Mining Sudanese Medicinal Plants for Natural Compounds against Malaria and Neglected Tropical Diseases

INAUGURALDISSERTATION

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Abdelhalim Babiker Mohamed Mahmoud

aus dem Sudan

Basel, 2020

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

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Basel, den 23.06.2020

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Dekan

To my father

Who taught me that perseverance and hard work always pays off.

May your inspiring soul rest in peace.

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Acknowledgments

First and foremost I offer my sincerest gratitude and heartfelt appreciation to Pascal Mäser, my supervisor, for giving me the opportunity to carry out the presented work. His unlimited support, open-minded leadership, and kindness that was reflected in both research and on a personal level at all times of my PhD gave me the courage, inspiration, and motivation to accomplish this thesis. I could not have imagined having a better advisor and mentor for my PhD study.

I would like also to thank Prof. Dr. Matthias Hamburger, for accepting me as a visiting scientist and allowed me to carry out the phytochemical investigations. His long experience, his sense of precision and detail has broadend my knowledge and my sense of analysis.

Very personal thanks go to Prof. Dr. Sami Ahmed Khalid whose constant encouragement, experienced advice and in-depth discussions guided me along the way of my research. His thorough knowledge and expertise in phytochemistry and pharmacology is a constant source of inspiration for me.

I warmly thank Dr. Marcel Kaiser for the stimulating discussions and his generous assistance and advice on the biological assays, from which I have learned and benefited a lot.

I would like to express my gratitude to Prof. Thomas J. Schmidt for joining the PhD committee and accepting to be co-referee, as well as for his time and inputs.

I am very grateful to Prof. em. Marcel Tanner and Prof. Suad Sulaiman for their mentorship and bringing this fruitful collaboration and network between Khartoum and Basel. I would also like to thank them for their continuous encouragement and support.

Many thanks to Monica Cal, Sonja Keller and Romina Rocchetti for their helpful and expert technical assistance in the bioassays performed. I'm further very thankful to Shereen Abd Algaffar for the mycetoma work that has been done in Sudan and Ombeline Danton for the

assistance in NMR and structure elucidations. Without their precious support it would not be possible to conduct this research.

Deep thanks extend to Christine Mensch, from the department of education and training at the SwissTPH for her continuous support and making my stay in Basel very pleasant.

My warm thanks to all current and former colleagues of the Parasite Chemotherapy Unit of the Swiss TPH and of the division of Pharmaceutical Biology. It was a great pleasure to work with you in a positive and familiar atmosphere. Sincere thanks to Natalie Wiedemar, Anna Fesser, Teresa Faleschini and Antoine Chauveau for the friendship and all the joyful moments and interesting discussions.

Very special thanks and heartfelt gratitude to my family: my mother, without your love, encouragement and whole-hearted prayers, I would not have reached this far. To my brother Mohamed, his family, my two sisters Saba and Rana, my whole extended family, and my dear friends who have given me their unequivocal support and encouragement which have paved the way to reach this stage.

Last but not least, to my wife Amal for whom my sincere expression of thanks and gratitude does not suffice. Thank you for your love, care, constant support and embracing. Throughout this journey you were my light during dark nights and my hope in desperate moments. You are my angel.

Abbreviations

ACT	Artemisinin-based Combination Therapies
COSY	COrrelation SpectroscopY
DALYs	Disability-Adjusted Life Years
DNDi	Drugs for Neglected Diseases Initiative
ECD	Electronic Circular Dichroism
ELSD	Evaporative Light Scattering Detector
ESI	ElectroSpray Ionization
GDP	Gross Domestic Product
HAT	Human African Trypanosomiasis
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence
HTS	High Throughput Screening
IC50	50% Inhibitory Concentration
MMV	Medicines for Malaria Venture
MS	Mass Spectrometry
NCEs	New Chemical Entities
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhouser Enhancement SpectroscopY
NPs	Natural Products
NTDs	Neglected Tropical Diseases
PDA	Photodiode Array Detector
RP	Reverse Phase
SAR	Structure-Activity Relationship
SI	Selectivity Index
UV	Ultraviolet
WHO	World Health Organization

Summary

Tropical parasitic diseases such as malaria, human African trypanosomiasis, Chagas disease, mycetoma, and leishmaniasis affect more than a billion people worldwide and have devastating consequences. There is no vaccine for any of these diseases, and the current drugs are problematic given their serious adverse effects and the emergence of drug-resistant parasites. Thus, there is an urgent need for the development of new, efficacious, safe, and cost-effective drugs.

Natural products have in many instances provided new leads to combat neglected tropical diseases. The aim of this work was to systemically evaluate Sudanese medicinal plants for their antiparasitic activity along with their cytotoxicity profile; followed by phytochemical investigation to identify bioactive compounds. A library of 235 plant extracts was prepared from over 60 plants used in Sudanese traditional medicine, and it was assessed for antiprotozoal activity against *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi, Leishmania donovani,* and *Plasmodium falciparum*.

Dereplication was performed for active extracts to enable a rapid identification of known active compounds and prioritization for follow-up isolation. Plants that displayed interesting activities, namely *Croton gratissimus*, *Cuscuta hyalina*, and *Haplophyllum tuberculatum*, were further pursued. HPLC-based activity profiling led to localization of activity and identification of the types of compounds in these plant extracts. Compound isolation and structure elucidation were achieved by a combination of analytical, preparative, and semipreparative chromatographic techniques such as HPLC-PDA-ELSD-MS and microprobe NMR.

HPLC-based activity profiling of *Croton gratissimus* allowed the identification of flavonoids, mainly quercetin derivatives, as responsible for the antileishmanial activity of the chloroform fraction of the crude ethanolic extract. Of these compounds, quercetin-3,7-dimethylether and ayanin were the most active against the protozoan parasites and with the highest selectivity indices.

Compounds that displayed moderate to higher antitrypanosomatid activity shared structural features, such as $\Delta^{2,3}$ unsaturation, presence of a hydroxyl group at C-3, a carbonyl group at C-4, and a catechol moiety in ring B. Phytochemical characterization of *Cuscuta hyalina* lead to the isolation of a unique flavonoid, pseudosemiglabrin, for the first time from *Cuscuta* species.

The antileishmanial activity of *Haplophyllum tuberculatum* was tracked by HPLC-based activity profiling, and eight compounds were isolated from the chloroform fraction. These included the lignans tetrahydrofuroguaiacin B, nectandrin B, furoguaiaoxidin, and 3,3'-dimethoxy-4,4'-dihydroxylignan-9-ol; and four cinnamoylphenethyl amides, namely dihydro-feruloyltyramine, N-trans-feruloyltyramine, N,N'-diferuloylputrescine, and 7'-ethoxy-feruloyltyramine. The water fraction yielded steroidal saponins. All these compounds were reported for the first time from *Haplophyllum* species and the family Rutaceae. Nectandrin B exhibited the highest activity against *L. donovani* (IC_{50} 4.5 µM) and the highest selectivity index (25.5).

Given the urgent need for better drugs and the fact that mycetoma is the most neglected of the neglected diseases, mycetoma has received special consideration. Different approaches were tackled to ultimately identify potential hits. With regard to antimycetomal natural products, several compounds were selected based on an educated-guess and were assessed accordingly. Of the tested natural compounds, magnolol possessed the highest activity (MIC of 15 µM) and selectivity (SI of 4.9).

In parallel, a drug repurposing (repositioning) strategy was pursued to find more promising hits. A series of nitroimidazole compounds were screened *in vitro* against the fungus *Madurella mycetomatis*. From this screening, niclosamide showed interesting activity with a minimal inhibitory concentration <5 µM. Furthermore, additional niclosamide analogues were tested for proof of concept. The tested compounds showed similar activity compared to niclosamide, not only against *M. mycetomatis* but also against the bacteria *Actinomadura* spp. The finding that a drug like niclosamide, which is on the WHO's list of Essential Medicines, exhibits *in vitro* activity against both fungal and bacterial mycetoma warrants the consideration of niclosamide or its ethanolamine salt as repurposing candidates for mycetoma.

1. INTRODUCTION:

Neglected Tropical Diseases, Drug Discovery, and the Sudan

1.1 Neglected Tropical Diseases

Neglected tropical diseases (NTDs), as classified by the WHO, are a group of 18 chronic disabling infections caused either by viruses, bacteria, fungi, protozoa or helminths. The diseases affect more than a billion people world-wide, mainly in Africa and mostly those living in remote rural areas, urban slums, or conflict zones [1]. The burden of these diseases has a high impact in terms of human suffering as well as contributing to poverty and under-development. NTDs account for 48 million disability-adjusted life years (DALYs) and 152 000 deaths per year [2,3]. Three of these diseases are Human African Trypanosomiasis (HAT) caused by *Trypanosoma rhodesiense* spp., Leishmaniasis caused by *Leishmania* spp., and Chagas disease (American trypanosomiasis) caused by *Trypanosoma cruzi* [4]. The three pathogens belong to the trypanosomatidae, a large family of flagellated protozoa. Although malaria, caused by the apicomplexan parasite *Plasmodium falciparum*, is no longer considered an NTD since 2000, owing to increased funding level globally by various international bodies and philanthropic organizations (e.g. the Global Fund, Bill and Melinda Gates Foundation, and the Medicines for Malaria Venture (MMV)), the disease still remains a major challenge due to the heavy death toll and its negative economic impact, which translate to 1.3% annual loss in gross domestic product (GDP) in malaria endemic African countries [5]. In addition, the occurrence of the disease among the poor is disproportionate with high mortality levels among pregnant women and children [6]. In the context of the present work, malaria will be addressed among the NTDs. General information about the above mentioned NTDs is summarized in Table 1.

Mycetoma was recently included in the WHO list of NTDs [7]. It is one of the most neglected diseases at all levels. Mycetoma is a chronic, progressively destructive morbid inflammatory disease acquired by traumatic inoculation of certain fungi (Eumycetoma) or bacteria (Actino-mycetoma) into the subcutaneous tissue [8]. The disease is geographically distributed through what is called as “the Mycetoma belt”, which includes India, Yemen, Somalia, Sudan, Senegal, Mexico, Venezuela, Colombia, and Argentina [9]. Usually the foot is the most affected part but any part of the body can be involved [10]. Late chronic stages of the disease result in destruction, deformity and loss of function and often lead to amputation.

Table 1: Summary of Neglected Tropical Disease under the scope of the study

	HAT	**Chagas disease**	**Leishmaniasis**	**Malaria**	**Mycetoma**
Causative agent	*Trypanosoma brucei rhodesiene, T. b. gambiense*	*Trypanosoma cruzi*	*Leishmania spp.* (~21 species)	*Plasmodium falciparum, P. vivax, P. malarie, P. ovale, and p. knowlesi*	*> 50 species, mainly Madurella mycetomatis* (Fungal type) *and Actinomadura madurae* (bacterial)
Vector	*Glossina spp.* (tsetse fly)	*Triatomine spp.*(Kissing bug)	*Phlebotomus spp.*(Sandflies)	*Anopheles spp.*	Unknown
Geographic distribution	Sub-saharan Africa	South and Central America	Africa, Asia, Europe, South and Central America	World-wide	Between the latitudes 15° S and 30° N
DALYs	560 000	546 000	3.32 million	82.67 million	Unknown
Deaths	9 100	10 300	51 600	445 000 [11]	Unknown
Treatment	Melarsoprol Eflornithine+ nifurtimox Fexnidazole	Nifurtimox Benznidazole	Liposomal amphotericin B Miltefosine Paromomycin	Artemisinin combination therapy	**Fungal**: Itraconazole **Bacterial**: Amikacin+ Co-trimoxazole

DALYs: Disability-adjusted life years

1.1.1 NTDs and Sudan

According to WHO reports, of the 17 neglected diseases, 9 are a recognized public health problem in Sudan (Figure 1). These include: leishmaniasis, schistosomiasis, lymphatic filariasis, onchocerciasis, trachoma, guinea worm, mycetoma, soil transmitted helminths, and leprosy. Large populations living in rural areas are infected by one or more of these diseases, with the school-age children being the most affected [12]. Sudan had made large progress in the eradication of dracunculiasis (guinea-worm disease). However, the country is still endemic for schistosomiasis and trachoma (3.6 million cases), and it has the highest incidence for cutaneous and visceral leishmaniasis in sub-Saharan countries with 15,000–20,000 new cases annually

[13]. The situation for mycetoma is not any better. Sudan is considered among the highest infected countries with more than 6000 cases, of which, 64% are under the age of 30. Of the mycetoma cases reported, 70% are eumycetoma, stressing the high need for effective treatment and adequate preventive and control measures to reduce the disease morbidity and mortality [14]. For malaria, Sudan is considered a high-burden and high-risk country. In 2012, more than 5000 cases were reported [15]. Malaria accounted for a higher mortality burden than disability, with an estimated total number of 44000 deaths in 2002 in Sudan [16].

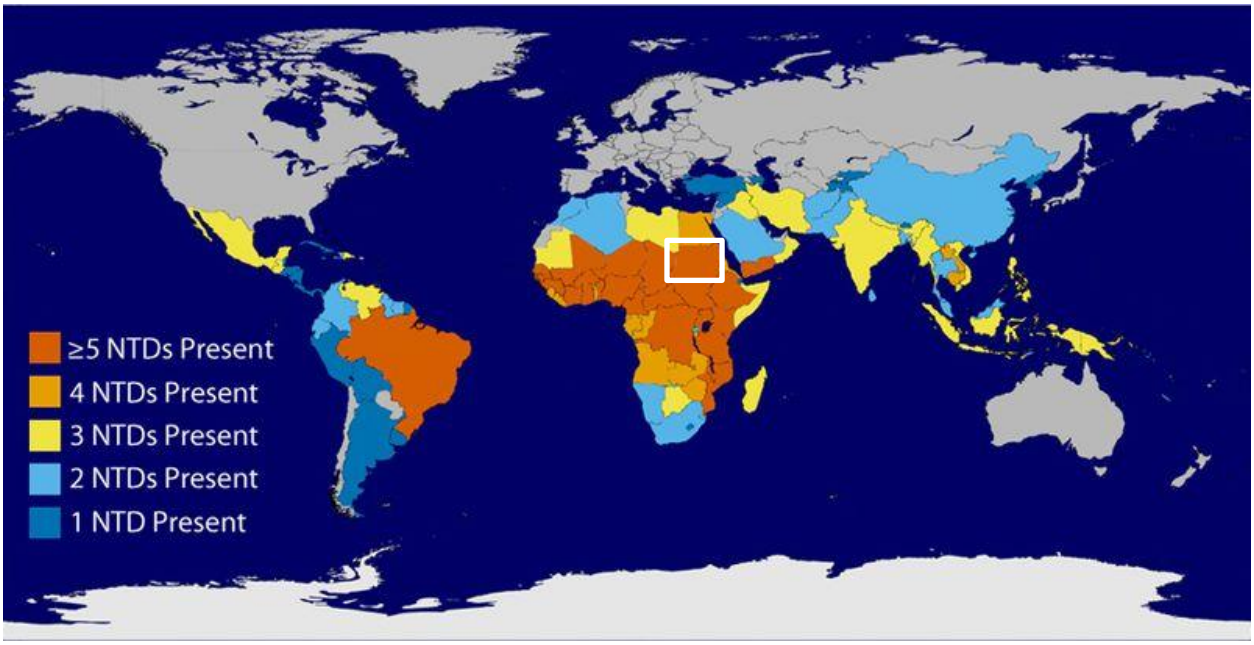

Figure 1: Burden of Neglected Tropical Diseases with emphasis on Sudan.

(*Source*: https://www.cdc.gov/globalhealth/ntd/diseases/ntd-worldmap-static.html)

1.2 Drug Discovery for NTDs:

1.2.1 Challenges and Gaps

Most of the currently available drugs for NTDs have drawbacks in terms of toxicity, limited availability of oral therapeutic dosage forms, development of resistance, or non-affordability, coupled with unavailability of vaccines for any of them. However, drug discovery and development of new and better medicines for NTDs is costly and of low-return. As already reported, of the 1602 new chemical entities (NCEs) that has been approved between 1981 and 2019, unfortunately, only 20 medicines out of the total were for the treatment of neglected diseases [17]. The creation of public-private partnerships like DNDi (Drugs for Neglected Diseases initiative) has helped to overcome this bottleneck. A sustainable solution for new drug development became feasible with the backing support of international pharmaceutical policy and collaboration [18]. Nevertheless, there are still major gaps and so far, with the exceptions of tafenoquine for malaria and fexinidazole for HAT, these initiatives have brought to market mainly new formulations and combinations of already existing drugs.

While the incidence of human African trypanosomiasis is at a historic low and a new oral drug, fexinidazole [19], has recently received positive opinion by the European Medicines Agency, the prospects are gloomier and less good for Chagas' disease and leishmaniasis. In Eastern Africa, a high-burden region of visceral leishmaniasis [20], sodium stibogluconate is still the mainstay of leishmaniasis chemotherapy [21], a pentavalent antimonial that can cause hepatotoxicity and cardiotoxicity [22]. There is one efficient and safe antileishmanial drug, AmBisome, a liposomal formulation of amphotericin B that was developed as a fungicide and repurposed for leishmaniasis [23]. However, AmBisome is expensive and requires a cold chain to delivery. Hence, many reports have outlined research priorities for kinetoplastids parasites regarding the need of new cost-effective therapies as a key element of the fight against protozoal neglected tropical diseases [24,25].

The treatment of malaria still relies globally on artemisinin-based combination therapies (ACT). Alarmingly, artemisinin-resistant isolates of *P. falciparum* have been described from south-east Asia [26–28] and most recently also from Africa [29]. Thanks to an increased funding level

globally by various international bodies and philanthropic organizations such as Medicine for Malaria Venture (MMV), there is a series of new molecules in the drug discovery pipeline. However, none of these has reached registration yet.

The bacterial type of the mycetoma, actinomycetoma, is readily cured by antibiotics combination therapy [30]. In contrast, management of the fungal type (Eumycetoma) is much more difficult. Treatment usually involves surgical excision combined with long term use of azole antifungals, which have limited efficacy, toxic adverse effects, are expensive, and have with high percentage of treatment failures [31]. DNDi together with the Mycetoma Research Center in Khartoum are currently running a phase II/III clinical trial to assess the efficacy and safety of fosravuconazole in comparison to the currently used itraconazole. Nevertheless, there is a dire need to find new therapeutic agents for eumycetoma that are efficient, affordable, safe, and decrease treatment period and surgical interventions [32].

1.2.2 Drug Development Strategies for NTDs:

Strategies for hit discovery usually involve two opposing, yet complementary, screening pathways; target-based (typically a protein or an enzyme), or phenotypic screening (whole organism, cell-based). Despite the paradigm shift in the pharmaceutical industry to move from whole-cell to target-based screening, in the case of antiparasitic drugs, the phenotypic approach has proven to be more successful. This success could be explained by the facts that (i) it does not require prior knowledge of the molecular target, which is the case of most neglected parasites where there are very few validated molecular targets; (ii) it enables high throughput screening of chemical libraries and identification of chemical entities without a known target or mechanism of action; (iii) target deconvolution is possible with the aid of genomic tools; (iv) successful drug candidates are likely to involve interaction with a number of different target enzymes ("unspecific" mode of action), which is the case of most of the currently used antiparasitics [33–35].

Alternative approaches include structure-based drug discovery, re-purposing of drugs from other disease areas, and in silico methods. Each strategy has its own advantages and disadvantages.

Of the many avenues and possibilities of drug discovery for neglected tropical diseases [36,37], two strategies will be highlighted and discussed in the scope of this work to fill the drug pipeline against these devastating and global diseases. These are; i) repurposing, and ii) natural products.

1.2.2.1 Repurposing

Since drug development is lengthy and expensive, the drug repurposing strategy (i.e. finding new uses for existing drugs) offers an attractive shortcut between the bench and the clinic, particularly where the resources for R&D are limited [38]. The concept of repurposing is actively pursued for NTDs, and many drugs that are currently used for the treatment of neglected tropical diseases have been 'repositioned' (Table 2). Concurrently, most of the repurposed compounds have arisen from phenotypic screening campaigns rather than target-based strategies [39], Owing to the limited number of fully validated targets in NTDs, as discussed earlier. Repurposing screening campaigns for NTDs have revealed potential molecules of different drug classes, like tricyclic antidepressants for antipalsmodial activity, tadalafil and the antispasmodic mebeverine for Chagas' disease, along with other molecules that fit established criteria of Target Product Profiles (TPP) for NTDs [40]. Interestingly, not only drugs used in other diseases are repurposed for NTDs, but also the other way around: suramin, developed for Nagana and sleeping sickness, was repurposed for the treatment of cancers and autism [41], and an exciting case is the antimalarial chloroquine in clinical trials for the treatment of the recent virus pandemic COVID-19 [42]. In the context of this work, nitroimidazoles were tested for their in vitro activity against *Madurella mycetomatis*, the major causative agent of Eumycetoma.

Table 2: Drugs repurposed for NTDs

Drug	Original use	Repurpose	Reference
Eflornithine	Anticancer	HAT	[43]
Ivermectin	*Onchocerca* in horses	River blindness	[44]
Fosmidomycin	Antibiotic	Malaria	[45]
Doxycycline	Antibiotic	Filariasis and Malaria	[46]

Amphotericin B	Antifungal	Visceral leishmaniasis	[47]
Miltefosine	Anticancer	Visceral leishmaniasis	[47]
Paromomycin	Antibiotic	Visceral leishmaniasis	[47]
Pentamidine	Equine trypanosomiasis	HAT- Stage 1	[43]
Albendazole	Anthelmintic for livestock	Lymphatic filariasis	[48]
Nifurtimox	Chagas disease	HAT	[49]

1.2.2.2 Natural Products

Natural products (NPs) remain a successful source of inspiration for the discovery of new drugs. A recent comprehensive review by Newman and Cragg, covering approved drugs during the period 1981-2019, revealed that one third of the small molecules launched over the last four decades were derived directly or indirectly from natural resources. Moreover, of the 20 approved antiparasitic drugs, 9 were of natural origin [17], some of them are summarized in Table 3. Many chemo-informatic studies showed that natural products cover a much wider and larger chemical space than combinatorial and synthetic compounds, due to their diversity in terms of chiral centers and richness in functional groups, which render them viable for a wider ligand affinity and better specificity to biological targets [50,51].

Table 3: Examples of antiparasitic drugs of natural or derived from natural origin

Drug	Natural origin	Source	Classification	Reference
Artemisinin	*Artemisia annua*	Plant	N	[52]
Ivermectin	*Streptomyces avermitilis*	Bacteria	N	[53]
Quinine	*Cinchona succirubra*	Plant	N	[54]
Moxidectin	Milbemycin derivative from *Streptomyces cyanogriseus* spp.	Bacteria	ND	[55]
Eflornithine	Difluoromethyl derivative of ornithine	Amino acid	ND	[56]

N: natural product; ND: natural product derivative

Many secondary metabolites with a wide variety of scaffolds, namely alkaloids, terpenes, and phenolic compounds (e.g. lignans, tannins, coumarins, flavonoids) have shown potent inhibition

of parasites responsible for NTD [37,57–59]. However, the potent activities displayed by some of these NPs are hampered by their toxicity and pharmacokinetic profiles that prevent their use in the clinic. Nonetheless, these hits can be modified by medicinal chemistry and drug delivery approaches to enhance the pharmacokinetic and safety characteristics.

1.2.2.3 Challenges and opportunities of Natural Products Drug Discovery

Despite the success of NPs, the interest of several major pharmaceutical companies has waived, and they cut down the use of natural products in their drug discovery programs. A major concern is that NPs are incompatible with high throughput screening (HTS), laborious to handle, highly complex, have non-specific activities, and issues with accessibility, logistics, and patentability [60]. A more rational and economic search for new lead structures from nature must therefore be a priority in order to overcome these problems. Key factors to achieve this competitiveness include employment of technological advances like robotics, bioassay miniaturization, and developments in spectroscopy in the NPs-based lead discovery processes such as speed of dereplication, bioassay-guided isolation, and structure elucidation [61]. Innovative omics-based approaches integrated with molecular networking enabled the prioritization and targeted isolation of novel natural products, and provided means to bridge the gap between ethnopharmacological drug discovery and industrial biotechnology for monitoring fermentation or other production processes [61–63].

1.2.2.4 Strategies for identification and Isolation of Bioactive Natural Products

A variety of approaches are being used for identification of bioactive secondary metabolites from plant, fungal, microbial, or marine sources, such as: i) traditional medicine and ethno-pharmacological knowledge (antimalarials, quinine and artemisinin), ii) taxonomical - chemotaxonomical (anticancer, taxol), iii) ecology-based (marine natural products, insecticides, antifeedants), and iv) pharmacophore-based or virtual screening (computer-based) [64].

The process of “bioassay-guided fractionation” starts once an extract has shown favorable activity in a screening. Then, it is necessary to isolate the compound(s) responsible for the pharmacological properties. Since extracts are complex matrices, there is the challenge of

localizing activity in the extract by overlaying biological data and chemo-analytical information in order to identify the active principles at an early stage [65]. One of these approaches is dereplication. Dereplication was initially defined as "the process of quickly identifying known chemotypes'' [66]. The definition has developed and extended over the years to include many strategies with the ultimate goal of accelerating the discovery of bioactive substances by improving the characterization methods of natural resources. Dereplication allows elimination and prioritization of extracts by comparing the chemical and biological characteristics of unknown compounds to that of previously identified compounds in databases. Hence, comprehensive databases are crucial for high performance dereplication workflows [67,68].

