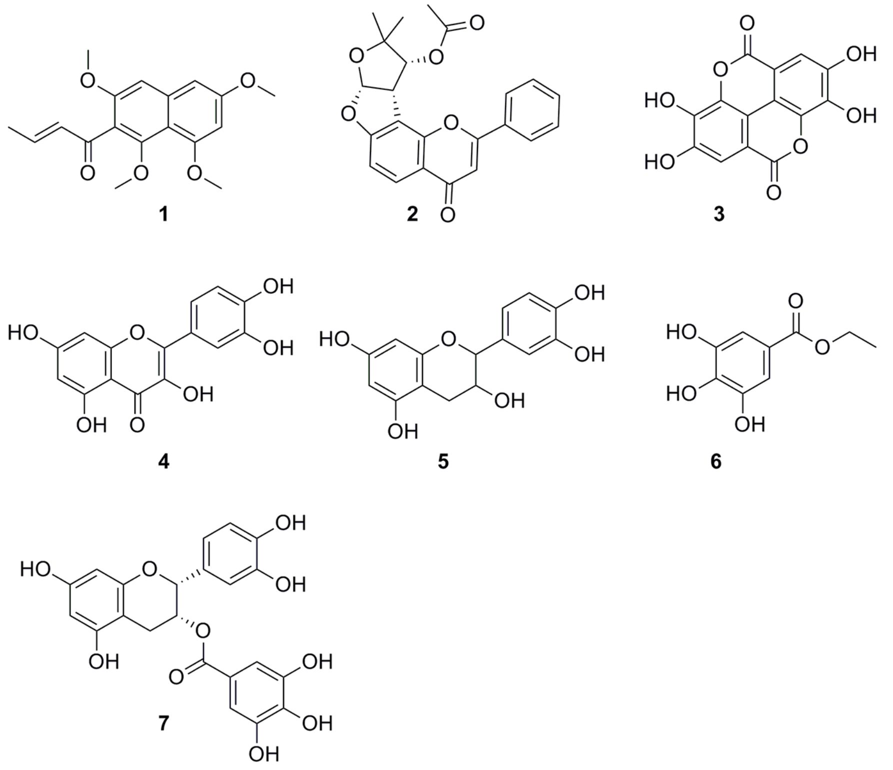

FIGURE 7 | Chemical structures of compounds identified by dereplication. Guieranone A (**1**), pseudosemiglabrin (**2**), ellagic acid (**3**), quercetin (**4**), catechin (**5**), ethyl gallate (**6**), and epicatechin gallate (**7**).

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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