Another approach is HPLC-based activity profiling, which has been successfully used for tracking bioactive compounds in crude mixtures [69]. The principle of this approach consists in the analytical scale HPLC separation of bioactive extracts. UV and MS data are recorded online in parallel to collection of fractions into microplates or deep-well plates, via a T-split of the column effluent. The fractions are dried, re-dissolved in a small amount of a suitable solvent (usually DMSO) and assayed for bioactivity. The chromatogram and the activity profile are then matched to identify active peaks. On-line spectroscopic information in combination with database searches can be used to dereplicate known compounds and facilitates prioritization of samples for follow-up activities [70,71]. (Figure 2)

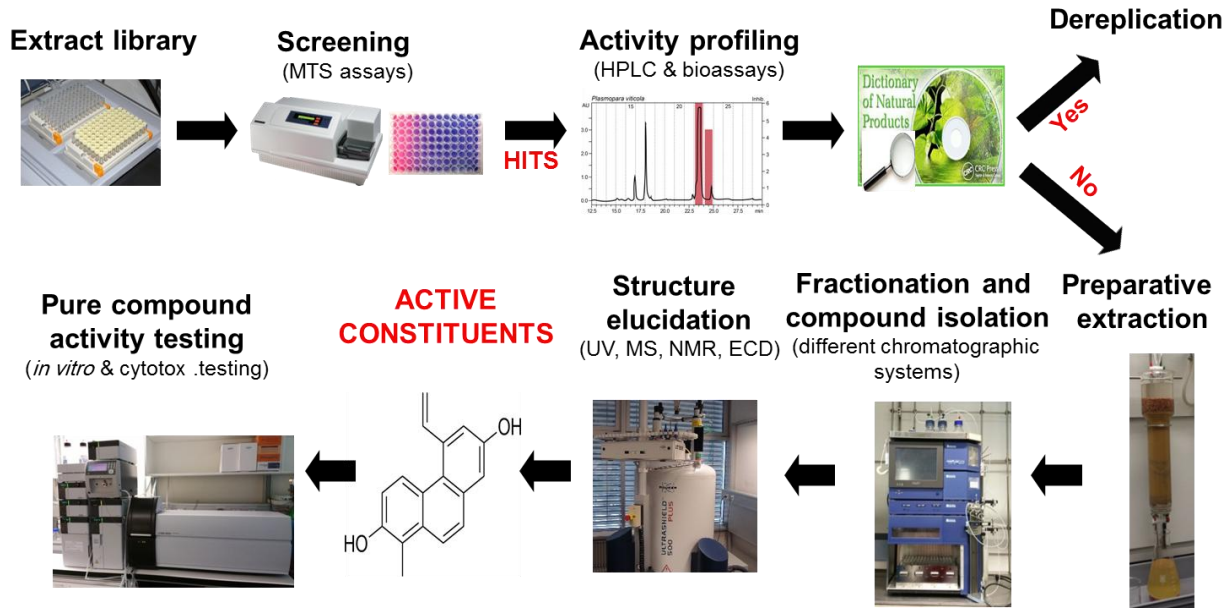

Figure 2: HPLC-based activity profiling approach

1.3 Ethnomedicine in Sudan and Biodiversity

Sudan is the third largest country in the African continent (after Algeria and Democratic Republic of the Congo). Located in east central Africa with a total surface area of 1.8 million km^2, Sudan encompasses different terrains and climatic zones, ranging from desert and semi-arid in the north to tropical savanna in the south. The country consists of a vast flat landscape bordered by mountains on the north east (the Red Sea Hills) and the west (the Marrah Mountains). The northern part features the Nubian Desert (part of the Sahara desert) and the east part reaches out to the red sea (Figure 3). This matchless geographical topography of Sudan, with its variable climates, makes it a unique place with different ecosystems and a richness of plant biodiversity.

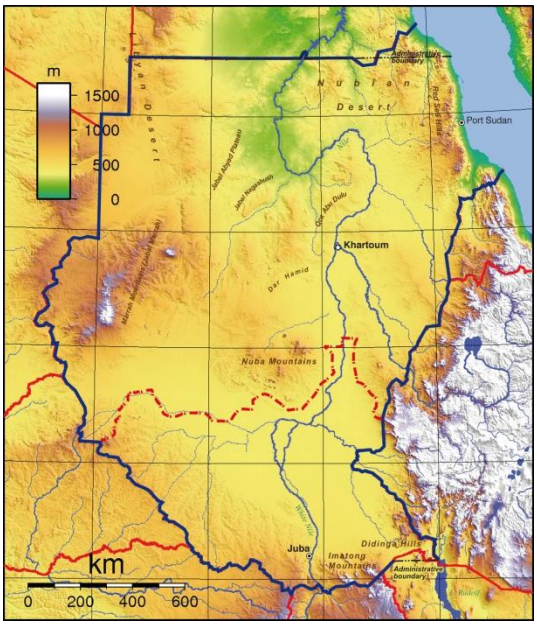

Figure 3: Topographical map of the Sudan, desert in the North and main mountain ranges: Red sea hills in northeast and Marrah mountains in the west.
(Source: https://www.nationsonline.org/oneworld/map/sudan-topographic-map.htm)

The flora of Sudan consists of 3137 species of flowering plants belonging to 170 families and 1280 genera [72]. It is estimated that 15% of these plants are endemic to Sudan. The intersection of diverse African, Arabic and Islamic cultures influenced the uniqueness of folkloric and herbal medicine [72]. Sudanese traditional medicine is an indigenous form of holistic health care system involving both mind and body (i.e. psychosomatic medicine). It is a unique combination of natural, cultural and religious background that prevalent in the community. However, there is limited published data available on the biological activities of the Sudanese medicinal plants.

1.4 Objectives

Sudan's biodiversity of medicinal plants coupled with deeply rooted ethno-botanical heritage remains a promising untapped reservoir for the discovery of diverse chemical entities. The main goals of this Ph.D. thesis are:

1. Better characterization of Sudanese medicinal plants and a rationale for their use.
2. Finding promising antiparasitic hit compound(s) that have the potential to generate novel leads.
3. Exploring different approaches of drug discovery for the neglected disease Mycetoma.

Chapter 2 starts with a comprehensive overview on medicinal plants that are being used traditionally in Sudan for tropical illnesses, with a focus on protozoal diseases and plants that were pharmacologically investigated. On the basis of this survey, a library consisting of 235 extracts andfractions thereof, representing 62 plants reputed as antiparasitics that belong to 35 different plant families, was assembled, prepared, and screened phenotypically for *in vitro* activity against the following panel of protozoal 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* proliferative erythrocytic stages grown in human erythrocytes. For selected active extracts, HPLC-based activity profiling in combination with on-line spectroscopy enabled a rapid identification of some of the bioactive compounds by dereplication.

Chapter 3 and **Chapter 4** continue with phytochemical characterization using the HPLC- activity profiling approach for plant extracts of promising antiprotozoal activity, followed by the isolation of their bioactive compounds by different preparartive and semipreaparative chromatographic techniques, and finally elucidation of the chemical structures. The activity of the isolated compounds was determined *in vitro* (whole-cell assays), alongside with cytotoxicity testing in mammalian cells. The main purpose of the cytotoxicity test was to calculate the

selectivity index (SI), which allows discriminating attractive compounds from poorly selective ones.

Chapter 5 investigates the *In vitro* antimycetomal activity, together with cytotoxicity profiling of compounds isolated from the chloroform and the water fractions of the ethanolic extract of *Haplophyllum tuberculatum* roots (Forsskal) A. Juss. (Rutaceae). In addition, various natural compounds of different calsses of plant secondary metabolites obtained from different plant species, and that have been previously reported for their antifungal and anti-infective activities, were also screened for their activity and cytotoxicity against *Maduralla mycetomatis*.

Chapter 6, the repurposing approach, was pursued for finding potential drug candidates that are active against fungal mycetoma. Selection and testing of nitroimidazoles and other redox-active molecules was performed based on their potential antifungal mechanism of action. The hypothesis that redox-active molecules will also be active against *Madurella mycetomatis*, the principal causative agent of eumycetoma, was not confirmed. Nevertheless, niclosamide was identified as a potential drug candidate.

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2. **Mining Sudanese Medicinal Plants for Antiprotozoal Agents**

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Published in

Frontiers in Pharmacology-Ethnopharmacology 2020;11:865.

doi:10.3389/fphar.2020.00865

I have performed all experiments and analysis and wrote the manuscript

2.1 Abstract

Neglected tropical diseases are major health hazards in developing countries. Annually, up to thirty 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 *Anogeissus leiocarpa* (DC.) Guill. & Perr.; and catechin, ethyl gallate, and epicatechin gallate from *Acacia nilotica* (L.) Delile. 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.

2.2 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 [1,2]. 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) [3]. 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 Malaria Report, WHO 2018). These diseases disproportionally affect the poor and vulnerable populations [4], 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 [5], 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 [6], and leishmaniasis as well [7]. Sudan has the highest incidence of leishmaniasis in sub-Saharan countries, with 15,000–20,000 new cases per annum [8]. The successful treatment of malaria is threatened by artemisinin-resistant mutants of *P. falciparum*, first reported from Southeast Asia [9–11] and, more recently, also from Africa [12].

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 [13–15].
A retrospective analysis showed that approx. 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 [16].

Sudan's biodiversity coupled with a deeply rooted ethno-botanical 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.

2.3 Results

2.3.1 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 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.	Malvaceae	Humbuk, Gargadan	Leaves	Malaria, hepatoprotective, antibacterial [17]
Acacia nilotica (L.) Delile	Fabaceae	Sunt	Leaves	Malaria [18], respiratory infections, diarrhoea, haemorrhage [19]
Ambrosia maritima L.	Asteraceae	Damsissa	Leaves	Malaria, kidney stones, renal colic, hypertension [20]
Anethum graveolens L.	Apiaceae	Shabat, Dill	Fruit, seeds, oil	Colic, carminative, flatulence and dyspepsia, joint swelling, sedative for babies, lactogenic [21]
Annona muricata L.	Annonaceae		Leaves	Antitumor, antiparasitic [22]
Anogeissus Leiocarpa (DC.) Guill. & Perr.	Combretaceae	Sahab	Bark	Cough, dysentry, giardiasis [23]
Argemone mexicana L.	Papaveraceae		Leaves	Malaria, early-stage trypansomiasis [24]
Aristolochia bracteolata Lam.	Aristolochiaceae	Irg el Agrrab, Um Galagil	Root	Malaria, scorpion stings [25]
Azadirachta indica A.Juss.	Meliaceae	Neem	Oil	Malaria, antihelminthic [26]
Boswellia papyrifera (Caill. ex Delile) Hochst	Burseraceae	Luban	Gum	Cough, respiratory infections [27]
Cardiospermum halicacabum L.	Sapindaceae		Leaves	Malaria, antiparasitic [28]
Combretum glutinosum Perr. ex DC.	Combretaceae	Habeil	Seeds	Fever, rheumatism [29]
Combretum hartmannianum Schweinf.	Combretaceae		Wood	Jaundice, diabetes, rheuma, wound healing, anthelminthic [30]

Plant species	Family	Vernacular name	Plant part	Traditional medicinal use
Croton gratissimus var. *gratissimus*	Euphorbiaceae	Um-Geleigla	Fruit	Malaria, hypertension, menstrual pain [31]
Cymbopogon citratus (DC.) Stapf	Poaceae	Lemon grass	Leaves	Kidney stones and infections, malaria [32]
Cyperus rotundus L.	Cyperaceae		Rhizome	Fever, stomach disorders, bowel irritation [33]
*Grewia tenax (*Forssk.) Fiori	Tiliaceae	Godeim	Fruits	Malaria, iron deficiency [34]
Guiera senegalensis J.F.Gmel.	Combretaceae	Gubeish	Leaves	Jaundice, malaria, hyperglycemia [25]
Haplophyllum tuberculatum (Forssk) A.Juss.	Rutaceae	Haza	Leaves	Malaria, asthma, kidney diseases, gynecological and bowel disorders [30,35]
Jatropha curcas L.	Euphorbiaceae	Habat El Muluk	Leaves	Malaria [36]
Lupinus albus subsp. *graecus* (Boiss. & Spruner) Franco & P.Silva (syn. *Lupinus termis* Forssk.)	Leguminosae	Tormos	Seeds	Paste for eczema and herpes zoster [37]
Moringa oleifera Lam.	Moringaceae	Shagarat al Rawag	Leaves	Antimicrobial, antipyretic, antihypertensive, antispasmodic, antiinflammatory [38,39]
Nauclea latifolia Sm.	Rubiaceae	Karmadoda	Fruit, root bark	Malaria, abdominal disease, antimicrobial [40,41]
Piper cubeba L. f.	Piperaceae		Fruits	Respiratory and intestinal disorders, nephroprotective, anticancer, antimicrobial [42]
Prosopis chilensis (Molina) Stuntz	Leguminosae	Miskeet	Leaves	Antiinflammatory, analgesic [43]
Senna occidentalis (L.) Link (syn. Cassia occidentalis L.)	Leguminosae	Soreib	Aerial part	Malaria, jaundice [25]
Striga hermonthica (Delile) Benth.	Orobanchaceae	Al-buda	Stem	Malaria [44]
Tephrosia apollinea (Delile) DC	Leguminosae	Dhawasi; Dhafra	Leaves	Antiangiogenic, antioxidant antiproliferative, anticancer [45]
Terminalia laxiflora Engl.	Combretaceae	Darout	Bark	Fever and respiratory infections [46]
Typha angustifolia L.	Typhaceae	Si'da	Stem	Leprosy wound bleeding, diarrhoea, anthelminthic, diuretic [47]
Xanthium Strumarium subsp. *brasilicum* (Vell.) O.Bolòs & Vigo (syn. *Xanthium brasilicum* Vell.)	Compositae		Leaves	Malaria [48]
Tinospora bakis (A.Rich.) Miers	Menispermaceae	Irg alhagar	Root	Fever, diarrhoea, abdominal pain [35]
Ziziphus spina-christi (L.) Desf.	Rhamnaceae	Sidir	Leaves	Fever, spasmolytic and anti-diarrhea [30]

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
Acacia nilotica (L.) Delile	Seed	Antiplasmodial	1.5 µg/mL	Terpenoids and tannins.	[18]
Anethum graveolens L.	Leaves	Antiplasmodial	-	Volatile oils	[24]
Annona muricata L.	Leaves	Antileishmanial	25 µg/mL	Acetogenins	[49]
Anogeissus Leiocarpa (DC.) Guill. & Perr.	Bark	Antiplasmodial	19 µg/mL	Ellagic acid, gallic acid, and gentisic acid	[50]
Argemone mexicana L.	Leaves	Antiplasmodial	1.7 µg/mL	Protopine, allocryptopine, and berberine	[51]
Aristolochia bracteolata Lam.	Root	Antiplasmodial	< 5 µg/mL	-	[18]
Azadirachta indica A.Juss.	Leaves	Antiplasmodial	2.5 µg/mL	Gedunin	[52,53]
Cardiospermum halicacabum L.	Leaves	Antiplasmodial	42 µg/mL	-	[54]
Combretum glutinosum Perr. ex DC.	Leaves	Trypanocidal	26.5 µg/mL	-	[29]
Combretum hartmannianum Schweinf.	Bark	Antiplasmodial	0.2 µg/mL	-	[38]
Commiphora myrrha (Nees) Engl.	Gum resin	Trypanocidal	8.1 µg/mL	-	[56]
Croton gratissimus var. *gratissimus*	Root	Antiplasmodial	-	Sesquiterpenes, monoterpenes, and alkaloids	[57]
Curcuma longa L.	Rhizome	Antiplasmodial	3- 4.2 µg/mL	Curcumin, demethoxycurcumin, and bis- demethoxycurcumin.	[58]
Cymbopogon citratus (DC.) Stapf	Leaves	Antiplasmodial	-	Essential oils	[59]
Cyperus rotundus L.	Whole plant	Antiplasmodial	-	Terpenes, monoterpenes and sesquiterpenes.	[60]
Guiera senegalensis J.F.Gmel.	Leaves and roots	Antiplasmodial	4.08 µM	Guiranone A	[23]
Haplophyllum tuberculatum (Forssk.) A.Juss.	Leaves	(1) Antileishmanial (2) Trypanocidal	(1) 16.59 µg/mL. (2) 0.2 µg/mL	(1) R-(+)-limonene (2) Justicidin B	[63–65]
Jatropha curcas L.	Seeds	Trypanocidal	1.9 µg/mL *(T. brucei) and* 7.4 µg/mL , (*T. cruzi)*	Phorbol esters	[66]
Mangifera indica L.	Stem bark	Antiplasmodial	> 50 µg/mL	-	[67]
Moringa oleifera Lam.	Leaves	Antileishmanial	5.25 µM	Niazinin	[68]
Nauclea latifolia Sm.	Stem and root	Antiplasmodial	0.9-3 µg/mL	Alkaloids tetrahydrodesoxycordifoline and 19-O-methylangustoline	[41,69]

Plant species	Part	Tested activities	IC_{50} value	Active metabolite(s)	Ref
Piper cubeba L. f.	Fruits	*Antitrypanosomal against T. cruzi* amastigotes	87.9 µg/mL	Essential oil	[70]
Senna occidentalis (L.) Link (syn.*Cassia occidentalis* L.	Leaves	Antiplasmodial	<3 µg/mL	Anthraquinones, terpenes and flavonoids.	[55]
Striga hermonthica (Delile) Benth.	Whole plant	Antiplasmodial	274.8 µg/mL	-	[44]
Tinospora bakis (A.Rich.) Miers	Roots	Antiplasmodial	28.6 µg/mL	Alkaloids	[71]
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	[72]
Ziziphus spina-christi (L.) Desf.	Leaves	Antileishmanial	>30 µg/mL	-	[38]

2.3.2 Testing for antiparasitic activity

The original extracts and all fractions obtained by partitioning were tested at two concentrations, 2 µg/mL 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).

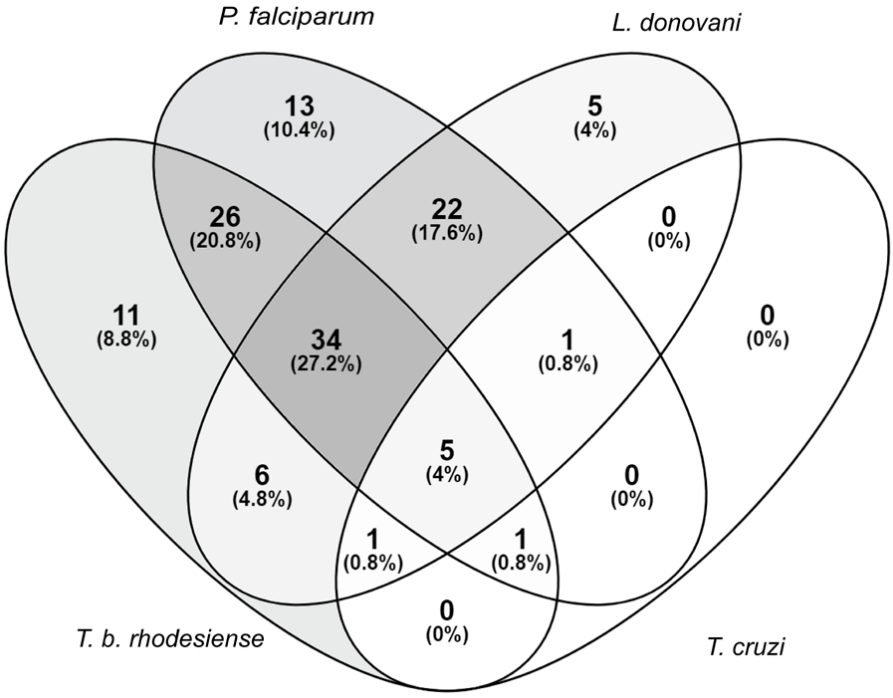

Figure 1. Susceptibility of parasites against a library of Sudanese medicinal plants. Activity criteria: >80% growth inhibition at 10 µg/mL or >50% growth inhibition at 2 µg/mL against one or more of the four included parasites. Venn diagram drawn with (https://bioinfogp.cnb.csic.es/tools/venny/).

2.3.3 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).

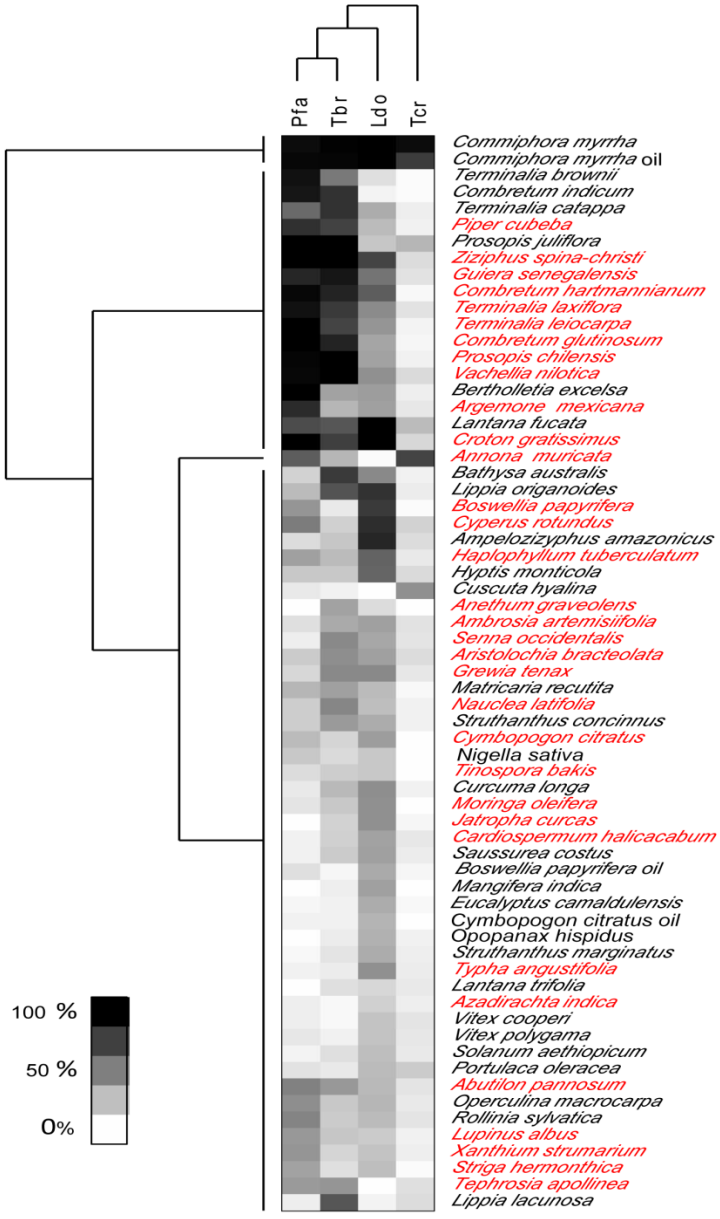

Figure 2. Heat map showing two-way clustering of bioactivity of extracts, and of parasites.

2.3.4 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 (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.

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}
Acacia nilotica (L.) Delile	Leaves	Ethyl acetate	21.5	83.0
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 (Nees) Engl.	Gum	Methanol	5.5	9.9
Croton gratissimus var. *gratissimus*	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
Xanthium Strumarium subsp. *brasilicum* (Vell.) O.Bolòs & Vigo (syn. *Xanthium brasilicum* Vell.)	Leaves	Petroleum ether	13.3	28.8

[a]N/A = Not Achievable

2.3.5 Extracts with selective anti-trypanosomal 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* (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 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, Table S1).

2.3.6 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).

2.3.7 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 (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).

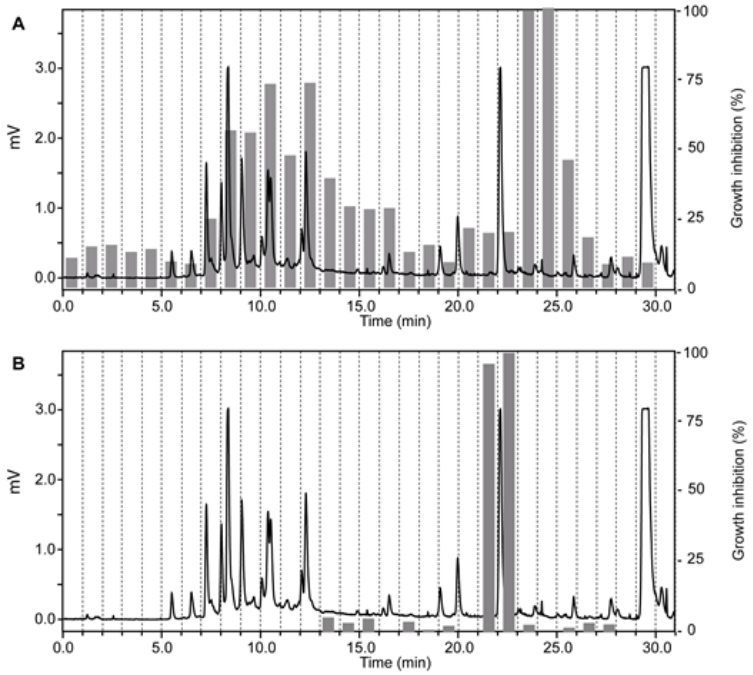

Figure 3. HPLC-based activity profiling of an ethyl acetate fraction from leaves of *Ziziphus spina-christi* (L.) Desf. The ELSD chromatogram of the fraction separation on an analytical RP-HPLC column is shown. Activity of the one minute micro-fractions is indicated for trypanocidal **A**) and antileishmanial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

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).

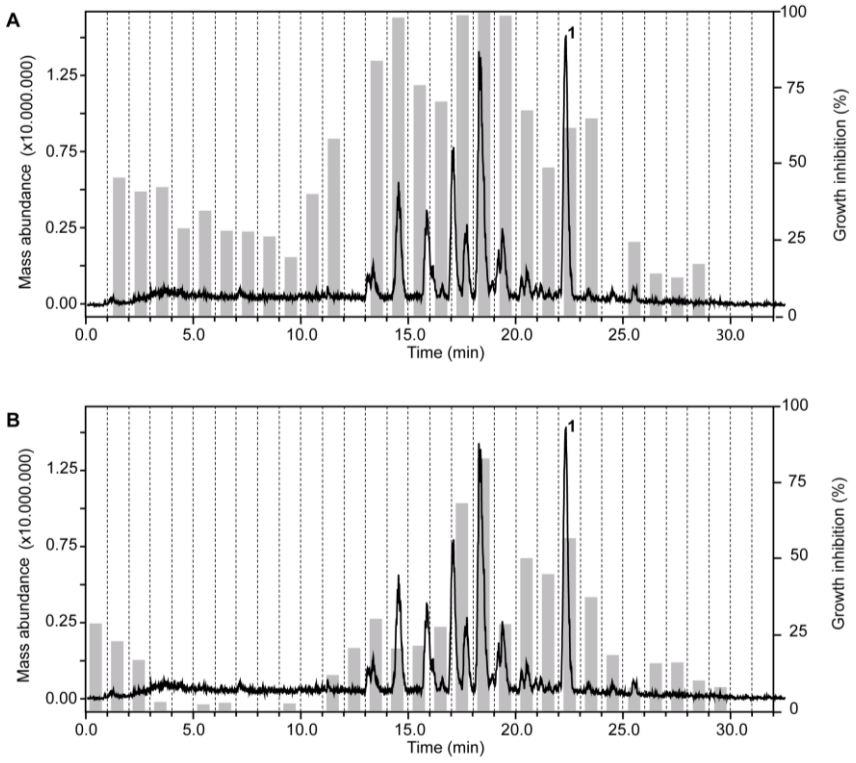

Figure 4. HPLC-ESIMS (base peak chromatogram) and activity profile of a chloroform fraction from leaves of *Guiera senegalensis* J.F.Gmel. Similar time window for trypanocidal (**A**) and antiplasmodial activity (**B**) were found. Peak **1** refers to guieranone A.

2.3.8 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 [73]. The chloroform fraction of *Tephrosia apollinea* (Delile) DC. leaves was active against three parasites (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 [74], of known antioxidant and anti-inflammatory activity.

The ethyl acetate fraction of the leaves, roots and seeds of *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 *A. Leiocarpa* [50,75]. Ellagic acid has been previously shown to possess antiplasmodial activity [76,92] which has been attributed to the inhibition of beta-haematin formation in the parasite [77]. The antiplasmodial activity of quercetin [78] has been associated with the inhibition of a parasite protein kinase [79].

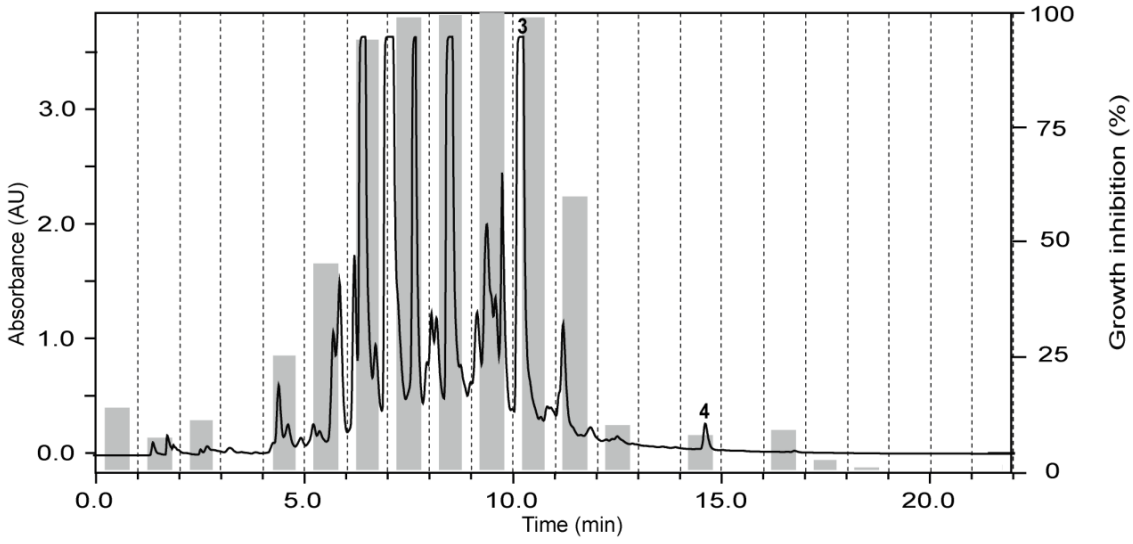

Figure 5. HPLC-PDA antiplasmodial activity profiling of the ethyl acetate fraction of the leaves of *Anogeissus Leiocarpa* (DC.) Guill. & Perr. recorded at 254 nm. Peaks **3** and **4** refer to ellagic acid and quercetin, respectively.

The leaf extract of *Acacia nilotica* (L.) Delile 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 nm and 280 nm in the UV spectra were detected in the active time window. These peaks corresponded to catechin [80] and epicatechin gallate [81], respectively. The occurrence of these compounds in *A. nilotica* has been reported [82,83]. 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 [84]. 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 [85]. However, we cannot exclude that ethyl gallate was formed from gallic acid during ethanol extraction.

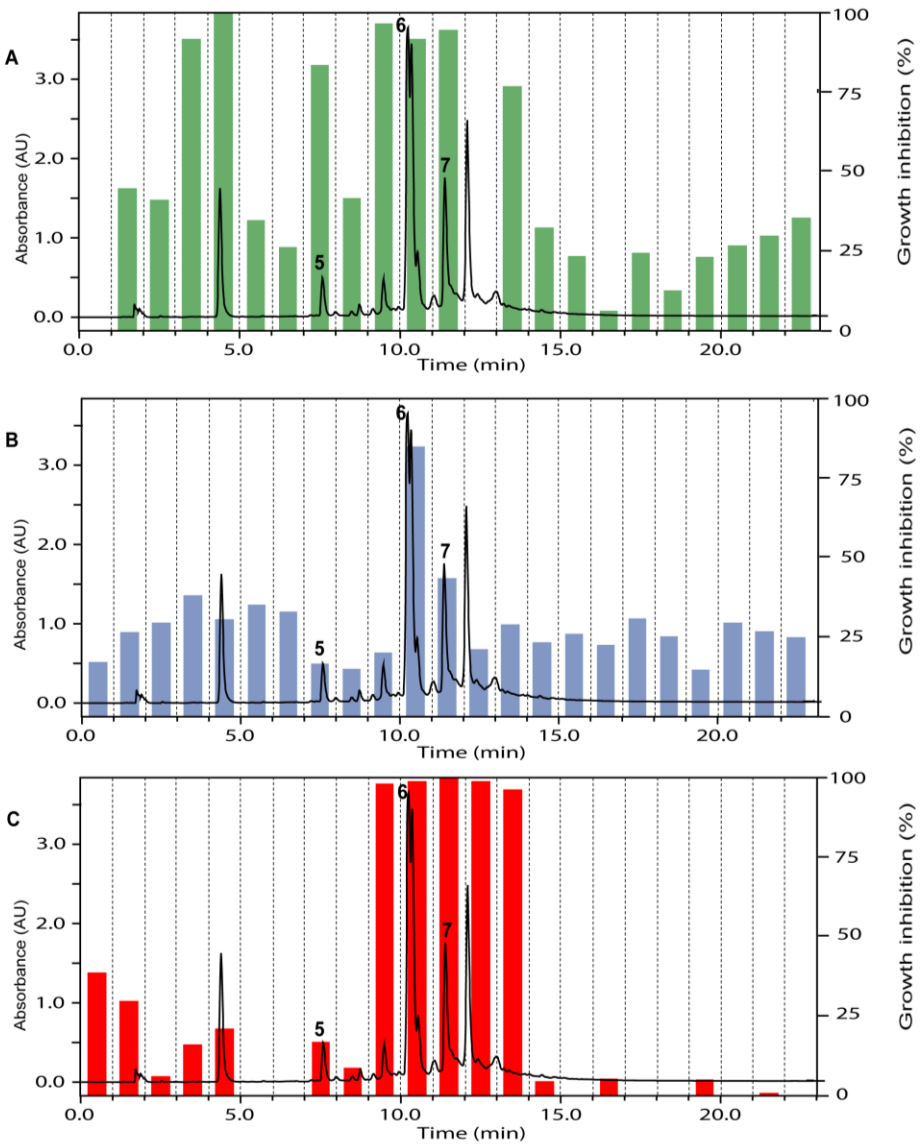

Figure 6. HPLC-UV trace of the ethyl acetate fraction of *Acacia nilotica* (L.) Delile leaves detected at 254 nm, and the % of inhibition of the one minute micro-fractions against *Trypanosoma brucei rhodesiense* (**A**), *Leishmania donovani* (**B**), and *Plasmodium falciparum* (**C**). Peaks **5**-**7** refer to catechin, ethyl gallate, and epicatechin gallate, respectively.

2.4 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 trypanosomatid parasites. This somehow surprising result is in agreement with previous screening campaigns reports [86–88]. The lack of overlap between activity against *T. cruzi* and *L. donovani* is not unusual and has been documented previously [89,90]. 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*., Anogeissus Leiocarpa* (DC.) Guill. & Perr., *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.

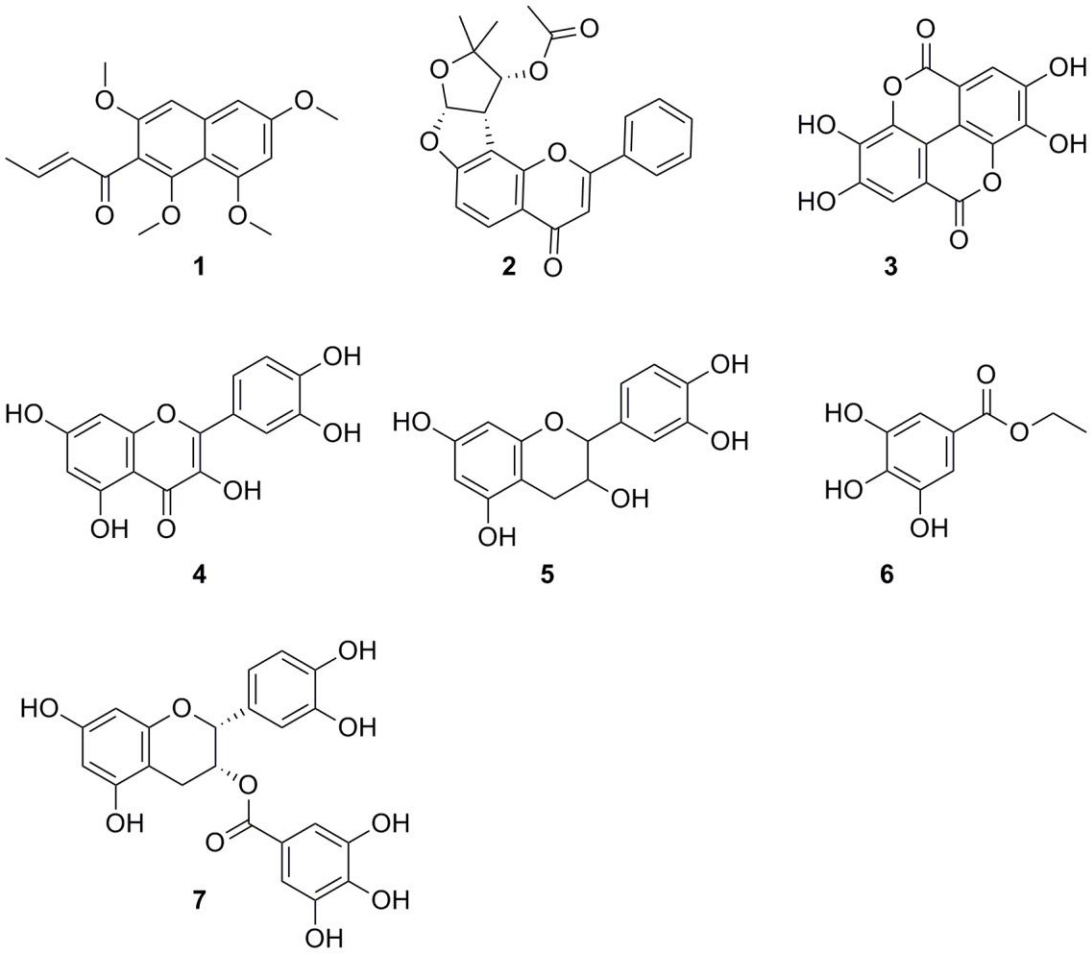

Figure 7. Chemical structures of compounds identified by dereplication. Guieranone A (**1**), pseudosemiglabrin (**2**), ellagic acid (**3**), quercetin (**4**), catechin (**5**), ethyl gallate (**6**), and epicatechin gallate (**7**).

Our findings corroborate previously reported activities of some plants, e.g. for *Z. spina-christi* [91], *A. Leiocarpa* [33,60], *G. senegalensis* [61], *Terminalia spp*. and *X. strumarium* [72,93]. Antiprotozoal activities of some other plants are reported here for the first time, e.g. the antitrypanosomal activity of *Cuscuta hyalina , Combretum indicum ,* and

Croton 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 *A. Leiocarpa*, and catechin (**5**), ethyl gallate (**6**), and epicatechin gallate (**7**) from *A. nilotica*. HPLC-based activity 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.

2.5 Material and Methods

Preparation of a library of plant extracts

A total of 62 plants reputed as antiparasitic in traditional medicine in Sudan were sollicited 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 ionisation mass spectrometer (ESIMS) (LCMS-8030, Shimadzu). LabSolutions software was used for data acquisition and processing. The separation was performed on a C_{18} 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 [94,95].

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 [96]. 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, 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 10^4 *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. 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 [97]. 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 uL 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, 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 was 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 [98]. 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 [99]. 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 2.5x CPRG/Nonidet solution was added to all wells. A colour 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 µg /mL 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 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 (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* [100] 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.

2.6 References

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2.7 Supporting Information:

Table S1. Screening results of protozoan activity of plant extracts at two concentrations, 10 µg/ml and 2 µg/ml.

Plant species	Family	Part	Fraction	*T. b. rhodesiense*		*L. donovani*		*T. cruzi*		*P. falciparum*	
				% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml
Abutilon pannosum var. *figarianum* (Webb) Verdc.	Malvaceae	Leaves	Crude	50.5	23.4	0.0	0.0	22.3	19.1	48.3	9.5
			Petroleum ether	75.7	42.1	106.0	37.1	19.8	9.6	88.1	9.5
			Chloroform	83.9	42.7	42.1	22.4	4.5	1.2	77.2	13.6
			Ethyl acetate	62.4	39.9	52.1	27.1	0.9	10.5	89.2	48.7
			Water residue	28.0	26.6	16.0	25.1	5.4	4.5	19.3	7.6
Acacia nilotica (L.) Delile	Fabaceae	Flowers	Crude	102.4	83.9	20.9	29.8	11.8	2.0	99.6	76.1
			Petroleum ether	89.5	27.8	48.2	31.6	0.0	0.8	95.3	10.6
			Chloroform	103.1	79.3	30.2	21.4	2.2	2.1	97.7	42.6
			Ethyl acetate	44.1	42.3	10.3	19.6	11.2	8.7	44.4	32.7
			Water residue	72.2	72.7	7.9	7.3	2.5	4.2	72.4	67.0
		Leaves	Crude	99.3	83.2	3.1	5.4	20.0	6.8	99.0	68.2
			Petroleum ether	96.7	67.5	91.0	0.0	45.4	17.2	81.5	7.6
			Chloroform	102.9	104.6	83.8	43.1	66.7	13.6	103.8	97.3
			Ethyl acetate	101.5	80.6	24.9	26.4	3.8	12.8	103.2	65.1
			Water residue	74.9	11.9	33.3	31.7	0.1	9.8	24.8	5.4
Ambrosia maritima L.	Asteraceae	Leaves	Crude	29.6	9.7	35.2	14.8	9.9	5.8	21.4	5.9
			Petroleum ether	60.7	21.4	107.0	36.6	13.1	0.0	84.5	5.4
			Chloroform	58.9	18.5	91.8	36.9	6.2	8.7	54.0	5.7
			Ethyl acetate	101.1	32.5	91.4	37.1	13.2	11.2	36.5	11.6
			Water residue	7.6	4.2	27.6	29.1	10.2	7.9	15.8	3.2

Plant species	Family	Part	Fraction	*T. b. rhodesiense*		*L. donovani*		*T. cruzi*		*P. falciparum*	
				% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml
Ampelozizyphus amazonicus Ducke	Rhamnaceae	Leaves	Ethyl acetate	62.6	21.7	105.5	85.1	15.5	12.6	99.7	13.4
			Butanol	55.5	23.9	38.5	18.1	0.9	4.4	48.0	3.3
Anethum graveolens L.	Apiaceae	Leaves	Crude	8.9	36.2	21.5	13.3	2.2	0.0	0.0	0.0
			Hexane	4.9	0.0	19.9	26.4	5.2	5.9	2.1	0.0
			Chloroform	1.4	1.7	11.7	12.7	9.0	3.3	3.1	0.0
			Ethyl acetate	38.9	22.1	44.3	34.1	2.1	0.0	10.5	13.8
Annona muricata L.	Annonaceae	Leaves	Crude	90.8	28.2	77.9	1.1	81.7	72.7	94.9	62.9
			Petroleum ether	93.8	28.3	99.6	44.2	82.7	30.9	102.4	31.4
			Chloroform	76.8	25.6	98.6	34.7	81.1	55.2	98.1	69.1
			Ethyl acetate	55.4	27.1	71.6	39.9	63.9	40.5	89.3	44.6
			Water residue	13.8	12.3	41.4	32.4	3.8	7.1	43.3	5.0
Anogeissus Leiocarpa (DC.) Guill. & Perr.	Combretaceae	Bark	Ethyl acetate	99.8	69.3	14.3	0.0	0.9	3.1	101.8	45.8
		Leaves	Chloroform	100.0	68.5	94.2	12.1	11.6	0.0	102.1	27.9
			Ethyl acetate	100.2	75.0	68.2	8.8	17.5	2.2	102.9	103.4
		Root	Ethyl acetate	100.8	64.9	29.2	3.8	12.3	4.2	103.2	67.3
		Seeds	Chloroform	99.7	50.2	94.1	46.0	15.3	6.0	99.5	34.8
			Ethyl acetate	98.6	73.3	90.4	40.5	18.1	4.3	102.5	106.4
		Wood	Ethyl acetate	97.0	76.8	81.2	0.0	18.0	12.3	102.3	98.2
Argemone mexicana L.	Papaveraceae	Leaves	Crude	33.4	11.1	76.2	33.8	0.0	6.9	97.6	7.1
			Petroleum ether	65.1	9.7	107.0	32.1	7.0	7.6	98.3	6.8
			Chloroform	75.2	28.0	108.0	37.1	11.3	8.7	98.2	82.6
			Ethyl acetate	56.5	14.5	106.6	40.4	2.8	9.5	98.4	7.9
			Water residue	2.1	3.1	24.4	22.6	6.7	9.9	62.2	4.4
		Bark	Crude	26.5	9.4	27.6	23.9	9.1	1.9	89.7	15.1
Aristolochia bracteolata Lam.	Aristolochiaceae	Root	Ethyl acetate	103.1	44.4	88.2	36.7	40.0	12.7	76.0	20.4

Plant species	Family	Part	Fraction	*T. b. rhodesiense*		*L. donovani*		*T. cruzi*		*P. falciparum*	
				% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml
Azadirachta indica A.Juss.	Meliaceae	Leaves	Oil	7.3	2.4	13.8	17.9	39.3	6.0	71.2	6.1
Bathysa australis (A.St.-Hil.) K.Schum.	Rubiaceae	Leaves	Hexane	91.3	58.4	83.8	1.0	36.7	15.4	82.6	10.6
			Methylene chloride	100.2	77.1	96.5	46.2	33.7	5.4	101.1	17.0
			Ethyl acetate	44.6	16.8	36.5	32.2	10.8	14.2	64.5	4.6
			Butanol	41.3	10.5	39.2	32.1	4.2	5.1	34.0	5.0
Bertholletia excelsa Bonpl.	Lecythidaceae	Fruit (endocarp)	Hexane	83.7	21.3	69.3	34.4	5.1	9.4	98.6	14.4
		Bark (trunk)	Crude	104.6	36.2	88.2	37.8	13.2	6.5	97.8	100.3
		Seeds (tugument)	Crude	81.3	19.9	40.5	42.5	2.4	9.0	46.3	5.3
Boswellia papyrifera (Caill. ex Delile) Hochst	Burseraceae	Gum resin	Crude	23.7	4.4	104.8	47.0	4.8	3.5	91.8	15.4
			Petroleum ether	71.6	8.2	116.8	76.9	9.8	0.8	100.0	41.5
			Chloroform	29.0	9.7	117.1	49.9	6.2	2.5	94.1	9.5
			Ethyl acetate	10.3	7.0	46.4	40.5	7.0	5.2	0.2	3.9
			Water residue	19.8	11.4	73.0	35.8	8.7	5.1	83.6	0.0
			Oil	10.8	2.8	49.3	33.0	3.0	2.9	2.4	12.2
Cardiospermum halicacabum L.	Sapindaceaee	Leaves	Crude	58.4	18.4	56.1	36.2	7.8	9.3	32.8	5.0
			Petroleum ether	42.0	7.2	79.7	35.4	4.9	6.1	35.0	5.7
			Chloroform	51.9	18.5	64.6	29.3	11.7	12.3	51.7	7.4
			Ethyl acetate	28.1	14.9	30.6	15.2	24.2	10.9	83.2	13.8
			Water residue	0.5	2.2	0.0	0.0	7.7	14.9	10.7	0.1
Combretum glutinosum Perr. ex DC.	Combretaceae	Seeds	Chloroform	51.6	41.7	12.0	24.4	9.6	7.1	18.3	9.0
			Ethyl acetate	102.6	85.7	96.5	35.2	13.6	2.9	103.5	103.5
		Wood	Chloroform	103.9	60.5	106.3	53.6	7.7	6.0	104.1	85.3
			Ethyl acetate	103.2	82.6	93.3	53.6	9.7	9.6	102.4	99.6

Plant species	Family	Part	Fraction	*T. b. rhodesiense*		*L. donovani*		*T. cruzi*		*P. falciparum*	
				% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml
		Bark	Ethyl acetate	86.2	75.0	95.3	24.3	12.6	1.8	102.9	99.4
Combretum hartmannianum Schweinf.	Combretaceae	Seeds	Chloroform	101.4	84.0	64.7	10.7	18.7	4.6	102.3	68.1
			Ethyl acetate	101.0	85.9	94.8	63.0	20.1	2.1	102.9	97.4
		Wood	Chloroform	94.6	49.1	101.1	42.1	52.8	22.8	102.4	32.6
			Ethyl acetate	95.1	57.0	83.4	22.3	33.2	5.3	102.5	61.2
Combretum indicum (L.) DeFilipps (syn. Quisqualis indica L.)	Combretaceae	Leaves	Chloroform	84.3	53.2	80.8	36.9	3.2	16.4	72.3	15.5
			Ethyl acetate	80.8	21.4	52.8	29.7	12.0	11.4	92.7	27.0
		Flowers	Chloroform	34.2	12.7	17.2	4.0	26.9	1.0	10.3	0.7
			Ethyl acetate	98.6	80.3	100.5	4.0	37.1	1.0	98.5	91.3
Commiphora myrrha (Nees) Engl.	Burseraceae	Gum resin	Methanol	85.0	99.1	103.2	104.4	104.2	95.0	98.3	94.8
			Oil	83.3	97.9	101.5	103.8	101.0	76.3	97.6	97.2
Croton gratissimus var. *gratissimus*	Euphorbiaceae	Fruits	Crude	49.6	45.6	92.7	40.2	2.9	3.6	88.8	9.3
			Petroleum ether	99.8	74.8	100.8	103.0	88.5	14.6	103.0	103.9
			Chloroform	63.9	12.5	107.1	55.9	38.4	11.0	97.8	24.9
			Ethyl acetate	100.1	32.9	25.3	17.8	9.4	0.1	76.8	32.5
			Water residue	21.0	2.2	20.9	15.9	6.9	10.3	18.1	0.2
		Leaves	Petroleum ether	75.1	9.7	8.6	0.0	47.8	6.9	57.4	0.0
			Chloroform	85.1	16.0	96.1	9.3	41.9	16.1	43.7	13.0
			Ethyl acetate	34.9	6.5	55.2	20.9	10.9	7.5	57.9	4.9
Curcuma longa L.	Zingiberaceae	Rhizome	Crude	29.3	14.3	54.1	38.3	3.8	0.0	1.6	0.0
			Petroleum ether	11.3	6.9	46.2	35.8	6.3	0.0	1.7	3.3
			Chloroform	54.2	26.7	88.0	43.5	6.3	4.6	92.7	7.7
			Ethyl acetate	33.0	13.7	39.0	27.1	4.4	5.2	1.2	1.9
			Water residue	16.3	11.3	46.3	35.4	0.0	0.0	6.4	7.5

Plant species	Family	Part	Fraction	*T. b. rhodesiense*		*L. donovani*		*T. cruzi*		*P. falciparum*	
				% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml
Cuscuta hyalina Roth ex Schult.	Convolvulaceae	Stem	Crude	66.9	4.7	2.5	0.0	59.1	42.5	58.8	7.7
			Petroleum ether	55.5	21.8	85.2	33.7	27.1	6.5	85.5	6.3
			Chloroform	17.1	15.7	65.7	31.2	67.8	7.8	103.7	4.8
			Ethyl acetate	95.3	24.9	58.5	36.9	50.7	32.6	100.6	7.2
			Water residue	3.4	10.8	39.3	27.6	9.6	12.3	29.9	3.5
Cymbopogon citratus (DC.) Stapf	Poaceae	Leaves	Crude	24.1	15.4	43.5	32.4	0.3	0.0	12.6	5.4
			Chloroform	37.2	25.6	51.0	35.6	0.5	1.4	96.2	19.9
			Ethyl acetate	39.9	16.0	73.2	38.0	2.4	0.0	85.0	25.7
			Water residue	16.8	3.6	56.5	37.7	3.5	0.0	0.0	2.0
			Oil	12.5	5.5	62.4	28.7	1.9	0.0	0.6	5.4
Cyperus rotundus L.	Cyperaceae	Rhizome	Crude	19.0	10.2	63.6	35.5	7.9	11.9	44.9	4.5
			Petroleum ether	72.6	17.8	106.0	82.0	73.4	17.4	103.5	50.7
			Chloroform	55.8	18.8	106.6	45.4	15.1	12.2	100.7	40.5
			Ethyl acetate	29.4	4.5	29.6	33.1	7.3	14.5	34.1	3.3
			Water residue	0.3	0.9	27.8	25.3	4.2	14.1	17.8	5.4
Eucalyptus camaldulensis Dehnh.	Myrtaceae	Leaves	Oil	8.9	4.5	57.5	32.2	0.0	1.7	5.3	3.5
Grewia tenax (Forssk.) Fiori	Malvaceae	Bark	Methanol	79.1	45.7	68.7	45.8	6.6	9.4	44.7	14.7
		Leaves	Petroleum ether	84.5	42.0	93.6	35.0	9.8	4.7	94.0	11.1
			Chloroform	84.7	29.7	41.3	34.2	5.3	8.4	81.5	9.3
			Ethyl acetate	65.9	22.5	50.1	36.6	14.7	6.3	67.2	33.8
		Root	Methanol	33.8	18.8	15.7	0.0	17.4	6.9	17.3	6.5
		Stem	Methanol	55.1	23.9	42.1	39.0	7.8	5.1	27.9	12.3
Guiera sengalensis J.F.Gmel.	Combretaceae	Leaves	Crude	101.2	46.2	58.8	16.7	8.6	14.5	84.0	5.1
			Petroleum ether	95.8	66.7	96.8	49.4	57.3	16.4	100.1	39.1

Plant species	Family	Part	Fraction	*T. b. rhodesiense*		*L. donovani*		*T. cruzi*		*P. falciparum*	
				% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml
			Chloroform	104.3	91.2	109.1	54.3	66.9	11.4	103.0	84.9
			Ethyl acetate	103.1	79.5	108.5	34.3	46.2	15.1	100.9	67.9
			Water residue	76.7	33.1	31.4	29.1	0.6	6.0	80.2	3.4
Haplophyllum tuberculatum (Forssk.) A.Juss.	Rutaceae	Root	Crude	23.8	16.1	39.5	28.5	12.9	9.8	35.9	7.2
			Petroleum ether	71.1	26.0	105.0	61.2	26.8	8.3	99.2	36.8
			Chloroform	62.1	23.7	106.4	40.0	67.4	15.7	102.1	25.3
			Ethyl acetate	35.1	16.7	40.8	30.3	8.2	11.6	39.2	2.7
			Water residue	16.5	3.7	38.7	29.2	7.1	5.9	20.6	4.4
Hyptis monticola Mart. ex Benth.	Lamiaceae	Leaves	Crude	0.0	0.0	23.1	22.1	10.2	14.3	22.3	2.7
		Flowers	Crude	74.6	20.9	103.0	60.4	35.6	14.4	100.3	20.7
Jatropha curcas L.	Euphorbiaceae	Leaves	Crude	32.9	17.1	61.9	43.2	2.8	2.6	0.0	0.0
			Petroleum ether	21.3	4.5	45.8	21.2	6.2	4.7	21.7	0.0
			Chloroform	32.2	18.7	47.4	30.0	3.9	0.0	22.7	11.0
			Ethyl acetate	19.5	8.5	47.2	37.2	4.2	0.0	4.3	3.0
			Water residue	16.9	7.1	49.8	35.1	0.0	0.0	1.6	0.0
Lantana fucata Lindl.	Verbenaceae	Aerial parts	Crude	97.8	66.2	106.9	103.6	90.9	26.2	101.9	70.1
Lantana trifolia L.	Verbenaceae	Branches	Butanol	71.5	11.8	11.6	15.1	5.3	8.2	23.0	0.0
Lippia lacunosa Mart. & Schauer	Verbenaceae	Leaves	Crude	81.4	20.9	62.2	2.5	3.1	9.2	46.5	5.5
			Ethyl acetate	96.3	66.2	15.5	3.6	27.4	13.3	30.7	5.6
			Butanol	71.0	11.2	6.1	0.0	2.2	13.0	31.1	2.6
Lippia origanoides Kunth	Verbenaceae	Leaves	Crude	99.5	67.1	105.2	79.9	35.8	7.1	103.2	26.2
Lupinus albus subsp. *graecus* (Boiss. & Spruner) Franco & P.Silva (syn. *Lupinus termis* Forssk.)	Leguminosae	Seeds	Crude	17.8	4.0	2.0	1.0	4.7	3.4	28.5	3.5
			Petroleum ether	35.0	14.4	7.4	3.2	6.3	8.2	24.7	5.0
			Chloroform	35.3	24.3	31.5	27.7	2.8	5.9	24.9	0.0
			Ethyl acetate	57.9	22.6	0.0	0.0	23.8	18.9	41.2	20.4

Plant species	Family	Part	Fraction	*T. b. rhodesiense*		*L. donovani*		*T. cruzi*		*P. falciparum*	
				% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml
			Water residue	44.2	21.9	22.2	20.2	9.6	5.0	32.4	39.6
Mangifera indica L.	Anacardiaceae	Peels	Crude	18.0	8.8	48.2	33.9	3.9	0.0	5.3	0.8
			Petroleum ether	11.7	6.4	52.8	37.2	6.3	0.0	0.0	0.0
			Chloroform	43.4	15.2	52.9	35.0	6.5	0.0	3.4	0.0
			Ethyl acetate	101.9	19.9	44.6	28.8	6.0	1.1	67.1	1.5
			Water residue	8.4	2.3	32.8	22.3	11.8	0.2	0.0	0.0
Matricaria chamomilla L.	Compositae	Flower	Crude	41.4	32.0	37.7	27.8	17.0	1.9	29.1	6.1
			Petroleum ether	75.8	24.6	106.6	22.1	10.2	3.7	91.1	5.0
			Chloroform	82.5	36.5	83.3	26.5	6.7	1.6	94.6	28.5
			Ethyl acetate	79.1	29.5	69.8	30.3	12.6	0.0	53.4	9.0
			Water residue	24.4	20.4	31.0	17.1	14.3	0.0	11.8	4.7
Moringa oleifera Lam.	Moringaceae	Leaves	Crude	36.2	17.0	43.9	31.4	1.7	0.0	48.6	7.3
			Hexane	12.2	5.3	45.5	35.0	0.9	0.0	1.5	7.6
			Chloroform	22.1	11.8	48.0	35.8	0.0	0.0	7.2	11.1
			Ethyl acetate	33.1	20.9	57.5	43.3	0.0	0.0	2.2	9.7
			Water residue	23.7	8.6	54.3	38.2	4.1	0.0	1.4	3.2
Nauclea latifolia Sm.	Rubiaceae	Unprocessed Fruit	Crude	61.9	21.0	26.2	20.2	10.7	1.2	17.5	5.1
			Petroleum ether	68.8	21.0	34.0	3.4	8.3	0.0	30.5	5.6
			Chloroform	103.1	57.0	91.7	31.2	56.0	3.2	83.5	30.9
			Ethyl acetate	70.9	6.0	0.0	0.0	18.6	6.9	43.4	6.9
			Water residue	26.1	12.5	31.3	26.6	3.4	4.3	33.4	39.7
		Root Bark	Crude	53.1	19.1	41.1	34.2	15.5	6.3	38.9	5.0
			Petroleum ether	64.1	15.6	76.3	33.9	33.7	6.4	66.0	8.3
			Chloroform	86.0	35.5	95.9	41.6	18.2	5.5	93.6	19.3
			Ethyl acetate	87.5	47.1	43.9	25.1	11.8	5.2	61.9	18.5

Plant species	Family	Part	Fraction	*T. b. rhodesiense*		*L. donovani*		*T. cruzi*		*P. falciparum*	
				% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml
			Water residue	24.9	7.0	37.5	25.9	8.8	2.7	21.2	3.4
		Processed Fruit	Crude	47.6	15.4	43.0	29.3	8.9	8.2	35.3	12.7
			Petroleum ether	26.0	7.3	36.9	23.6	12.5	2.0	23.6	1.5
			Chloroform	51.8	14.2	40.3	19.7	7.6	0.0	35.1	7.2
			Ethyl acetate	45.5	4.2	25.4	14.9	4.0	0.1	21.9	8.0
			Water residue	81.1	66.0	0.0	0.0	16.9	9.1	18.2	9.8
Nigella sativa L.	Ranunculaceae	Seeds	Oil	6.5	13.9	36.6	21.0	3.9	0.0	9.9	20.7
Operculina macrocarpa (L.) Urb.	Convolvulaceae	Roots	Crude	43.9	20.5	61.1	28.4	43.3	7.6	102.8	43.6
Opopanax hispidus (Friv.) Griseb.	Apiaceae	Fruits	Oil	59.7	6.5	57.8	30.1	5.5	5.1	0.0	0.0
Piper cubeba L. f.	Piperaceae	Fruits	Crude	79.6	16.2	33.0	35.3	12.9	3.1	53.7	9.9
			Petroleum ether	71.1	16.2	103.4	29.5	5.2	3.3	45.0	10.9
			Chloroform	86.0	29.2	64.0	28.0	9.6	5.3	101.2	21.3
			Ethyl acetate	101.9	74.2	47.4	26.1	13.8	5.0	103.2	81.1
			Water residue	33.0	3.0	36.5	24.1	10.9	13.6	31.5	7.6
Portulaca oleracea L.	Portulacaceae	Aerial part	Crude	29.1	9.1	36.5	23.6	8.8	2.2	29.1	12.8
			Petroleum ether	69.0	8.5	103.2	26.4	15.2	19.9	58.9	11.1
			Chloroform	81.2	13.9	56.2	22.2	7.6	1.1	73.7	7.7
			Ethyl acetate	77.4	7.6	23.9	19.6	8.1	0.9	28.1	3.3
			Water residue	8.2	0.4	0.0	0.0	26.1	11.0	24.2	7.5
Prosopis chilensis (Molina) Stuntz	Leguminosae	Leaves	Crude	102.5	103.5	36.7	29.8	29.8	2.3	99.6	103.5
			Hexane	102.9	0.8	77.5	36.1	17.2	5.5	95.8	98.0
			Chloroform	104.5	102.8	49.2	36.3	25.6	4.6	103.4	98.2
			Ethyl acetate	8.4	7.9	51.9	30.7	11.7	3.5	32.0	6.3
Prosopis juliflora (Sw.) DC.	Leguminosae	Leaves	Crude	103.0	102.3	40.1	21.9	14.9	28.2	101.1	105.0

Plant species	Family	Part	Fraction	*T. b. rhodesiense*		*L. donovani*		*T. cruzi*		*P. falciparum*	
				% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml
Rollinia sylvatica (A. St.-Hil.) Martius	Annonaceae	Leaves	Crude	85.3	20.3	66.2	26.4	50.9	9.8	90.5	47.5
Saussurea costus (Falc.) Lipsch.	Compositae	Leaves	Methanol	99.4	19.6	105.2	36.5	26.1	7.4	45.3	5.0
Senna occidentalis (L.) Link (syn. Cassia occidentalis L.)	Leguminosae	Aerial part	Crude	25.8	15.5	30.4	31.1	4.9	8.9	27.4	5.1
			Petroleum ether	54.4	17.3	105.6	35.9	19.0	6.7	91.6	3.0
			Chloroform	40.4	46.0	58.2	34.4	12.1	9.5	53.7	6.1
			Ethyl acetate	21.9	15.1	51.9	37.1	8.5	7.4	25.5	5.1
			Water residue	8.3	8.0	45.7	32.6	4.4	8.7	16.0	2.0
Solanum aethiopicum L.	Solanaceae	Fruits	Butanol	15.2	11.7	36.2	24.8	17.6	7.6	26.8	4.2
Striga hermonthica (Delile) Benth.	Orobanchaceae	Stem	Crude	20.1	1.5	42.6	20.8	7.4	8.3	36.5	9.2
			Petroleum ether	78.9	12.3	102.4	24.8	12.8	0.6	99.5	36.2
			Chloroform	66.9	7.8	77.6	0.0	16.1	12.1	51.8	9.2
			Ethyl acetate	85.9	22.4	88.3	30.7	16.3	6.5	36.9	5.2
			Water residue	13.8	4.9	42.8	34.2	7.7	1.9	20.1	2.4
Struthanthus concinnus Mart.	Loranthaceae	Branches	Ethyl acetate	89.6	38.7	80.4	32.4	10.6	5.2	102.0	18.8
			Butanol	11.6	1.9	41.9	25.1	3.1	5.4	23.7	8.3
Struthanthus marginatus (Desr.) G.Don	Loranthaceae	Branches	Ethyl acetate	17.4	10.3	51.9	31.5	7.9	5.9	31.8	2.3
Tephrosia apollinea (Delile) DC.	Leguminosae	Leaves	Crude	44.5	17.1	41.4	23.4	18.2	3.8	46.7	10.7
			Petroleum ether	80.8	28.8	78.6	24.9	79.6	19.4	91.2	14.9
			Chloroform	96.8	31.4	99.8	23.2	69.9	6.5	100.0	30.1
			Ethyl acetate	43.8	15.6	38.6	20.3	9.7	5.9	87.9	10.4
			Water residue	94.8	41.3	91.6	0.0	83.2	12.0	66.1	38.7
Terminalia brownii Fresen	Combretaceae	Seeds	Ethyl acetate	103.0	52.4	88.0	12.3	5.5	1.1	97.5	92.9

Plant species	Family	Part	Fraction	*T. b. rhodesiense*		*L. donovani*		*T. cruzi*		*P. falciparum*	
				% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml	% inhib. 10 ug/ml	% inhib. 2 ug/ml
Terminalia catappa L.	Combretaceae	Leaves	Chloroform	98.4	80.1	61.9	27.5	12.5	4.8	78.1	17.4
			Ethyl acetate	102.1	80.5	55.2	32.2	9.1	5.9	101.6	58.2
Terminalia laxiflora Engl.	Combretaceae	Seeds	Chloroform	72.3	33.8	39.8	40.3	9.3	7.7	44.7	12.7
			Ethyl acetate	102.1	77.5	96.6	45.2	17.9	11.2	99.8	93.4
		Bark	Chloroform	86.4	57.8	100.3	49.9	16.0	3.6	100.3	19.3
			Ethyl acetate	102.5	82.6	73.6	29.6	11.3	5.9	99.8	68.6
Tinospora bakis (A.Rich.) Miers	Menispermaceae	Root	Chloroform	17.7	4.2	26.3	20.0	7.0	7.4	17.3	4.6
			Methanol: Acetone (7:3)	28.5	18.8	44.9	20.9	1.2	0.0	89.1	13.4
Typha angustifolia L.	Typhaceae	Stem	Crude	57.0	16.6	98.9	26.5	8.4	3.7	64.2	6.7
			Petroleum ether	27.2	7.0	104.5	43.4	51.7	6.7	23.4	5.0
			Chloroform	78.2	19.0	86.0	33.1	23.0	9.0	93.3	24.8
			Ethyl acetate	41.3	11.7	48.7	28.7	7.8	4.1	61.2	17.2
			Water residue	30.1	4.5	41.7	25.0	13.8	5.5	46.8	6.5
Vitex cooperi Standl.	Lamiaceae	Barks	Methanol	1.0	1.9	38.6	23.3	16.4	10.2	29.5	6.2
Vitex polygama Cham.	Lamiaceae	Leaves	Water residue	16.8	5.3	37.1	23.4	2.3	9.0	37.2	8.9
Xanthium Strumarium subsp. *brasilicum* (Vell.) O.Bolòs & Vigo (syn. *Xanthium brasilicum* Vell.)	Compositae	Leaves	Crude	70.5	5.4	36.6	18.4	14.9	11.5	41.3	9.3
			Petroleum ether	102.8	16.2	98.8	23.1	45.9	6.2	100.3	41.1
			Chloroform	101.1	20.0	52.1	15.1	8.4	4.0	85.8	10.2
			Ethyl acetate	94.7	14.3	58.5	0.0	35.8	5.7	62.5	8.8
			Water residue	9.4	5.6	25.5	21.5	11.7	4.5	30.3	0.0
Ziziphus spina-christi (L.) Desf.	Rhamnaceae	Leaves	Chloroform	96.4	83.1	99.2	6.4	48.1	8.3	100.9	12.4
			Ethyl acetate	101.5	102.8	99.0	72.8	65.5	13.5	101.5	102.6
		Root	Chloroform	100.8	77.8	99.6	56.1	70.3	6.4	103.1	106.2

3. HPLC-Based Activity Profiling for Antiprotozoal Compounds in *Croton gratissimus* and *Cuscuta hyalina*

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Published in

Frontiers in Pharmacology-Ethnopharmacology 2020;11:1246.

doi:10.3389/fphar.2020.01246

I have performed the extraction of plant material, microfractionation, compounds isolation and some of their structure elucidation. Structure elucidation for most of the compounds was done by Ombeline Danton. Compounds testing were done by the PCU at SwissTPH. I prepared the figures and wrote the manuscript.

3.1 Abstract

In a screening of Sudanese medicinal plants for antiprotozoal activity, the chloroform fraction obtained by liquid-liquid partitioning from ethanolic extracts of fruits of *Croton gratissimus* var. *gratissimus* and stems of *Cuscuta hyalina* Roth ex Schult. exhibited *in vitro* activity against axenically grown *Leishmania donovani* amastigotes. This antileishmanial activity was localized by HPLC-based activity profiling. Targeted preparative isolation afforded flavonoids **1**-**6**, 3-methoxy-4-hydroxybenzoic acid (**7**), and benzyltetrahydroisoquinoline alkaloids laudanine (**8**) and laudanosine (**9**) from *C. gratissimus*, and pinoresinol (**10**), isorhamnetin (**11**), (-)-pseudosemiglabrin (**12**), and kaempferol (**13**) from *C. hyalina.*

The antiprotozoal activity of **1-13** against *Leishmania donovani* (axenic and intracellular amastigotes), *Trypanosoma brucei rhodesiense* (bloodstream forms), and *Plasmodium falciparum* (erythrocytic stages), and cytotoxicity in L6 murine myoblast cells were determined *in vitro*. Quercetin-3,7-dimethylether (**6**) showed the highest activity against axenic *L. donovani* (IC_{50} 4.5 µM, selectivity index (SI) 12.3), *P. falciparum* (IC_{50} 7.3 µM, SI 7.6) and *T. b. rhodesiense* (IC_{50} 2.4 µM, SI 23.2). The congener ayanin (**2**) exhibited moderate antileishmanial (IC_{50} 8.2 µM, SI 12.2), antiplasmodial (IC_{50} 7.8 µM, SI 12.9) and antitrypanosomal activity (IC_{50} 11.2 µM, SI 8.9). None of the compounds showed notable activity against the intramacrophage form of *L. donovani*.

Keywords: *Croton gratissimus*, *Cuscuta hyalina*, antiprotozoal activity, HPLC-activity profiling, Flavonoids

3.2 Introduction

Parasitic protozoa are the causative agents of devastating, yet often neglected diseases. The kinetoplastids, a group of flagellated protozoa, cause neglected tropical diseases that put more than one billion people around the globe at risk [1,2]. These diseases are human African trypanosomiasis (HAT) caused by *Trypanosoma brucei* spp., Chagas' disease caused by *Trypanosoma cruzi*, and Leishmaniasis caused by *Leishmania* spp. [3]. The apicomplexan parasite *Plasmodium falciparum* is the causative agent of malaria tropica which claims more than 400,000 lives every year [4].

These infections are of high public health relevance and socio-economic impact. Most of the currently available drugs have drawbacks in terms of toxicity, limited availability of oral therapeutic dosage forms, development of resistance, or non-affordability.

Natural products have in many instances provided new leads to combat neglected tropical diseases [5]. As part of an ongoing screening project of Sudanese medicinal plants for antiprotozoal activity [6,7], the chloroform extract of *Croton gratissimus* var. *gratissimus* (Euphorbiaceae), and *Cuscuta hyalina* Roth ex Schult. (Convolvulaceae) showed promising activity against *P. falciparum* and *Leishmania donovani*.

The genus *Croton* comprises over 1300 species that are widely distributed throughout tropical and subtropical regions of the world. *Croton* species have been used traditionally in Africa, South Asia and Latin America for the treatment of infections and digestive disorders [8,9]. In Sudan, *C. gratissimus*, locally known as *Um-Geleigla*, has been used traditionally for the treatment of hypertension and malaria [10]. The main secondary metabolites include flavonoids, terpenoids and essential oil [11–13]. Previous studies have demonstrated that the roots of *C. gratissimus* possessed antiplasmodial activity *in vivo* [14]. Cembranolide diterpenes isolated from the leaves were found to be active when tested against *P. falciparum* [15].

The genus *Cuscuta* comprises over 200 species distributed worldwide. They are stem obligate holoparasitic plants possessing neither roots nor fully expanded leaves. The interaction

between parasite and host is established through haustoria [16]. Different *Cuscuta* species have been used in traditional Indian and Chinese medicine. Cytotoxic, antioxidant, and antimicrobial activities have been reported [17]. Previous phytochemical investigations of the genus *Cuscuta* identified flavonoids, lignans, alkaloids, fatty acids, and essential oil [17,18]. The phytochemistry and antiparasitic activity of *C. hyalina* has not been studied.

In an earlier screening of Sudanese medicinal plants for antiprotozoal activity, the ethanolic extracts of *Croton gratissimus* fruits and *Cuscuta hyalina* stems had been found to exhibit *in vitro* antiprotozoal activity against axenic *L. donovani* (MHOM/ET/67/L82). Subsequent liquid liquid partitioning against petroleum ether, chloroform and ethyl acetate located the activity in the chloroform portion [6]. We here report on the targeted isolation and structure elucidation of compounds responsible for the activity, and on their *in vitro* activity against *T. b. rhodesiense* (STIB 900), axenic and intramacrophage amastigotes of *L. donovani* (MHOM/ET/67/L82), and *P. falciparum* (NF54).

3.3 Results and Discussion

3.3.1 Extraction and HPLC-based Activity Profiling

The methanolic extracts of *Croton gratissimus var. gratissimus* fruits and *Cuscuta hyalina* Roth ex Schult. stems had been previously found to exhibit antiprotozoal activity [6]. The antileishmanial activity displayed by the chloroform fractions of the two plants was tracked by HPLC-based activity profiling, a procedure combining analytical separation with on-line spectroscopy and time-based microfractionation for bioactivity testing [19,20]. One-minute microfractions were collected and tested for *L. donovani* growth inhibition. The HPLC‑ESIMS (positive base peak chromatograms) trace and the corresponding antileishmanial activity profiles for *C. gratissimus* and *C. hyalina* are shown in Figures 1 and 2. Major antileishmanial activity and a series of distinct peaks in the HPLC-ESIMS trace were observed in the time window between 18 and 24 min for *C. gratissimus*, and between 13 and 17 min for *C. hyalina*.

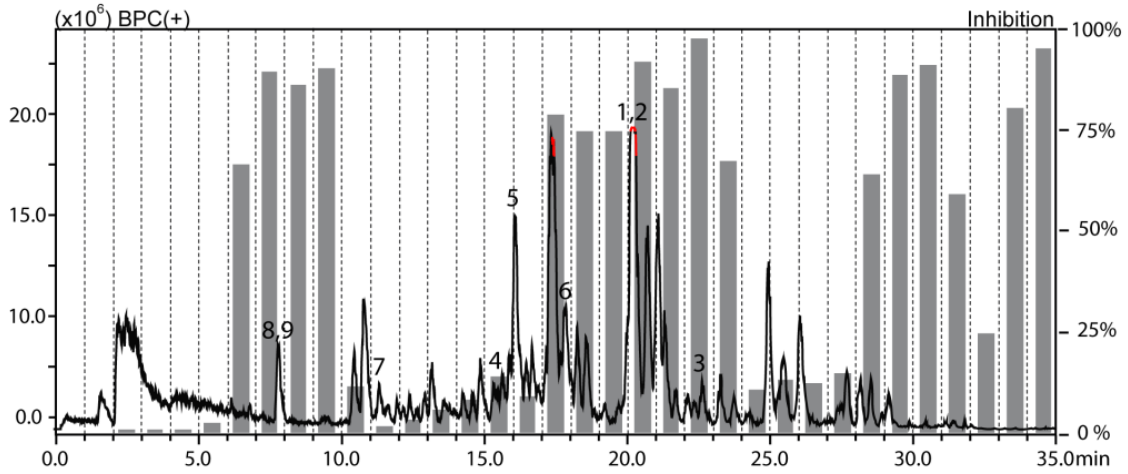

Figure 1. HPLC-based activity profiling of the chloroform fraction of *Croton gratissimus* var. *gratissimus* against axenic amastigotes of *L. donovani*. The ESIMS (positive base peak chromatogram) of a separation of 300 µg of fraction on an analytical RP-HPLC column is shown. Activities of one-minute microfractions are shown with grey columns, and are expressed as percent growth inhibition compared to untreated parasites. Bold numbers in the chromatogram refer to compounds **1**-**9**.

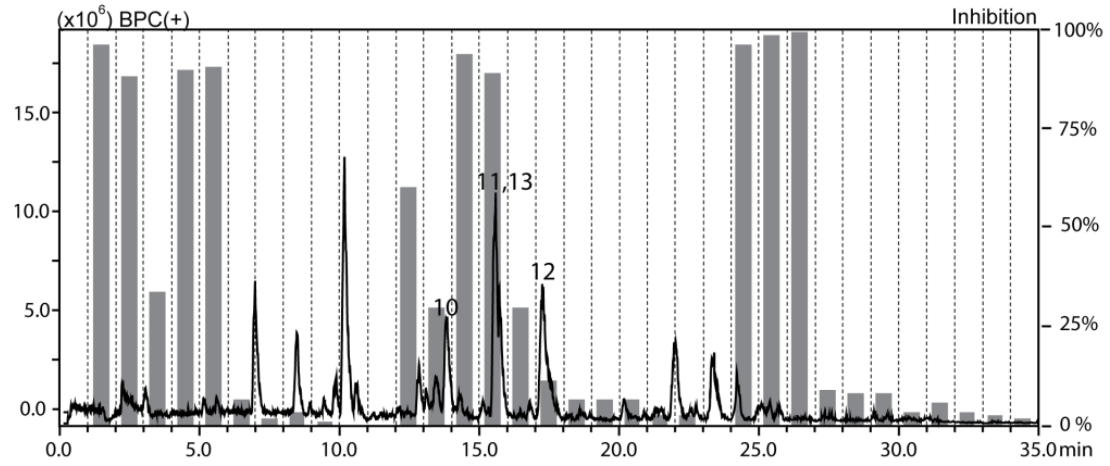

Figure 2. HPLC-based activity profiling of the chloroform fraction of *Cuscuta hyalina* Roth ex Schult. against axenic amastigotes of *L. donovani*. The ESIMS (positive base peak chromatogram) and activity profile (gray bars) are shown. Bold numbers in the chromatogram refer to compounds **10**-**13**.

3.3.2 Compound Isolation and Structure Elucidation

Separation of the chloroform fraction of *C. gratissimus* on a Sephadex LH-20 column yielded 19 subfractions (A-S). Based on the HPLC‑PDA-ESIMS analysis, subfractions M, O, K, and C were found to contain peaks associated with the active time window. Further purification by semipreparative RP-HPLC afforded compounds **1**-**3** from subfraction M, **4**-**6** from subfraction O, **7** from subfraction K, and **8** and **9** from subfraction C.

By means of 1D and 2D NMR data (Tables S1-S4; Supporting Information), six flavonoids were identified as quercetin-3,3',4'-trimethylether (**1**) [21], ayanin (**2**) [22], retusin (**3**) [22], naringenin (**4**) [23], quercetin-3,4'-dimethyl ether (**5**) [24], quercetin-3,7-dimethylether (**6**) [25], along with 3-methoxy-4-hydroxybenzoic acid (**7**) [26], and the two benzyltetrahydroisoquinoline alkaloids laudanine (**8**) [27] and laudanosine (**9**) [27]. For naringenin (**4**), an optical rotation close to 0 and the absence of a Cotton effect (CE) in the ECD indicated a 1:1 mixture of *R*- and *S*-stereoisomers. The absolute configuration of **8** and **9** was determined as *R* based on the optical rotation ($[\alpha]^{25}_{D}$ -6.6 (*c* 0.04, MeOH) for **8** [28] and $[\alpha]^{25}_{D}$ -62.5 (*c* 0.04, MeOH) for **9** [29]. Moreover, the ECD spectra of both compounds showed two negative cotton effects (CEs) at

210-215 and 240-242 nm which were in good agreement with calculated spectra of the *R*-stereoisomers (Figure S1 and S2, Supporting Information).

Compounds **1-9** are reported here for the first time from *C. gratissimus*, but some have been previously identified in other *Croton* species, such as ayanin (**2**) and quercetin-3,7-dimethylether (**6**) from *C. schiedeanus* [30], quercetin-3,4'-dimethylether (**5**) from *C. arboreus* [31], 3-methoxy-4-hydroxybenzoic acid (**7**) from *C. tonkinensis* [32], and *R*-laudanine (**8**) and *R*-laudanosine (**9**) from leaves and stems of *C. celtidifolius* [33].

Preparative chromatography on silica gel of the chloroform fraction of *C. hyalina* yielded 16 subfractions (A-P). Peaks associated with the active time window were detected in subfraction B. Further separation by semipreparative RP-HPLC afforded compounds **10**-**12.**

Based on the NMR data (Tables S5 and S6, Supporting Information), compounds were identified as the lignan pinoresinol (**10**) [34] and as flavonoids isorhamnetin (**11**) [35] and (-)-pseudosemiglabrin (**12**) [36]. In addition, kaempferol (**13**) was identified by dereplication with a reference compound. The absolute configuration of **10** and **12** was established based on their optical activity and ECD spectra. For compound **10**, the optical rotation $[\alpha]^{25}_{D}$ + 69.0 (*c* 0.10, MeOH) and the positive cotton effect at 207 nm (Δε +21.78) in the ECD spectrum indicated a (+)-(7*S*,7'*S*,8*R*,8'*R)* configuration of pinoresinol (Figure S3, Supporting Information).The optical rotation $[\alpha]^{25}_{D}$ -410.0 (*c* 0.05, MeOH) of **12** indicated (-)-pseudosemiglabrin. The ECD spectrum showed four negative CEs at 206 (Δε -12.84), 226 (Δε -10.40), 257 (Δε -11.01), 275 (Δε -7.99) nm, and a positive CE at 215 nm (Δε +3.69). This was in agreement with calculated spectra for the 3''*S*,4"*R*,5''*S* stereoisomer (Figure S4, Supporting Information), and opposite to the ECD data published for (+)-pseudosemiglabrin [37]. However, the assignment of C-5'' as *S* by Pirrung and Lee was incorrect. The absolute configuration of (-)-pseudosemiglabrin (**12**) was thus assigned as 3''*S*,4"*R*,5''*S.*

Kaempferol (**13**) and isorhamnetin (**11**) have been previously reported from different *Cuscuta* species [17], while pinoresinol has been identified in *C. chinensis* [38]. To the best of our knowledge, this is the first report on isolation of pseudosemiglabrin (**12**) from *Cuscuta* species.

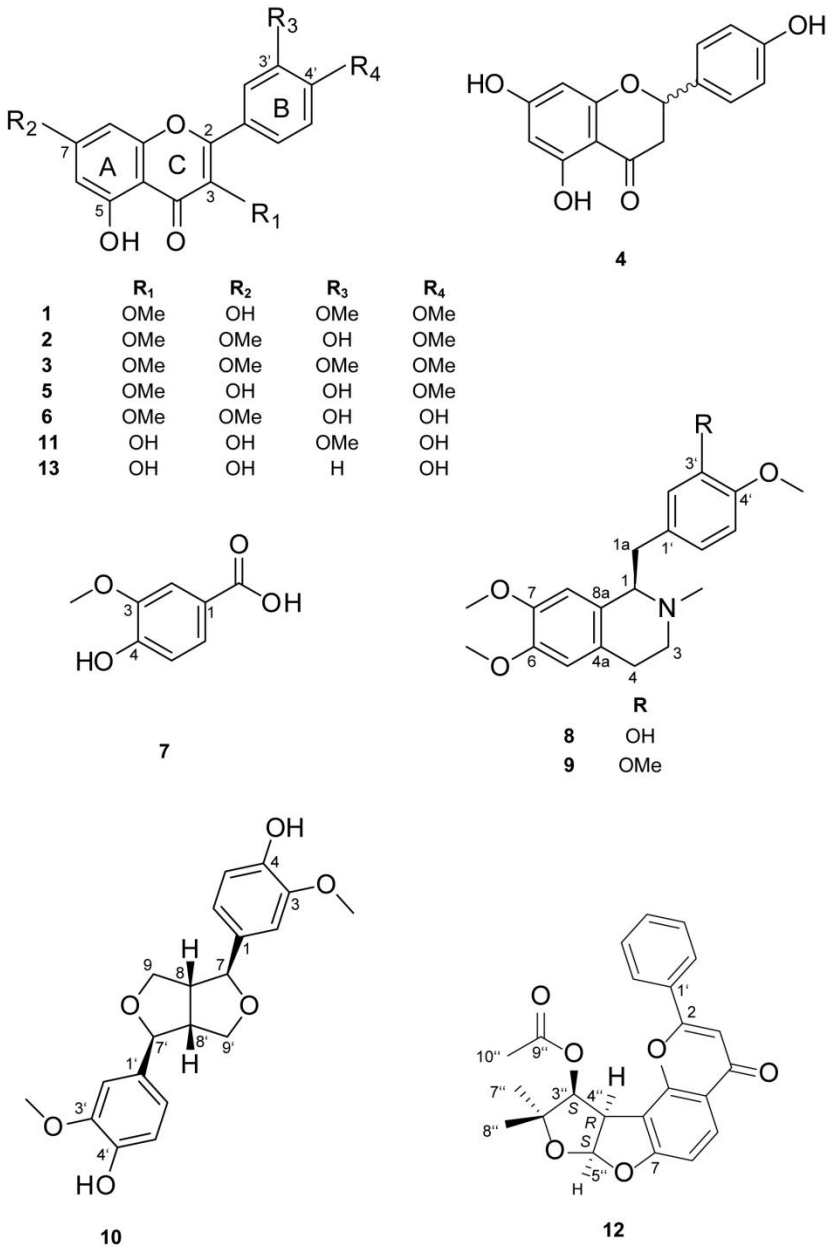

Figure 3. Chemical structures of compounds **1**-**13**

3.3.3 Activity against *Leishmania donovani* Axenic and Intracellular Amastigotes

All compounds were tested for their activity against *L. donovani* (MHOM/ET/67/L82) axenic amastigotes (Table 1). Quercetin-3,7-dimethylether (**6**) has shown the highest activity (IC_{50}

4.5 µM), followed by ayanin (**2**) (IC_{50} 8.2 µM). Both compounds exhibited similar selectivity indices (SI 12.3), which were the highest among the tested compounds.

Compounds **1** and **5** from *C. gratissimus*, and **11**-**13** from *C. hyalina* exhibited IC_{50} values in the range of 15-22 µM against axenic amastigotes. These compounds showed varying degrees of cytotoxicity in L6 rat skeletal cells. Moderate selectivity (selectivity indices (SI) of 4 – 6) towards *L. donovani* axenic amastigotes was observed for compounds **11**-**13**, while quercetin-3,4'-dimethylether (**5**) showed the highest toxicity (SI 1.3).

The benzyltetrahydroisoquinoline alkaloids laudanine (**8**) and laudanosine (**9**), and the furofurano lignan pinoresinol (**10**) showed only marginal activity against *L. donovani* axenic amastigotes (IC_{50} > 150 µM). The weak activity of the alkaloids was in agreement with previous reports [39].

After a first testing against axenic amastigotes, compounds were tested against *L. donovani* amastigotes in mouse macrophages. However, in this more elaborate and more physiological model none of the compounds showed activity (Table 1). In general, IC_{50} values for the intramacrophage form are higher than those for the axenic amastigotes [40]. This loss of activity in the intracellular model could be due to poor cellular permeability of the compounds, binding to cytosolic proteins in the host cell, or metabolism in the host cell phagolysosome [40–42].

3.3.4 Activity against *Trypanosoma brucei rhodesiense*

All compounds were also tested for their *in vitro* activity against the blood stream form of *T. brucei rhodesiense* (STIB 900) (Table 1). As for *L. donovani*, quercetin-3,7-dimethylether (**6**) was the most active (IC_{50} 2.4 µM) and most selective (SI 23.2). Quercetin-3,4'-dimethyl ether (**5**) was also active (IC_{50} 6.6 µM) but had a low selectivity (SI 2.9). Ayanin (**2**) had an IC_{50} of 11.2 µM with moderate cytotoxicity (SI 8.9), and isorhamnetin (**11**) an IC_{50} of 25.9 µM and a SI of 5.0. Kaempferol (**13**), pseudosemiglabrin (**12**) and 3-methoxy-4-hydroxybenzoic acid (**7**) showed marginal activity (IC_{50} 36-39 µM), and IC_{50} > 80 µM were determined for retusin (**3**), naringenin (**4**), laudanine (**8**), laudanosine (**9**) and pinoresinol (**10**).

3.3.5 Activity against *Plasmodium falciparum*

In vitro activity against the erythrocytic stages of the *P. falciparum* drug sensitive strain NF54 was determined for all compounds (Table 1). Ayanin (**2**) and quercetin-3,7-dimethylether (**6**) showed IC_{50} values of 7 to 8 µM, but **2** exhibited a higher selectivity index (SI 12.9) than **6** (SI 7.6). Quercetin-3,4'-dimethylether (**5**) was also rather active (IC_{50} <20 µM) but equally showed cytotoxic in L6 cells. Isorhamnetin (**11**) and pseudosemiglabrin (**12**) showed IC_{50}'s around 20 µM against *P. falciparum* and a moderate degree of selectivity towards the parasite (SI ~ 5). 3-Methoxy-4-hydroxybenzoic acid (**7**), and pinoresinol (**10**) were the least active among the tested compounds.

3.3.6 Correlation between Chemical Structure of Isolated Flavonoids and Antiprotozoal Activity

Of the isolated compounds, only flavones showed notable activity (Table 1). From a comparison of flavones **1**-**3**, **5**, **6**, **10** and **13** the following conclusions can be drawn: Compounds with a hydroxyl group at C-3' (**2**, **5**, and **6**) were the most active against the three parasites, whereby a catechol moiety as in **6** further increased the activity. Free hydroxyl groups at C-3 or C-7 (as in **5**, **11** and **13**) had not resulted in significant *in vitro* activity. Compounds **2** and **6** exhibited the highest selectivity, while **5** showed significant cytotoxicity in L6 cells leading to a low SI.

Naringenin (**4**) displayed the weakest antiparasitic activity among the tested flavonoids. The presence of a double bond between C-2 and C-3 has been previously found to be essential for antiparasitic activity [41]. Overall, our results were in agreement with previous structure-activity studies of flavonoids [43].

The influence of a balance between antioxidant and prooxidant properties of flavonoids on antiparasitic activity, and a correlation with their chemical structure has been investigated with the aid of QSAR models [44]. Compounds that displayed moderate to higher antitrypanosomal activity shared structural features, such as $\Delta^{2,3}$ unsaturation, presence of a hydroxyl group at C-3, a carbonyl group at C-4, and a catechol moiety in ring B. Our results were in line with these findings. To the best of our knowledge, the antitrypanosomal activities of quercetin-3,7-dimethylether (**6**) and ayanin (**2**) are here reported for the first time.

Table 1: *In vitro* activity of compounds **1**–**13** against *T. b. rhodesiense* (STIB 900), *L. donovani* (MHOM-ET-67/L82) axenic and intracellular amastigotes, *P. falciparum* (NF54), and cytotoxicity in L6 cells.

No.	Compound	IC_{50} [a] (μM)				
		L. donovani		***T. b. rhodesiense***	***P. falciparum***	**L6 cells**
		Axenic	Intramacrophage			
1	Quercetin-3,3',4'-trimethylether	18.8 (9.8) [b]	>79.9	55.1 (3.4) [b]	42.1 (4.4) [b]	186.3
2	Ayanin	8.2 (12.3) [b]	>95.9	11.2 (8.9) [b]	7.8 (12.9) [b]	100.9
3	Retusin	34.1 (6.3) [b]	>185.8	83.8 (2.6) [b]	23.4 (9.2) [b]	215.1
4	Naringenin	41.8 (5.6) [b]	>121.3	184.2 (1.3) [b]	73.2 (3.2) [b]	233.3
5	Quercetin-3,4'-dimethylether	15.2 (1.3) [b]	>33.3	6.6 (2.9) [b]	18.2 (1.1) [b]	19.2
6	Quercetin-3,7-dimethylether	4.5 (12.3) [b]	>66.7	2.4 (23.2) [b]	7.3 (7.6) [b]	55.4
7	3-Methoxy-4-hydroxybenzoic acid	153.3 (3.9) [b]	>595.2	38.6 (15.4) [b]	138.4 (4.3) [b]	595.2
8	Laudanine	193.3 (1.5) [b]	>291.5	143.1 (2.0) [b]	78.6 (3.7) [b]	291.5
9	Laudanosine	185.4 (1.5) [b]	>186.3	101.5 (2.8) [b]	76.1 (3.7) [b]	280.1
10	Pinoresinol	151.4 (1.6) [b]	>279.3	116.8 (2.1) [b]	>139.7	241.9
11	Isorhamnetin	21.7 (6.0) [b]	>210.4	25.9 (5.0) [b]	21.6 (6.0) [b]	130.2
12	Pseudosemiglabrin	18.9 (4.1) [b]	>84.2	37.5 (2.1) [b]	16.3 (4.8) [b]	78.1
13	Kaempferol	20.8 (5.3) [b]	>115.4	36.4 (3.1) [b]	53.1 (2.1) [b]	111.4
	Positive control	0.5 [c]	6.6 [c]	0.01 [d]	0.01 [e]	0.03 [f]

[a] The IC_{50}s are mean values from at least two independent replicates (the variation is a maximum of 20%).
[b] Selectivity index (SI): IC_{50} in L6 cells divided by IC_{50} in the titled parasitic strain.
[c] Miltefosine, [d] Melarsoprol, [e] Chloroquine [f] Podophyllotoxin.

3.4 Materials and Methods

General experimental procedures

HPLC-grade methanol and acetonitrile from Macron Fine Chemicals (Avantor Performance Materials), and water from a Milli-Q water purification systems (Merck Millipore) were used for HPLC separations. For fractionation and preparative separation, technical grade solvents from Scharlau (Scharlab S. L.) were used after distillation. Silica gel 60 F_{254} coated aluminum TLC plates were obtained from Merck. Silica gel (230-400 µm, Merck) and Sephadex LH-20 (25-100 µm, Sigma-Aldrich) were used for open column chromatography. Optical rotation was measured in methanol using a JASCO P-2000 digital polarimeter equipped with a sodium lamp (589 nm) and a temperature-controlled microcell (10 cm). UV and ECD spectra were recorded in methanol on a Chirascan CD spectrometer (Applied Photophysics) using 110 QS 1 mm path precision cells (Hellma Analytics). NMR spectra were recorded on a Bruker Avance III NMR spectrometer operating at 500.13 MHz for ^{1}H and 125.77 MHz for ^{13}C. ^{1}H NMR, COSY, HSQC, HMBC, and NOESY spectra were measured at 23 °C in a 1 mm TXI probe with a z-gradient, using standard Bruker pulse sequences. Spectra were analyzed by Bruker TopSpin 3.5 pl 7 and ACDLabs Spectrus Processor. NMR spectra were recorded in DMSO-d_6 (99.9 atom % D; Armar Chemicals).

HPLC-PDA-ELSD-ESIMS data were recorded in positive- and negative-ion mode (scan range of m/z 200−1500) on a Shimadzu LC-MS/MS 8030 triple quadrupole MS system, connected via a T-splitter (1:10) to a Shimadzu HPLC system consisting of degasser, binary mixing pump, autosampler, column oven, and a diode array detector and to an Alltech 3300 ELSD detector. Separation was achieved on a SunFire C_{18} (3.5 µm, 150 × 3.0 mm i.d.) column equipped with a guard column (10 mm × 3.0 mm i.d.) (Waters). Data acquisition and processing were performed with LabSolution software.

Microfractionation was carried out with the same HPLC instrument connected via a T split to an FC204 fraction collector (Gilson) with only UV detection, using a SunFire C_{18} (3.5 µm, 150 × 3.0 mm i.d.) column equipped with a guard column (10 mm × 3.0 mm i.d.) (Waters).

Semipreparative HPLC separations were carried out with an Agilent HP 1100 Series system consisting of a quaternary pump, autosampler, column oven, and a diode array detector. SunFire C_{18} (5 µm, 10 × 150 mm i.d.) columns (Waters) were used for separations. Chemstation software was used for data acquisition and processing. Preparative separations were carried out on a Puriflash 4100 system (Interchim) or a Reveleris PREP purification system (Büchi). Sephadex LH-20 (110 × 3 cm; 25-100 µm) and silica gel (40 × 5 cm, 230−400 mesh) columns were used.

All handling of infectious agents (*L. donovani*, *T. b. rhodesiense*, *P. falciparum*) was performed under strict biosafety level 2 conditions under notification A000275 to the Swiss Federal Office of Public Health.

Plant Material

Croton gratissimus var. *gratissimus* fruits and *Cuscuta hyalina* Roth ex Schult. stems were obtained from the Herbarium of the Faculty of Pharmacy, University of Science and Technology, Omdurman, Sudan. The taxonomic identity was confirmed by the Medicinal and Aromatic Plants Research Institute, Sudan and voucher specimens (CZFCHL02 and ChSCHL 02) were deposited. Plant materials were dried at room temperature and milled before extraction.

Extraction

Powdered materials of *C. gratissimus* fruits and *C. hyalina* stems (500 g each), respectively, were extracted with 1 Litre of 70% ethanol and kept in a magnet rod shaker for 24 h. The extraction procedure was repeated three times for each herbal drug. Extracts were filtered and dried under reduced pressure. For each plant, the ethanolic extract was suspended in water and partitioned successively with petroleum ether, chloroform, and ethyl acetate. Three repetitive partitioning procedures, each with 500 mL of either solvent were performed. This

afforded 3.5 g and 1.2 g of the chloroform extracts of *C. gratissimus* fruits and *C. hyalina* stems, respectively.

Microfractionation

HPLC-based microfractionation of the chloroform extracts of *C. gratissimus* fruits and *C. hyalina* stems was performed [H_2O + 0.1% formic acid (A), MeCN + 0.1% formic acid (B); 0→100% B (0-30 min), 100% B (30-40 min); flow rate 0.4 mL/min; sample concentration 10 mg/mL in DMSO; injection volume twice 35 µL] by collecting one-minute fractions from minute 1 to minute 40 into a 96-deepwell plate. After drying of plates in a Genevac EZ-2 evaporator, microfractions were tested for their antiprotozoal activity according to previously established protocols [19,20].

Preparative Isolation

The chloroform fraction (3.5 g) of *C. gratissimus* fruits was fractionated by column chromatography (CC) on Sephadex LH-20 (110 × 3 cm; 25-100 µm) using methanol as eluent at a flow rate of 1mL/min. A total of 19 fractions (A-S) were combined based on TLC patterns (silica gel; CH_2CL_2–MeOH, 90:10, 75:25, and 50:50, respectively; detection with 1% ethanolic vanillin and 10% sulfuric acid, followed by heating at 105 ^oC). Fractions were submitted to HPLC-PDA-ELSD-MS analysis to track peaks previously detected in the active time windows of the activity profile.

Fraction M (36 mg) was submitted to semipreparative RP-HPLC [H_2O (A), CH_3CN (B); 43% B (0−22 min), 43→100% B (22−27 min), 100% B (27−30 min), flow rate 4 mL/min; sample concentration 50 mg/mL in DMSO; injection volume 50 µL], yielding quercetin-3,3',4'-trimethylether (**1**, 0.3 mg, t_R 10.2 min), ayanin (**2**, 21.9 mg, t_R 16.7 min), and retusin (**3**, 0.4 mg, t_R 28.8 min).

Fraction O (15 mg) was submitted to semipreparative RP-HPLC [H_2O (A), CH_3CN (B); 35% B (0−34 min), 35→100% B (34−40 min), 100% B (40−45 min) , flow rate 4 mL/min; sample concentration 50 mg/mL in DMSO; injection volume 50 µL], to afford naringenin (**4**, 0.51 mg, t_R

9.6 min), quercetin-3,4'-dimethylether (**5**, 1.9 mg, t_R 12.1 min), and quercetin-3,7-dimethylether (**6**, 7.1 mg, t_R 20.5 min).

Fraction K (26.6 mg) was purified by semipreparative RP-HPLC [H_2O (A), CH_3CN (B), both containing 0.1% formic acid; 10→32% B (0−30 min), 32→100% B (30−35 min), 100% B (35−40 min), flow rate 4 mL/min; sample concentration 50 mg/mL in DMSO; injection volume 50 µL], to afford 3-methoxy-4-hydroxybenzoic acid (**7**, 0.63 mg, t_R 10.9 min).

Fraction C (100.6 mg) was purified by semipreparative RP-HPLC [H_2O (A), CH_3CN (B), both containing 0.1% formic acid; 10→17% B (0−20 min), 17→100% B (20−25 min), 100% B (25−30 min), flow rate 4 mL/min; sample concentration 50 mg/mL in DMSO; injection volume 50 µL], to afford laudanine (**8**, 0.41 mg, t_R 9.1 min), and laudanosine (**9**, 0.63 mg, t_R 14.5 min).

The chloroform fraction (1.9 g) of *C. hyalina* stems was fractionated by CC on silica gel (40 × 5 cm, 230−400 mesh), using a gradient of CH_2CL_2−MeOH (99:1 to 0:100) as mobile phase. A total of 16 fractions (A-P) were combined based on TLC patterns (silica gel; CH_2CL_2−MeOH, 99:1, 90:10, and 80:20, respectively; detection with 1% ethanolic vanillin and 10% sulfuric acid, followed by heating). Fractions were submitted to HPLC-PDA-ELSD-MS analyses to track peaks previously detected in the active time windows of the activity profile.

Fraction B (52.7 mg) was purified by semipreparative RP-HPLC [H_2O (A), CH_3CN (B); 25→70% B (0−30 min), 70→100% B (30−33 min), 100% B (33−40 min) , flow rate 4 mL/min; sample concentration 50 mg/mL in DMSO; injection volume 50 µL], to afford pinoresinol (**10**, 7.8 mg, t_R 10.2 min), isorhamnetin (**11**, 3.5 mg, t_R 13.6 min), pseudosemiglabrin (**12**, 2.2 mg, t_R 23.5 min). Kaempferol (**13**) was identified by co-injection of a reference standard (Sigma-Aldrich)**.**

Quercetin-3,3',4'-trimethylether (**1**): amorphous solid; ^1H and ^{13}C NMR, see Table S1, Supporting Information; ESIMS *m/z* 345 $[M + H]^+$.

Ayanin (**2**): amorphous solid; ^1H and ^{13}C NMR, see Table S1, Supporting Information; ESIMS *m/z* 345 $[M + H]^+$.

Retusin (**3**): amorphous solid; ^1H and ^{13}C NMR, see Table S1, Supporting Information; ESIMS *m/z* 359 $[M + H]^+$.

Naringenin (**4**): amorphous solid; ^{1}H and ^{13}C NMR, see Table S2, Supporting Information; ESIMS *m/z* 273 $[M + H]^+$.

Quercetin-3,4'-dimethylether (**5**): amorphous solid; ^{1}H and ^{13}C NMR, see Table S2, Supporting Information; ESIMS *m/z* 331 $[M + H]^+$.

Quercetin-3,7-dimethylether (**6**): amorphous solid; ^{1}H and ^{13}C NMR, see Table S2, Supporting Information; ESIMS *m/z* 331 $[M + H]^+$.

3-Methoxy-4-hydroxybenzoic acid (**7**): amorphous solid; ^{1}H and ^{13}C NMR, see Table S3, Supporting Information; ESIMS *m/z* 169 $[M + H]^+$.

R-Laudanine (**8**): amorphous solid; $[\alpha]^{25}_{D}$ -6.6 (*c* 0.04, MeOH); UV λ_{max} (MeOH) (log ε) 226 (0.07), 291 (0.01) nm; ECD (MeOH, *c* 3.5 x 10^{-4} M, 1 mm path length) $\lambda_{max}(\Delta\varepsilon)$ 214 (-0.56), 241 (-0.47), 290 (-0.39); ^{1}H and ^{13}C NMR, see Table S4, Supporting Information; ESIMS *m/z* 344 $[M + H]^+$.

R-Laudanosine (**9**): amorphous solid; $[\alpha]^{25}_{D}$ -62.5 (*c* 0.04, MeOH); UV λ_{max} (MeOH) (log ε) 201 (0.75), 226 (0.18), 279 (0.06) nm; ECD (MeOH, *c* 1.4 x 10^{-4} M, 1 mm path length) $\lambda_{max}(\Delta\varepsilon)$ 211 (-19.43), 241 (-6.67), 290 (-3.38); ^{1}H and ^{13}C NMR, see Table S4, Supporting Information; ESIMS *m/z* 358 $[M + H]^+$.

(+)-(7S,7'S,8R,8'R)-Pinoresinol (**10**): amorphous solid; $[\alpha]^{25}_{D}$ 69.0 (*c* 0.10, MeOH); UV λ_{max} (MeOH) (log ε) 202 (0.70), 232 (0.10) nm; ECD (MeOH, *c* 7.0 x 10^{-5} M, 1 mm path length) $\lambda_{max}(\Delta\varepsilon)$ 207 (+21.78) nm; ^{1}H and ^{13}C NMR, see Table S5, Supporting Information; ESIMS *m/z* 359 $[M + H]^+$.

Isorhamnetin (**11**): amorphous solid; ^{1}H and ^{13}C NMR, see Table S5, Supporting Information; ESIMS *m/z* 317 $[M + H]^+$.

(-)-(3''S,4"R,5''S)-Pseudosemiglabrin (**12**): amorphous solid; $[\alpha]^{25}_{D}$ -410.0 (*c* 0.05, MeOH); UV λ_{max} (MeOH) (log ε) 212 (0.73), 255 (0.49), 309 (0.43) nm; ECD (MeOH, *c* 2.6 x 10^{-4} M, 1 mm path length) $\lambda_{max}(\Delta\varepsilon)$ 206 (-12.84), 215 (+3.69), 226 (-10.40), 257 (-11.01), 275 (-7.99) nm; ^{1}H and ^{13}C NMR, see Table S6, Supporting Information; ESIMS *m/z* 393 $[M + H]^+$.

Kaempferol (**13**): identified by co-injection of a reference standard (Sigma-Aldrich)**.**

Sample preparation

Compounds were dissolved in DMSO (10 mg/mL) and warmed up to 40°C and/or sonicated if necessary. These DMSO stocks were kept at -20°C. For each assay, a fresh dilution to 100 □g/mL

in medium was prepared. This was used to prepare the serial dilutions directly in the 96-well assay plates. Since DMSO is cytotoxic, the maximum DMSO concentration in the test was 1%.

Activity against *Leishmania donovani* axenic amastigotes

Amastigotes of *L. donovani* strain MHOM/ET/67/L82 were grown under an atmosphere of 5% CO_2 in air in axenic culture at 37 °C in SM medium [45] at pH 5.4 supplemented with 10% heat-inactivated fetal bovine serum. 50 µL of culture medium was added in the wells of a 96-well plate and serial drug dilutions of eleven 3-fold dilution steps covering a final range from 100 to 0.002 µg/mL were prepared. 50 µL culture medium with $2x10^5$ amastigotes from axenic culture were added to each well. After 70 h of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. 10 µL of resazurin (12.5 mg resazurin dissolved in 100 mL distilled water) were added to each well and the plates incubated for another 2 h. Then the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analyzed using the software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA). Decrease of fluorescence (= inhibition) was expressed as percentage of the fluorescence of untreated control cultures and plotted against the drug concentrations. From the sigmoidal inhibition curves the IC_{50} values were calculated. Miltefosine was used as positive control drug. Assays were performed in two independent replicates at least.

Activity against *Leishmania donovani* intramacrophage amastigotes

Mouse peritoneal macrophages (4×10^4 in 100 µL RPMI 1640 medium with 10% heat-inactivated FBS) were seeded into wells of a 96-well plate. After 24 h, 2×10^5 amastigote *Leishmania donovani* in 100 µL were added. The amastigotes were taken from an axenic amastigote culture grown at pH 5.4. The medium containing free amastigote forms was removed after 24 h and replaced by fresh medium. The washing step was repeated and afterwards the serial drug dilution was prepared with at least 6 dilution steps. Compounds were dissolved in DMSO at 10 mg/mL and further diluted in medium. After 96 hours of incubation at 37 °C under a 5 % CO_2 atmosphere, the medium was removed and cells were fixed by adding

50µL 4% formaldehyde solution followed by a staining with a 5 µM DRAQ5 solution. Plates were imaged in ImageXpress XLS (MD) microscope using a 20x air objective (635 nm excitation: 690/50 emission). 9 images were collected per well. Automated image analysis was performed with a script developed on Meta Xpress Software (MD). Three outputs were provided for each well: i) number of host cell nuclei; ii) numbers of infected and non-infected host cells; iii) number of parasite nuclei per infected host cell. The IC_{50} values were calculated based on the infection rate and the numbers of intracellular amastigotes. The cytotoxicity to macrophages was determined in parallel, and IC_{50} values were calculated based on the numbers of surviving, uninfected macrophages. Miltefosine was used as control. Assays were performed in two independent replicates at least.

Activity against *Trypanosoma brucei rhodesiense* STIB900

The stock was originally isolated from a Tanzanian patient and adapted to axenic culture conditions after several mouse passages and cloned. Minimum Essential Medium (50 µL) supplemented with 25 mM HEPES, 1g/L additional glucose, 1% MEM non-essential amino acids (100x), 0.2 mM 2-mercaptoethanol, 1mM Na-pyruvate [46] and 15% heat inactivated horse serum was added to each well of a 96-well microtiter plate. Serial drug dilutions of eleven 3-fold dilution steps covering a range from 100 to 0.002 µg/mL were prepared. Then $4x10^3$ bloodstream forms of *T. b. rhodesiense* STIB 900 in 50 µL were added to each well and the plate incubated for 70 h at 37 °C and under a 5% CO_2 atmosphere. 10 µL resazurin solution (resazurin, 12.5 mg in 100 mL double-distilled water) was then added to each well and incubation continued for a further 2–4 h [47]. Plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wave length of 536 nm and an emission wave length of 588 nm. Softmax Pro programme (Molecular Devices Cooperation, Sunnyvale, CA, USA) was used for data analyses and IC_{50} values were calculated by linear regression [48], and 4-parameter logistic regression from the sigmoidal dose inhibition curves. Melarsoprol (Arsobal Sanofi-Aventis, received from WHO) was used as control. Assays were performed in two independent replicates at least.

Activity against *Plasmodium falciparum*

In vitro activity against the erythrocytic stages of *P. falciparum* was determined using a ^{3}H-hypoxanthine incorporation assay [49], using the drug sensitive NF54 strain [50]. Compounds were dissolved in DMSO at 10 mg/mL and further diluted in medium before addition to parasite cultures incubated in RPMI 1640 medium without hypoxanthine, supplemented with HEPES (5.94 g/L), $NaHCO_3$ (2.1 g/L), neomycin (100 U/mL), Albumax^{R} (5 g/L) and washed human red cells A^{+} at 2.5% haematocrit (0.3% parasitaemia). Serial drug dilutions of eleven 3-fold dilution steps covering a range from 100 to 0.002 µg/mL were prepared. The 96-well plates were incubated in a humidified atmosphere at 37 °C; 4% CO_2, 3% O_2, 93% N_2. After 48 h 50 µL of ^{3}H-hypoxanthine (=0.5 µCi) was added to each well of the plate. The plates were incubated for a further 24 h under the same conditions. The plates were then harvested with a Betaplate™ cell harvester (Wallac, Zurich, Switzerland), and the red blood cells transferred onto a glass fibre filter, and lysed with distilled water. The dried filters were inserted into a plastic foil with 10 mL of scintillation fluid and counted in a Betaplate™ liquid scintillation counter (Wallac, Zurich, Switzerland). IC_{50} values were calculated from sigmoidal inhibition curves by linear regression using Microsoft Excel. Chloroquine (Sigma C6628) was used as control. Assays were performed in two independent replicates at least.

In vitro cytotoxicity with L-6 cells

Assays were performed in 96-well microtiter plates, each well containing 100 µL of RPMI 1640 medium supplemented with 1% L-glutamine (200mM) and 10% fetal bovine serum, and 4000 L-6 cells (a primary cell line derived from rat skeletal myoblasts) [51]. Serial drug dilutions of eleven 3-fold dilution steps covering a range from 100 to 0.002 µg/mL were prepared 24 h post seeding L-6 cells. The plates were incubated for 70 h and inspected under an inverted microscope to assure growth of the controls and sterile conditions. 10 µL of resazurin was then added to each well and the plates incubated for another 2 hours. Then the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. The IC_{50} values were calculated by linear regression and 4-parameter logistic

regression from the sigmoidal dose inhibition curves using SoftmaxPro software (Molecular Devices Cooperation, Sunnyvale, CA, USA). Podophyllotoxin (Sigma P4405) was used as positive control. All assays were performed in two independent replicates at least.

Activities of all compounds were expressed in µM using the formula:

Activity (µM) = Activity (µg/mL)*1000 / Molecular weight.

Acknowledgments

We wish to thank M. Cal, R. Rocchetti and S. Märki for help with antiparasitic drug testing, S. Abdelgaffar for help with preparation of extracts, and professors Suad Sulaiman and Marcel Tanner for their mentorship. We gratefully acknowledge financial support by the Amt für Ausbildungsbeiträge Basel and the Emilia Guggenheim-Schnurr Foundation.

Author Contributions

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

Conflict of Interest

The authors declare no conflict of interest.

3.5 References

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3.6 Supporting Information

HPLC-Based Activity Profiling for Antiprotozoal Compounds in *Croton gratissimus* and *Cuscuta hyalina*

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Table S 1. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds **1**-**3** (DMSO-*d6*; 500.13 Hz for ^{1}H and 125.77 for ^{13}C NMR; δ in ppm)

	1		**2**		**3**	
Position	δ_C [a]	δ_H (mult *J* in Hz)	δ_C [a]	δ_H (mult *J* in Hz)	δ_C [a]	δ_H (mult *J* in Hz)
2	C [b]		155.2, C		155.4, C	
3	137.4, C		137.9, C		138.3, C	
4	C [b]		177.8, C		C [b]	
5	160.9, C		160.8, C		161.0, C	
6	101.4, CH	5.73, d (1.5)	97.2, CH	6.20, br s	97.8, CH	6.36, br s
7	C [b]		164.8, C		165.2, C	
8	95.3, CH	5.91, d (1.2)	91.7, CH	6.47, br s	92.4, CH	6.74, br s
9	157.2, C		155.9, C		156.3, C	
10	100.4, C		105.0, C		105.3, C	
1'	123.1, C		122.2, C		C [b]	
2'	111.4, CH	7.57 [c]	115.0, CH	7.57, br s	111.6, CH	7.66, br s
3'	148.4, C		146.2, C		148.6, C	
4'	150.6, C		150.1, C		151.4, C	
5'	111.8, CH	7.11, d (8.2)	111.5, CH	7.01, d (8.2)	111.8, CH	7.16, d (8.5)

6'	121.3, CH	7.58 [c]	120.1, CH	7.49, br d (8.2)	122.1, CH	7.71, d (8.5)
3-OMe	59.7, CH_3	3.77, s	59.3, CH_3	3.81, s	59.8, CH_3	3.83, s
7-OMe			55.5, CH_3	3.80, s	55.8, CH_3	3.87, s [c]
3'-OMe	55.7, CH_3	3.83, s			55.8, CH_3	3.86, s
4'-OMe	55.6, CH_3	3.84, s	55.4, CH_3	3.88, s	55.9, CH_3	3.87, s [c]

[a] ^{13}C NMR data extracted from HSQC and HMBC spectra, [b] Signal not visible in HMBC, [c] Overlapping signals.

Table S 2. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds **4**, **5** and **6** (DMSO-*d6*; 500.13 Hz for ^{1}H and 125.77 for ^{13}C NMR; δ in ppm)

	4		**5**		**6**	
Position	δ_C [a]	δ_H (mult *J* in Hz)	δ_C [a]	δ_H (mult *J* in Hz)	δ_C [a]	δ_H (mult *J* in Hz)
2	77.8, CH	5.29, dd (12.2, 3.1)	154.5, C		155.9, C	
3	42.0, CH_2	3.03, dd (16.9, 12.4) 2.56, dd (17.1, 3.1)	137.7, C		137.9, C	
4	192.8, C		177.4, C		178.0, C	
5	162.4, C		161.0, C		161.0, C	
6	96.8, CH	5.59, d (1.5)	99.0, CH	6.14, d (1.8)	97.5, CH	6.22, s
7	174.1, C		166.4, C		165.0, C	
8	97.4, CH	5.57, d (1.5)	93.7, CH	6.34, d (1.5)	92.0, CH	6.50, br s
9	163.7, C		156.3, C		156.1, C	
10	99.3, C		103.2, C		105.1, C	
1'	129.5, C		122.3, C		C [b]	
2'	128.0, CH	7.27, d (8.5)	114.8, CH	7.52, d (2.1)	115.6, CH	7.58, br s
3'	115.1, CH	6.78, d (8.5)	146.2, C		145.3, C	

4'	157.6, C		149.9, C		148.8, C	
5'	115.1, CH	6.78, d (8.5)	111.8, CH	7.05, d (8.5)	115.7, CH	6.92, br s
6'	128.0, CH	7.27, d (8.5)	119.9, CH	7.51, dd (8.2, 2.1)	120.6, CH	7.43, br d (7.9)
3-OMe			59.4, CH_3	3.78, s	59.5, CH_3	3.79, m
7-OMe					55.8, CH_3	3.79, s
4'-OMe			55.4, CH_3	3.85, s		

[a] ^{13}C NMR data extracted from HSQC and HMBC spectra, [b] Signal not visible in HMBC.

Table S 3. ^{1}H and ^{13}C NMR Spectroscopic Data for Compound **7** (DMSO-*d6*; 500.13 Hz for ^{1}H and 125.77 for ^{13}C NMR; δ in ppm)

	7	
Position	δ_C [a]	δ_H (mult *J* in Hz)
2	113.1, CH	7.46 b
3	147.0, C	
5	114.7, CH	6.84, br s
6	123.1, CH	7.43 b
3-OMe	55.5, CH3	3.79, s

[a] ^{13}C NMR data extracted from HSQC and HMBC spectra, [b] Overlapping signals, [c] broad signal due to concentrated sample.

Table S 4. ^{1}H and ^{13}C NMR Spectroscopic Data for Compound **8** and **9** (DMSO-*d6*; 500.13 Hz for ^{1}H and 125.77 for ^{13}C NMR; δ in ppm)

	8		**9**	
Position	δ_C [a]	δ_H (mult *J* in Hz)	δ_C [a]	δ_H (mult *J* in Hz)
1	63.8, CH	3.61, dd (5.8, 5.8)	63.9, CH	3.66 [b]
1a	39.6, CH_2	2.94, dd (13.7, 6.1) 2.71 [b]	39.8, CH_2	2.98, dd (13.9, 6.0) 2.78, dd (13.9, 6.3)
3	46.5, CH_2	3.04, ddd (12.7, 8.0, 5.2) 2.56, m	46.8, CH_2	3.05, ddd (12.4, 7.8, 4.9) 2.59 ddd (12.2, 4.6, 4.6)
4	24.9, CH_2	2.67 [b] 2.47, m	25.0, CH_2	2.68, m 2.46, m
4a	126.1, C		126.4, C	
5	111.7, CH	6.59, s	112.0, CH	6.60, s
6	146.8, C		147.1, C	
7	146.1, C		146.5, C	
8	111.5, CH	6.32, s	111.7, CH	6.35, s
8a	129.5, C		129.4, C	
1'	132.9, C		132.6, C	

2'	114.0, CH	6.62 [b]	114.0, CH	6.69, br s
3'	144.5, C		148.4, C	
4'	146.8, C		147.1, C	
5'	114.7, CH	6.63 [b]	111.9, CH	6.79, d (8.2)
6'	121.8, CH	6.51, dd (8.2, 1.2)	121.8, CH	6.64, d (7.6)
2-NMe	42.1, CH_3	2.37, s	42.4, CH_3	2.38, s
6-OMe	55.3, CH_3	3.68, s	55.6, CH_3	3.69, s
7-OMe	55.1, CH_3	3.53, s	55.4, CH_3	3.54 [b]
3'-OMe			55.5, CH_3	3.65, s [b]
4'-OMe	55.4, CH_3	3.65, s	55.7, CH_3	3.70, s

[a] ^{13}C NMR data extracted from HSQC and HMBC spectra, [b] Overlapping signals.

Table S 5. ^{1}H and ^{13}C NMR Spectroscopic Data for Compound 10 and 11 (DMSO-*d6*; 500.13 Hz for ^{1}H and 125.77 for ^{13}C NMR; δ in ppm)

	10		**11**	
Position	δ_C [a]	δ_H (mult *J* in Hz)	δ_C [a]	δ_H (mult *J* in Hz)
1	132.3, C			
2	110.6, CH	6.92, br s	146.1, C	
3	147.6, C			
4	146.0, C		175.5, C	
5	115.3, CH	6.77 [b]	160.4, C	
6	118.6, CH	6.77 [b]	98.0, CH	6.20, d (1.2)
7	85.2, CH	4.64, d (4.3)	164.0, C	
8	53.6, CH	3.05, m	93.2, CH	6.46, d (1.2)
9	70.9, CH_2	4.15, dd (8.90, 6.7) 3.76, dd (9.00, 3.5)	155.8, C	
10			102.5, C	
1'	132.3, C		121.7, C	
2'	110.6, CH	6.92, br s	111.7, CH	7.77, br s
3'	147.6, C		147.1, C	

4'	146.0, C		148.5, C	
5'	115.3, CH	6.77 [b]	115.2, CH	6.95, d (8.5)
6'	118.6, CH	6.77 [b]	121.3, CH	7.69, br d (7.6)
7'	85.2, CH	4.64, d (4.3)		
8'	53.6, CH	3.05, m		
9'	70.9, CH_2	4.15, dd (8.90, 6.7) 3.76, dd (9.00, 3.5)		
3-OMe	55.7, CH_3	3.78, s		
3'-OMe	55.7, CH_3	3.78, s	55.5, CH_3	3.85, s

[a] ^{13}C NMR data extracted from HSQC and HMBC spectra, [b] Overlapping signals.

Table S 6. ^{1}H and ^{13}C NMR Spectroscopic Data for Compound 12 (DMSO-*d6*; 500.13 Hz for ^{1}H and 125.77 for ^{13}C NMR; δ in ppm)

	12	
Position	δ_C [a]	δ_H (mult *J* in Hz)
2	162.2, C	
3	107.2, CH	6.92, s
4	176.6, C	
5	128.0, CH	7.97, d (8.5)
6	108.9, CH	7.00, d (8.5)
7	153.7, C	
8	112.6, C	
9	164.3, C	
10	118.2, C	
1'	131.3, C	
2'	126.6, CH	7.99 [b]
3'	129.4, CH	7.55, m
4'	132.2, CH	7.59, m

5'	129.4, CH	7.55, m
6'	126.6, CH	7.99 [b]
2''	85.0, C	
3''	76.7, CH	5.56, d (8.9)
4''	47.8, CH	4.85, dd (8.7, 6.6)
5''	112.5, CH	6.53, d (6.4)
7''	27.5, CH_3	1.33, s
8''	23.3, CH_3	1.05, s
9''	169.4, C	
10''	20.3, CH_3	1.42, s

[a] ^{13}C NMR data extracted from HSQC and HMBC spectra, [b] Overlapping signals.

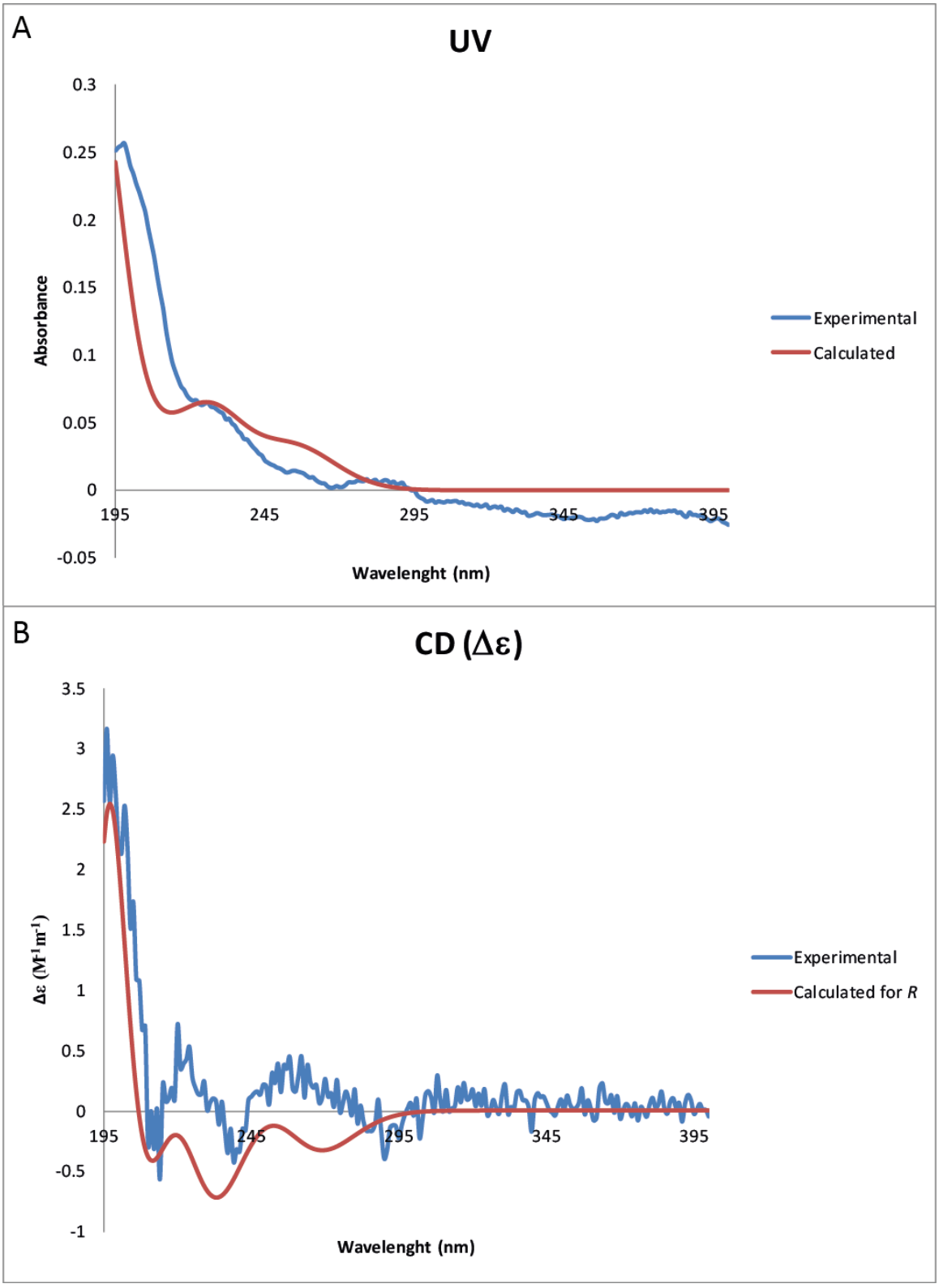

Figure S 1. Comparison of experimental and calculated UV (A) and ECD (B) spectra for compound **8** in MeOH (0.12 mg/mL).

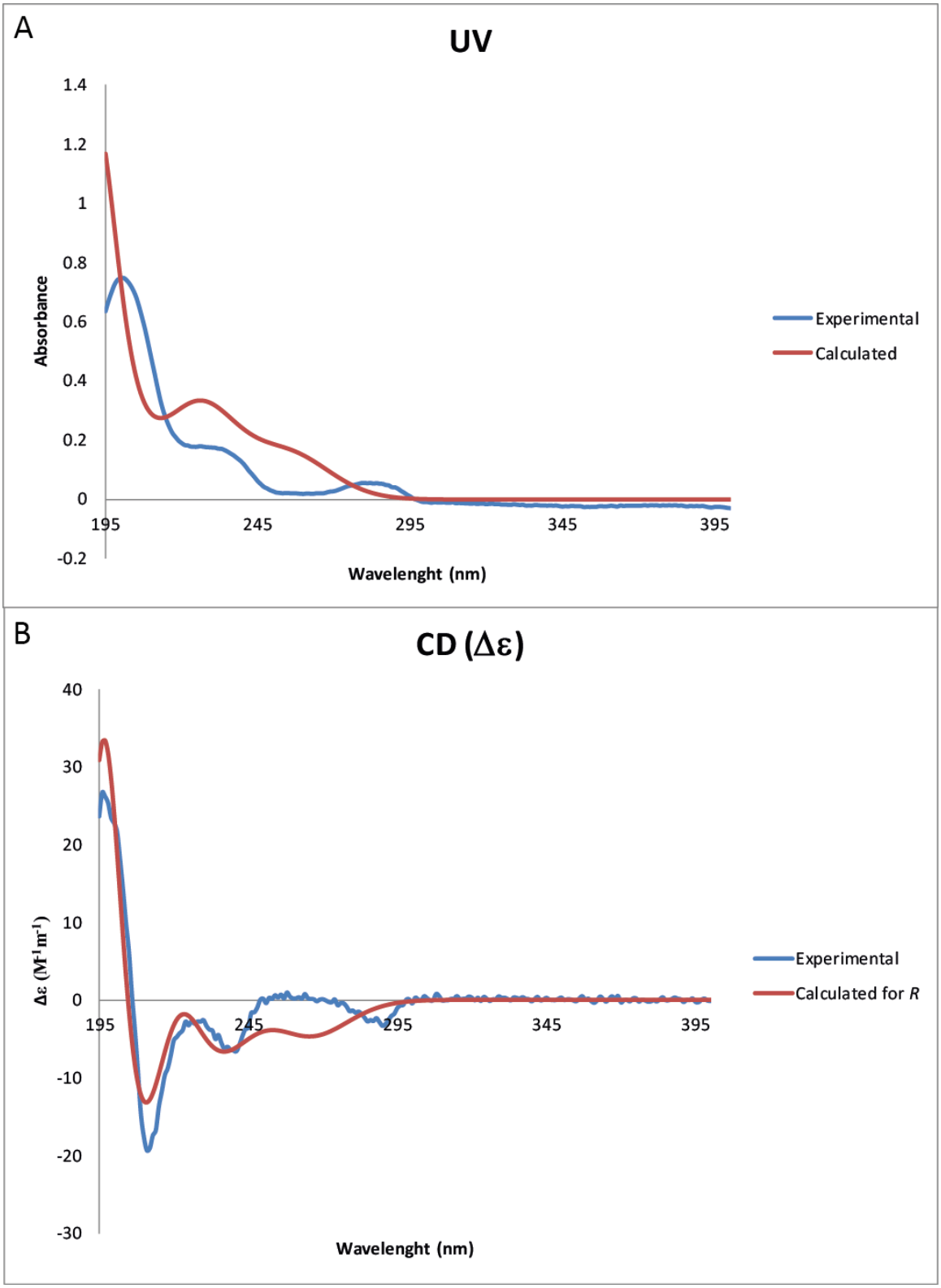

Figure S 2. Comparison of experimental and calculated UV (A) and ECD (B) spectra for compound **9** in MeOH (0.05mg/mL).

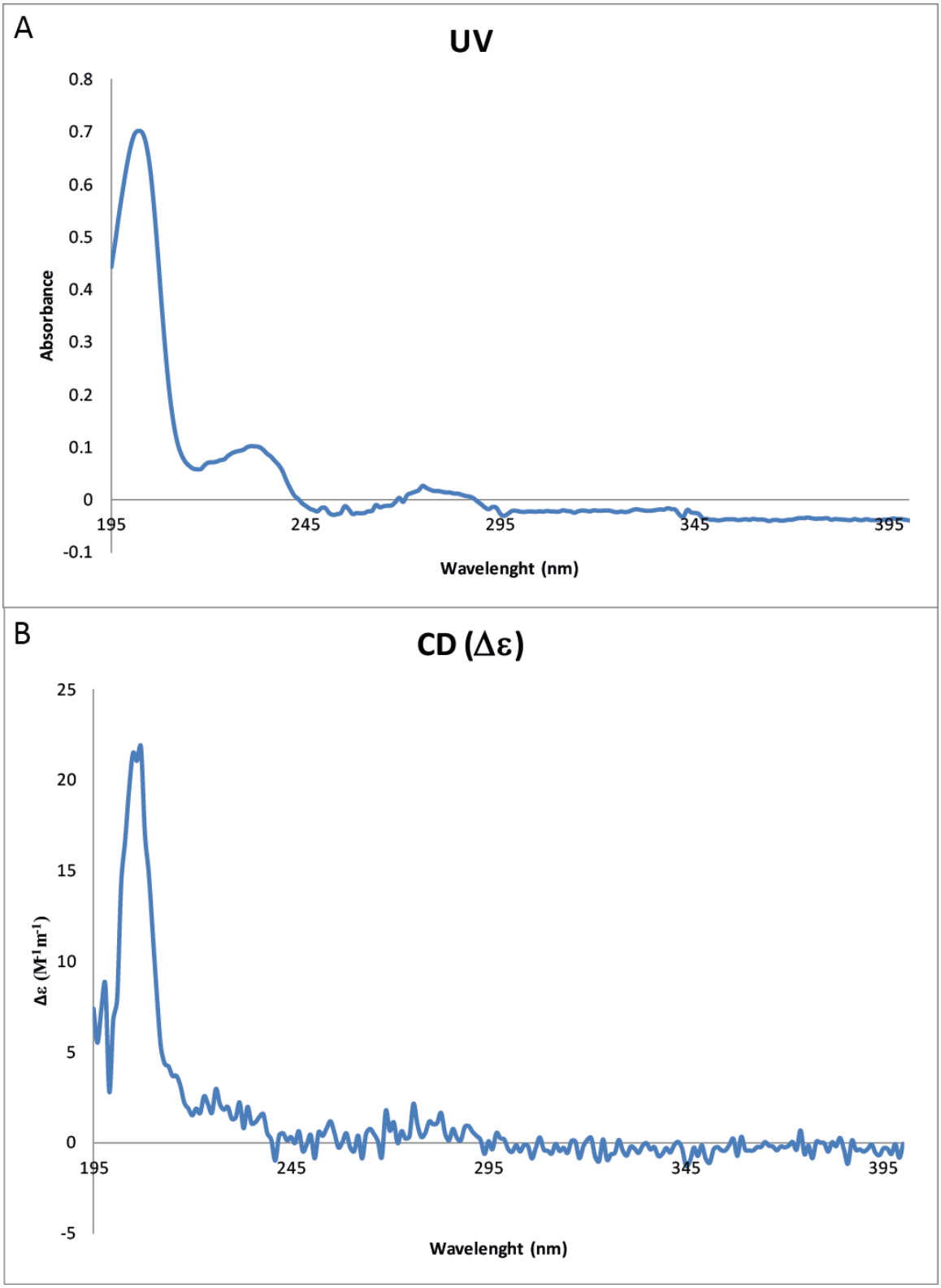

Figure S 3. Experimental UV (A) and ECD (B) spectra for compound **10** in MeOH (0.025 mg/mL).

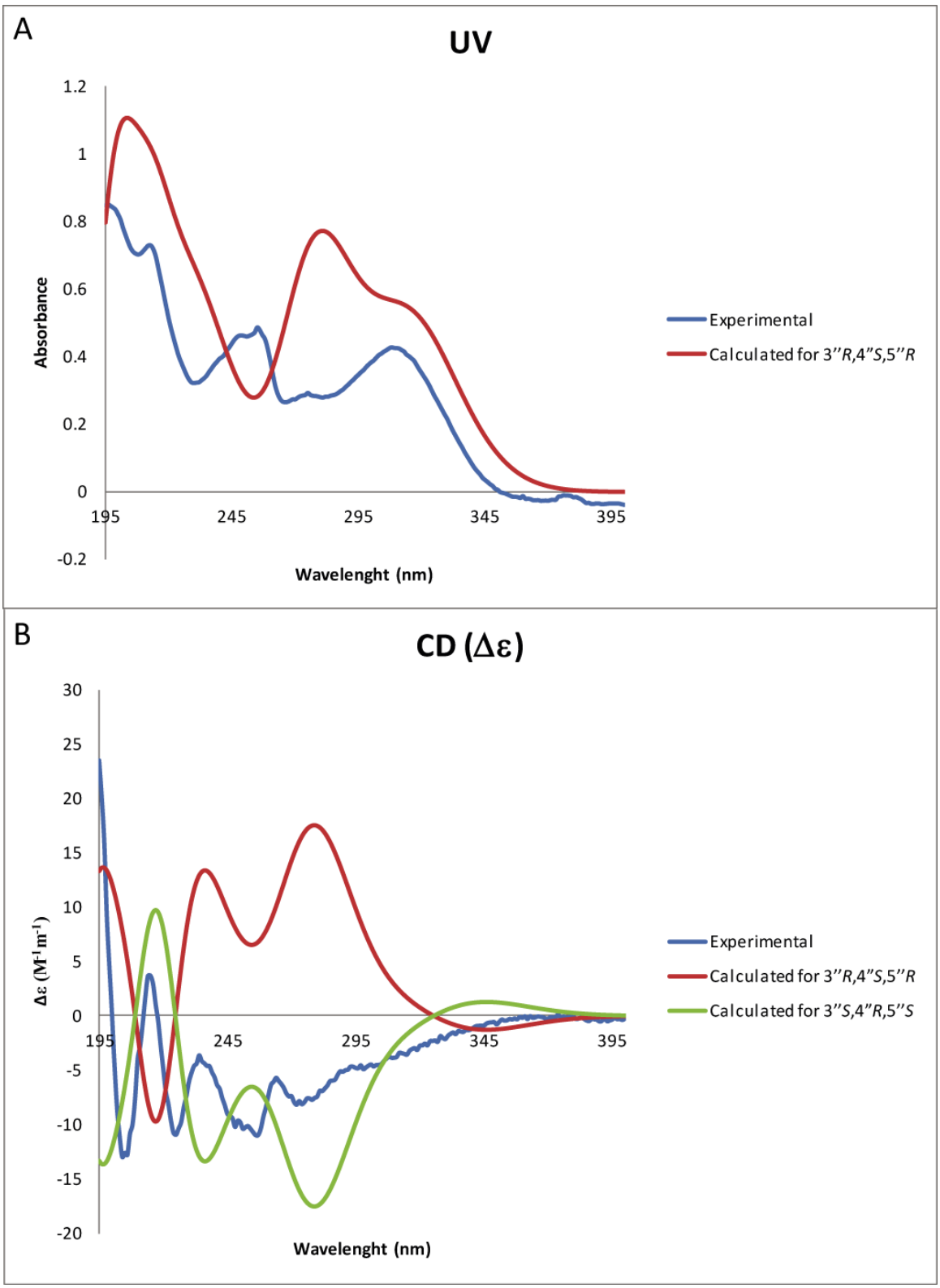

Figure S 4. Comparison of experimental and calculated UV (A) and ECD (B) spectra for compound **12** in MeOH (0.1mg/mL).

Computational Methods for ECD calculation.

Conformational analysis of compounds **8**, **9** and 1**2** was performed with MacroModel 9.8 software (Schrödinger LLC) employing the OPLS 2005 (Optimized Potential for Liquid Simulations) force field in H_2O. The five conformers with the lowest energy were submitted to geometrical optimization and energy calculation using Density Function Theory (DFT) with Becke's nonlocal three-parameter exchange and correlation functional and the Lee-Yang-Parr correlation functional level (B3LYP) using the B3LYP/6-31+G(d,p) basis set in MeOH with the Gaussian 09 program package [1]. Vibrational evaluation was done at the same level to confirm minima. Excitation energy (denoted by wavelength in nm), rotator strength (Rstr), dipole velocity (Rvel), and dipole length (Rlen) were calculated in MeOH by TD-DFT/B3LYP/6- 31G(d,p). ECD curves were obtained on the basis of rotator strengths with a half-band of 0.3 eV using SpecDis v1.71 [2]. ECD spectra were calculated from the spectra of individual conformers according to their contribution calculated by Boltzmann weighting.

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4. Lignans, Amides, and Saponins from *Haplophyllum tuberculatum* and their Antiprotozoal Activity

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Published in

Molecules 2020;25(12):2825.

doi:10.3390/molecules25122825

I have performed the extraction of plant material, microfractionation, compounds isolation and some of their structure elucidation. Structure elucidation for most of the compounds was done by Ombeline Danton. Compounds testing were done by the PCU at SwissTPH. I prepared the figures and wrote the manuscript.

4.1 Abstract

A screening of Sudanese medicinal plants for antiprotozoal activities, revealed that, the chloroform and water fractions from an ethanolic extract from roots of *Haplophyllum tuberculatum* were tested active against *Leishmania donovani*. The antileishmanial activity was tracked by HPLC-based activity profiling, and eight compounds were isolated from the chloroform fraction. These included lignans tetrahydrofuroguaiacin B (**1**), nectandrin B (**2**), furoguaiaoxidin (**7**), and 3,3'-dimethoxy-4,4'-dihydroxylignan-9-ol (**10**), and four cinnamoylphenethyl amides, namely dihydro-feruloyltyramine (**5**), N-trans-feruloyltyramine (**6**), N,N'-diferuloylputrescine (**8**), and 7'-ethoxy-feruloyltyramine (**9**). The water fraction yielded steroid saponins **11**-**13**. Compounds **1**, **2**, and **5**-**13** are reported for the first time from *Haplophyllum* species and the family Rutaceae. The antiprotozoal activity of the compounds plus two stereoisomeric tetrahydrofuran lignans fragransin B_2 (**3**) and fragransin B_1 (**4**) was determined against *Leishmania donovani* amastigotes, *Plasmodium falciparum,* and *Trypanosoma brucei rhodesiense* bloodstream forms, along with their cytotoxicity to rat myoblast L6 cells. Nectandrin B (**2**) exhibited the highest activity against *L. donovani* (IC_{50} 4.5 µM) and the highest selectivity index (25.5).

4.2 Introduction

Neglected tropical diseases (NTDs) are a group of infectious diseases that are prevalent in tropical and sub-tropical developing countries. NTDs are strongly associated with poverty and of high socio-economic impact. NTDs account for 48 million disability-adjusted life years (DALYs) and 152,000 deaths per year [1,2]. The NTD leishmaniasis, caused by *Leishmania* spp., imposes a global burden of 3.3 million DALYS and 51,600 annual deaths [1,2]. There is no vaccine, and current drugs are problematic given their serious adverse effects and the emergence of drug-resistant parasites [3]. There is one efficient and safe drug, AmBisome, a liposomal formulation of amphotericin B [4]. However, the high price of the drug and the need of an uninterrupted cold chain for delivery severely limit its use. In Eastern Africa, a high-burden region of visceral leishmaniasis [5], sodium stibogluconate is still the mainstay of leishmaniasis chemotherapy [3]. This pentavalent antimonial can cause hepatotoxicity and cardiotoxicity [6]. Thus, there is an urgent need for the development of new, efficacious, safe, and cost-effective drugs for the treatment of leihmaniasis and other diseases caused by kinetoplastid parasites [7,8].

A library of Sudanese medicinal plants traditionally used as anti-infectives was screened for antiprotozoal activity against *Leishmania donovani, Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, and *Plasmodium falciparum.* One of the most promising hits was *Haplophyllum tuberculatum* (Forssk.) A. Juss. (Rutaceae). Chloroform and aqueous fractions obtained by partitioning of an ethanolic extract from roots of the plant were active against *L. donovani* and *P. falciparum* (> 85% growth inhibition at 10 μg/mL) [9].

Haplophyllum tuberculatum is a perennial herb distributed throughout North Africa and the Middle East. In Sudan, the plant is locally known as *Haza,* and the aerial parts have been used traditionally to treat malaria, asthma, kidney diseases, gynecological and bowel disorders [10, 11]. Anti-inflammatory, antioxidant, antibacterial, and antifungal activities have been reported [12–14], and alkaloids, flavonoids, coumarins and lignans have been identified [15–17]. The methanolic extract of aerial part and roots of *H. tuberculatum* possessed activity against *P. falciparum* [18], and Justicidin A was found active [19]. The essential oil from the leaves had antileishmanial activity [20]. Justicidin B, a lignan isolated from the leaves, showed trypanocidal activity [21].

4.3 Results and Discussion

4.3.1 Extraction and HPLC-based Activity Profiling

The chloroform and the water fractions from roots of *H. tuberculatum* had been previously found to be active against *L. donovani* when tested at 10 µg/mL [9]. The antileishmanial activity was tracked by HPLC-based activity profiling, a procedure combining time-based microfractionation with bioactivity testing [22]. One-minute microfractions were collected and tested for growth inhibition of *L. donovani* axenic amastigotes. The HPLC-ESIMS traces (base peak chromatograms in positive ion mode) overlaid with the antileishmanial activity of microfractions are shown for the chloroform (Figure 1) and the water fraction (Figure 2). Pronounced antileishmanial activity was found in the chloroform fraction in the time window of 16-21 min, and moderate activity in the window of 12-15 min. For the water fraction, the antileishmanial activity was confined to a narrow time window between 19-20 min.

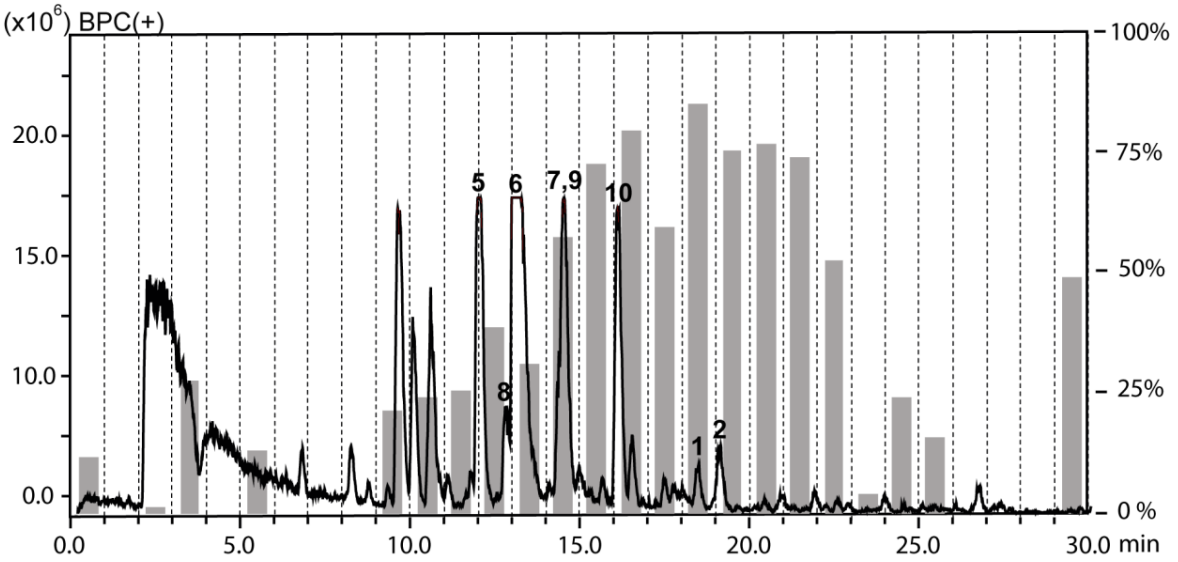

Figure 1. HPLC-based activity profiling of the chloroform fraction against axenic amastigotes of *L. donovani*. The ESIMS (base peak chromatogram in positive ion mode) from a separation of 300 µg of fraction is shown. For each microfraction the activity is expressed as percent growth inhibition in comparison to untreated cultures (grey bars). Bold numbers in the chromatogram refer to compounds **1, 2,** and **5**-**10**.

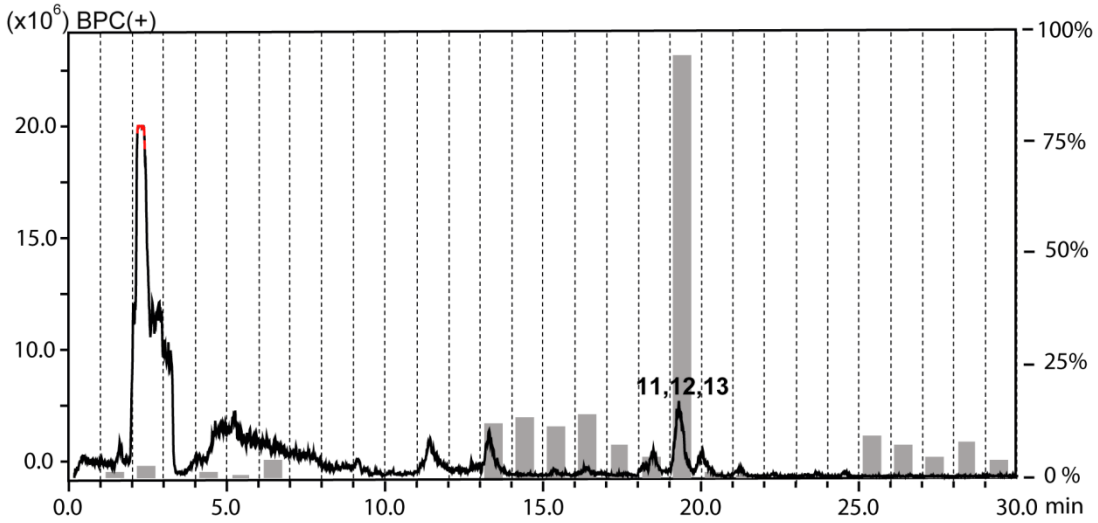

Figure 2. HPLC-based activity profiling of the water fraction against axenic amastigotes of *L. donovani*. The ESIMS (base peak chromatogram in positive ion mode) from a separation of 300 µg of fraction is shown. For each microfraction the activity is expressed as percent growth inhibition in comparison to untreated cultures (grey bars). Bold numbers in the chromatogram refer to compounds **11**-**13**.

4.3.2 Compound Isolation and Structure Elucidation

Preparative separation by MPLC of the chloroform fraction on C_{18} cartridge yielded 13 subfractions (A-M). HPLC‑PDA‑MS analysis showed that subfractions B and C contained peaks from the active time window. Further purification by semipreparative RP-HPLC afforded compounds **1** and **2** from subfraction B. Based on NMR data (Table **S1**, Supporting Information), the two compounds were identified as the known lignans tetrahydrofuroguaiacin B (**1**) and nectandrin B (**2**) (Figure 3) [23–25]. Semipreparative RP-HPLC of subfraction C afforded compounds **5**-**10.** By means of 1D and 2D NMR data (Tables **S2** and **S3**, Supporting Information), these were identified as four cinnamoylphenethyl amides, namely dihydro-feruloyltyramine (**5**) [26], (*E*)-N-feruloyltyramine (**6**) [27], N,N'-diferuloylputrescine (**8**) [28], and 7'-ethoxy-feruloyltyramine (**9**) [29], and two lignans, furoguaiaoxidin (**7**) [25] and 3,3'-dimethoxy-4,4'-dihydroxylignan-9-ol (**10**) [30] (Figure 3). Compounds **9** and **10** showed optical rotation close to zero, and no cotton effects in the ECD spectra. From a comparison with published data for the two compounds, we conclude that they most likely were racemic mixtures.

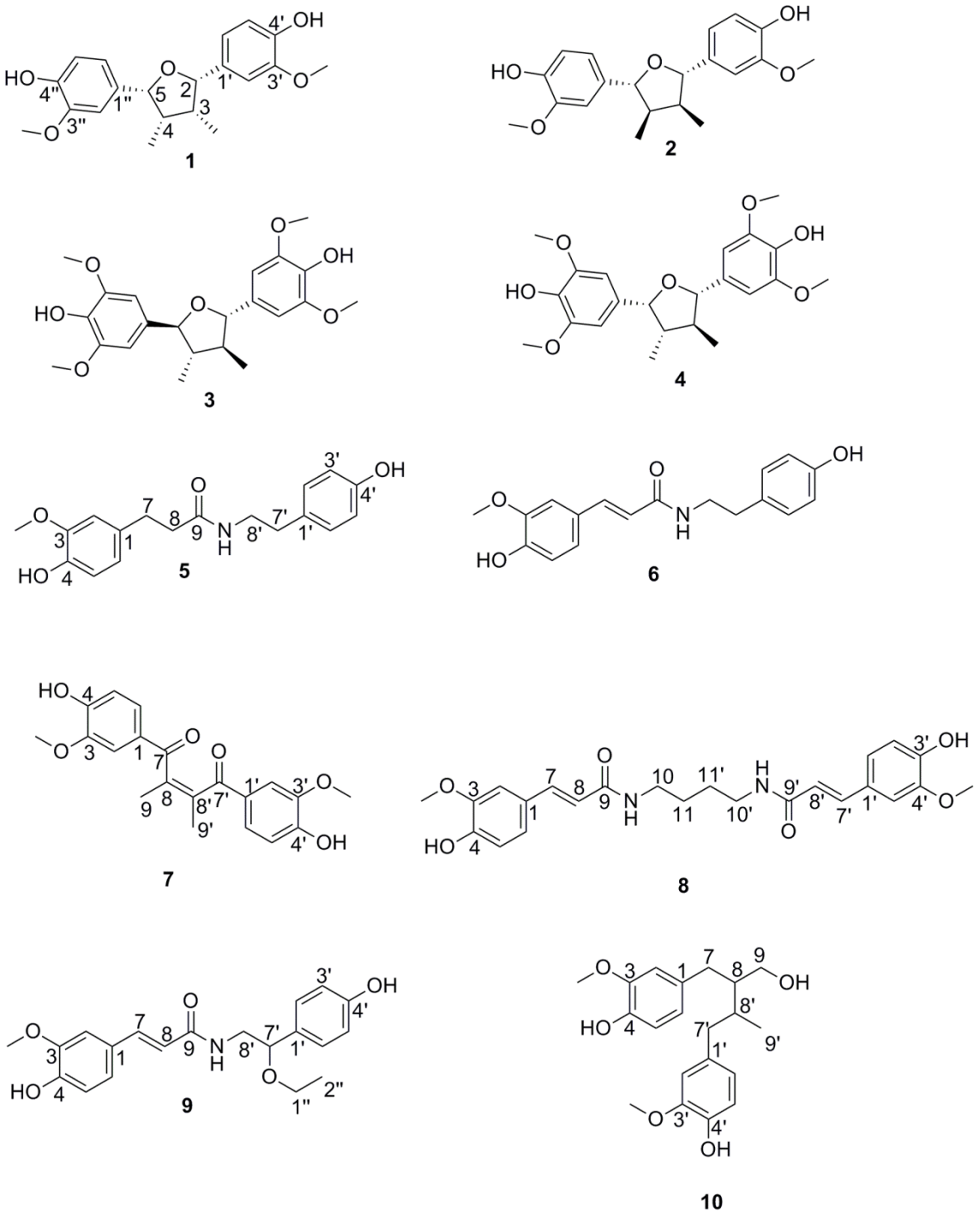

Figure 3. Structures of compounds **1**-**10**.

Preparative RP chromatography by MPLC of the water fraction on a C_{18} cartridge yielded 15 subfractions (A-O), whereby subfractions J and K contained the peaks from the active time window of the activity profile (Figure 2). Semipreparative HPLC on a Hilic column afforded inseparable mixtures of **11** and **12** from subfraction J, and **11** and **13** from subfraction K. The HRESIMS spectrum of 11 exhibited a sodium adduct ion (*m/z* 1039.5083 $[M + Na]^+$, calcd for

$C_{50}H_{80}O_{21}Na^+$ 1039.5085) indicative of a molecular formula of $C_{50}H_{80}O_{21}$. The NMR data (Table **S4**) pointed to a steroidal saponin bearing four sugars. A comparison of ^{13}C chemical shifts with literature data identified the aglycon as yamogenin [31]. This was corroborated by a detailed analysis of the 2D NMR spectra, in particular by cross peaks in the ROESY spectrum between H-1β (δ_H 1.78), H_3-19 (δ_H 0.94), H-8 (δ_H 1.54), H_3-18 (δ_H 0.72), and H-20 (δ_H 1.75), between H-1α (δ_H 0.96) and H-3 (δ_H 3.46), and between H_3-21 (δ_H 0.92), H-17 (δ_H 1.65), H-16 (δ_H 4.27) and H-14 (δ_H 1.07). After hydrolysis and derivatization the sugars were identified as D -glucose, L -rhamnose, and D -xylose [32]. The interglycosidic linkages and the attachment position of the sugar chain at the aglycon were established by HMBC correlations from δ_H 4.43 (1H, d, *J* = 7.5 Hz, H-1-Glc1) to δc 76.9 (C-3), from δ_H 5.04 (1H, br s, H-1-Rha1) to δc 76.4 (C-2-Glc1), from δ_H 4.34 (1H, d, *J* = 7.5 Hz, H-1-Glc2) to δc 81.1 (C-4-Glc1), and from δ_H 4.38 (1H, d, *J* = 7.1 Hz, H-1-Xyl) to δc 86.4 (C-3-Glc2). saponin **11** was identified as (*3*S,*20*S,*22R*,*25*S)-spirost-5-en-3-yl-(β-D-xylopyranosyl-(1 → 3)-β-D-glucopyranosyl-(1→4)[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside [33].

Compound **12** had a molecular formula of $C_{51}H_{82}O_{21}$ (HRESIMS data *m/z* 1053.5233 $[M + Na]^+$, calcd for $C_{51}H_{82}O_{21}Na^+$ 1053.5241). As for compound **11**, NMR data (Table **S4**, Supporting Information) indicated yamogenin bearing four sugars. The only difference was in the presence of a rhamnopyranose instead of a xylopyranose. Thus, saponin **12** was identified as (*3*S,*20*S,*22R*,*25*S)-spirost-5-en-3-yl-(β-D-rhamnopyranosyl-(1 → 3)-β-D-glucopyranosyl-(1 → 4)[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside [34]. Compound **13** had a molecular formula of $C_{50}H_{80}O_{21}$ (*m/z* 1039.5072 $[M + Na]^+$, calcd for $C_{50}H_{80}O_{21}Na^+$ 1039.5085). Based on the NMR data and especially the carbon chemical shifts (Table **S4**, Supporting Information), the aglycone was identified as diosgenin [31]. The sugar moiety was identical to that in saponin **11**. Compound **13** was therefore identified as (*3*S,*20*S,*22*R,*25*R)-spirost-5-en-3-yl-(β-D-xylopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside [33] (Figure 4). It is interesting to note that **11** and **13** are diastereoisomers with with C-25 methyl group bearing axial and equatorial orientations, respectively.

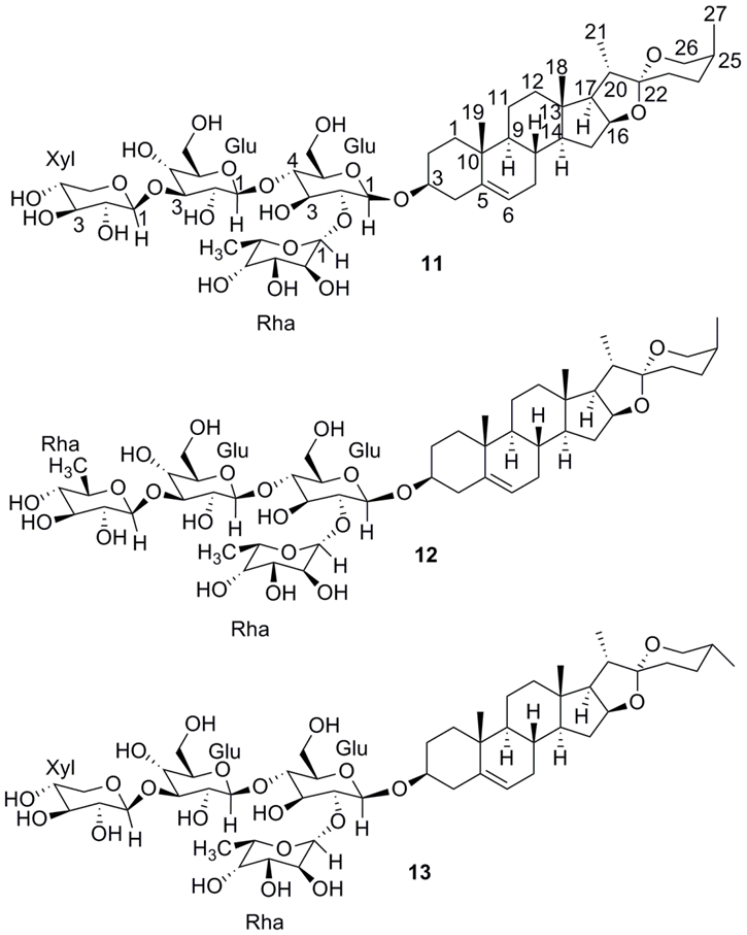

Figure 4. Structures of saponins **11**-**13**.

4.3.3 Comparison to previously reported compounds

The two tetrahydrofuran-type lignans **1** and **2** were previously reported from different Myristicaceae, Elaeagnaceae, Poaceae, and Piperaceae species [24,35].

Arylnaphthalen-type lignans had been identified in aerial part and roots of *H. tuberculatum* [36,37], but tetrahydrofuran-type lignans are reported here for the first time from *Haplophyllum* species.

Cinnamoylphenethyl amides such as **5**, **6**, **8** and **9** have been reported from over 30 families of flowering plants [38]. Amides **5** and **6** have been previously reported from different species of Annonaceae [26,39,40] and Lauraceae [41,42]. Compound **6** was also found in Papaveraceae [43], Cannabaceae [44], Solanaceae [27,45], and Portulacaceae [46,47], while **9** was identified in Portulacaceae [46,47] and Cactaceae [29]. Amide **7** has been previously reported from *Guaiacum officinale* [25], while compound **8** has been found in *Tribulus*

terrestris [48], *Peltophorum pterocarpum* [49], and *Zea mays* [50]. Amide **10** has been previously identified in *Schisandra bicolor* var. *tuberculata* [30].

Molluscicidal saponins **11**-**13** have been previously isolated, among a series of similar compounds (balanitins), from the Sudanese medicinal plant *Balanites aegyptiaca* (Zygophyllaceae) [33,34]. It can be assumed that these saponins contributed to the molluscicidal activity [51] that has been described for *H. tuberculatum* [52]. To the best of our knowledge, compounds **1**, **2**, and **5**-**13** have been isolated here for the first time not only from *Haplophyllum* species but also from plants of the Rutaceae family.

4.3.4 Biological testing

Compounds **1**-**10** were tested for their *in vitro* activities against the following protozoan parasites: *Leishmania donovani* (MHOM/ET/67/L82) axenic amastigotes, *Plasmodium falciparum* (NF54) proliferative erythrocytic stages, and *Trypanosoma brucei rhodesiense* (STIB 900) bloodstream forms (Table 1). In parallel, cytotoxicity of these compounds in rat skeletal myoblasts (L-6 cells) was determined in order to obtain an initial assessment of their selectivity. The results are reported in Table **1**. Subfractions **J** and **K** from the aqueous fraction containing steroid saponins **11**and **12** in **J**, and **11** and **13** in **K**, exhibited IC_{50} < 8 µg/mL across all parasites and a selectivity index < 2, indicative of general cytotoxicity.

Table 1: *In vitro* Activity of Compounds **1**–**10** against *L. donovani* (MHOM-ET-67/L82) axenic amastigotes, *P. falciparum* (NF54), *T. b. rhodesiense* (STIB 900), and Cytotoxicity in L6 Cells.

Compound	*L. donovani*		*P. falciparum*		*T. b. rhodesiense*		L6 cells
No.	IC_{50} [a] (µM)	SI [b]	IC_{50} [a] (µM)	SI [b]	IC_{50} [a] (µM)	SI [b]	
1	22.6 ± 6.5	4.2	53.1 ± 2.0	1.8	59.4 ± 2.5	1.6	95.2 ± 9.3
2	4.5 ± 1.0	25.5	9.5 ± 0.1	12.1	48.3 ± 1.3	2.4	115.0 ± 2.6
3	36.0 ± 7.3	4.7	36.1 ± 1.0	4.7	47.5 ± 4.5	3.6	170.5 ± 40.1
4	29.2 ± 6.0	4.8	17.4 ± 2.1	8.0	40.7 ± 0.4	3.4	138.9 ± 3.8
5	141.3 ± 1.9	n.d	158.7	n.d	206.0 ± 66.7	n.d	>317.5
6	99.4 ± 9.3	2.5	68.4 ± 3.8	3.6	120.9 ± 36.9	2.0	246.8 ± 3.7
7	136.5 ± 1.5	n.d	48.1 ± 10.9	n.d	114.0 ± 0.0	n.d	>280.9
8	97.0 ± 0.9	1.5	30.6 ± 8.3	4.8	18.9 ± 8.4	7.8	146.5 ± 18.8
9	69.6 ± 4.6	3.0	30.4 ± 8.0	6.8	72.7 ± 30.4	2.9	207.8 ± 21.6
10	55.3 ± 3.0	2.3	9.3 ± 1.3	13.7	27.5 ± 8.4	4.6	127.6 ± 3.9
Positive control	0.5 [d]		0.01 [e]		0.01 [c]		0.03 [f]

[a] The IC_{50}s are mean values from at least two independent replicates ± absolute deviation.
[b] Selectivity index (SI): IC_{50} in L6 cells divided by IC_{50} in the titled parasitic strain.
[c] Melarsoprol, [d] Miltefosine, [e] Chloroquine [f] Podophyllotoxin.
n.d: Not determined

4.3.5 Activity against *Leishmania donovani* axenic amastigotes

Nectandrin B (**2**) was the most active (IC_{50} of 5 µM) and the most selective (SI 26) of all compounds tested. This finding contrasts with a previous report on nectandrin B being inactive against *L. donovani* [53]. The discrepancy may come from the fact that, in the aforementioned study, Nectandrin B was tested against the promastigote form of the parasite (i.e. the proliferative form in the gut of the sandfly vector) while we tested against the amastigote form (i.e. the proliferative form in the mammalian host). The antileishmanial activity cannot be ascribed to any particular mode of action at this point. In general, some tetrahydrofuran lignans have been reported to inhibit trypanothione reductase [54], an enzyme of a thiol-redox system that is unique to trypanosomatid protozoa and essential for their survival [55,56]. Compared to **2**, tetrahydrofuroguaiacin B (**1**) was less active and more toxic. The two compounds only differ in their stereochemistry at the tetrahydrofuran ring which appears to play a crucial role in the antileishmanial activity of such lignans. To better understand the role of the stereochemistry at the central ring and the contribution of substituents at the aromatic rings, structurally related lignans **3** and **4** that had been previously reported from *Myristica fragrans* [23,24] were also included in the testing. However, both compounds were significantly less active than nectrandrin B (**2**). Unfortunately, the activity of nectandrin B (**2**) against *L. donovani* was lost in the intracellular amastigote assay tested up to a concentration of 30 µM.

4.3.6 Activity against *Plasmodium falciparum*

Lignans **2** and **10** were the most active and least toxic compounds (IC_{50} 9-9.5 µM, SI 12-14). Compound **10** was selectively active against *P. falciparum*. Compared to **2**, fragransin B_1 (**4**) (IC_{50} 17 µM, SI 8), was less active against *P. falciparum*, but was slightly more active and less cytotoxic than its stereoisomer fragransin B_2 (**3**). Amides **8** and **9** showed comparable activity (IC_{50} 30 µM, SI 8), while the congener **5** was the least active among all tested compounds.

A wide spectrum of biological activities of cinnamoylphenethyl amides has been described, including antiproliferative [57], antibacterial [58], antifungal [59] and antioxidant activities [60]. To the best of our knowledge, this is the first report of antileishmanial and antiplasmodial activities of such compounds.

4.3.7 Activity against *Trypanosoma brucei rhodesiense*

Of all compounds tested, diferuloylputrescine (**8**) exhibited the highest activity against *T. b. rhodesiense* and the highest selectivity (IC_{50} 19 µM, SI 8). The lignan **10** showed a lower activity and selectivity index (IC_{50} 28 µM and SI 5), while **5** and **6** were the least active among all tested compounds. None of the tetrahydrofuran lignans **1**-**4** exhibited significant activity against *T. b. rhodesiense.*

4.4 Materials and Methods

General experimental procedures

HPLC grade solvents from Sigma-Aldrich (St. Louis, MO, USA), and Macron Fine Chemicals (Avantor Performance Materials, Phillipsburg, NJ, USA), and ultrapure water from a Milli-Q water purification system (Merck Millipore, Darmstadt, Germany) were used for HPLC separations. For extraction and preparative separation, technical grade solvents (Scharlab S. L., Barcelona, Spain) were used after distillation. Silica gel 60 F_{254} coated aluminum TLC plates were obtained from Merck (Darmstadt, Germany).

Optical rotation was measured in methanol on a JASCO P-2000 digital polarimeter (Tokyo, Japan) equipped with a sodium lamp (589 nm) and a temperature-controlled microcell (10 cm). UV and ECD spectra were recorded in methanol on a Chirascan CD spectrometer (Applied Photophysics, Leatherhead, UK) using 110 QS 1 mm path precision cells (Hellma Analytics, Müllheim, Germany). NMR spectra of compounds **1**, **2**, and **5**-**10** were recorded on a Bruker AVANCE III 500 MHz spectrometer (Billerica, CA, USA) operating at 500.13 MHz for ^{1}H and 125.77 MHz for ^{13}C. ^{1}H, COSY, HSQC, HMBC, and NOESY spectra were measured at 23°C in a 1-mm TXI probe with a z-gradient. The sample volume was 10 µL. NMR spectra of **11**-**13** were recorded on a Bruker AVANCE NEO 600 MHz spectrometer operating at 600.18 MHz for ^{1}H and 150.92 MHz for ^{13}C with an inverse 1.7-mm TCI micro-cryoprobe (30 µL sample volume) at 23°C. This cryogenically cooled probe delivered a 4-fold gain of mass sensitivity over the 1 mm TXI room-temperature probe and enabled the NMR analysis of small sample amounts (µg range), usually obtained for natural products. Spectra were analyzed by Bruker TopSpin 3.5 pl 7 and ACDLabs Spectrus Processor. NMR spectra were recorded in DMSO-d_6 99.9 atom%D (Armar Chemicals, Döttingen, Switzerland).

HRESIMS data were measured in the positive ion mode on an Orbitrap LQT XL mass spectrometer (Thermo Scientific, Waltham, MA, USA). HPLC-PDA-ELSD-ESIMS data were recorded in positive- and negative-ion mode (scan range of *m/z* 200−1500) on a Shimadzu LC-MS/MS 8030 triple quadrupole MS system (Kyoto, Japan) connected via a T-splitter (1:10) to a photo diode array detector (PDA) (SPD-M20A, Shimadzu, Kyoto, Japan), and evaporative light scattering detector (ELSD) (3300, Alltech, Büchi, Flawil, Switzerland). For data acquisition and processing, LabSolutions software (Kyoto, Japan) was used.

Separations were performed on a C18 SunFire column (3.0 × 150 mm i. d., 3.5 µm) equipped with a precolumn (10mm× 3.0mm i. d.) (Waters).

Microfractionation was carried out with the same HPLC instrument connected via a T split to an FC204 fraction collector (Gilson, Mettmenstetten, Switzerland) with only UV detection, using a SunFire C_{18} (3.5 µm, 150 × 3.0 mm i.d.) column equipped with a guard column (10 mm × 3.0 mm i.d.) (Waters). Semipreparative HPLC were performed on an Agilent 1100 system (Santa Clara, CA, USA) with PDA detector. A SunFire C_{18} column (5 µm, 10 × 150mm i. d.) fitted with a guard column (10 × 10mm i.d.) (Waters) and a Nucleodur Hilic column (5 µm , 10 × 150mm i. d.) (Macherey-Nagel), were used for separations. ChemStation software (Agilent Technologies) was used for data acquisition and processing. Preparative HPLC was carried out on a PuriFlash 4100 system (Interchim, Montluçon, France). Separations were performed on Redi*Sep* Rf Gold ®-C18 MPLC cartridge 100g (Teledyne Isco).

Plant Material

Roots of *Haplophyllum tuberculatum* were collected in February 2018 in Khartoum, Sudan. The taxonomic identity was confirmed by the Medicinal and Aromatic Plants Research Institute, Sudan. A voucher specimen (HTR 02) is deposited at the Herbarium of the Faculty of Pharmacy, University of Science and Technology, Omdurman, Sudan. The plant material was dried at room temperature and milled with a hammer mill before extraction.

Extraction

The powdered roots of *H. tuberculatum* (300 g) were extracted with 1 Litre of 70% ethanol under stirring for 24 h. The extract was filtered through Whatman no. 1 filter paper and concentrated in a rotary evaporator to obtain 35.7 g of extract. The extract amount was suspended in water and partitioned consecutively with petroleum ether, chloroform, and ethyl acetate. Three repetitive partitioning procedures, each with 500 mL of either solvent, were performed. In total, 0.4 g of petroleum ether fraction, 4.0 g of chloroform fraction, 2.0 g of ethyl acetate fraction, and 16.4 g of the water fraction wer obtained after evaporation.

Microfractionation for activity profiling

HPLC-based microfractionation of the chloroform and the water fractions was performed [H_2O + 0.1% formic acid (A), CH_3CN + 0.1% formic acid (B); 0→100% B (0-30 min), 100% B

(30-40 min); flow rate 0.4 mL/min; sample concentration 10 mg/mL in DMSO; injection volume 2 x 35 µL] by collecting one-minute fractions from minute 1 to 40 into a 96-deep-well plate. Plates were then dried in a Genevac EZ-2 evaporator (Ipswich, UK) and prepared for antiprotozoal activity testing based on previously established protocols [22,61].

Preparative Isolation

The chloroform fraction was reconstituted in DMSO and separated on a Redi*Sep* Rf Gold ® RP-C18, 100 gm cartridge [H_2O (A), CH_3CN (B); 5→100% B (0-120 min), flow rate 20 mL/min]. A total of 13 subfractions (A-M) were combined based on TLC patterns. The subfractions were analyzed by HPLC-PDA-MS to track peaks previously detected in the active time windows of the activity profile.

Subfraction B (247 mg) was submitted to semipreparative HPLC on a C_{18} column [H_2O (A), CH_3CN (B); 56% B (0−35 min), 56→100% B (35−40 min), 100% B (40−45 min), flow rate 4 mL/min; sample concentration 50 mg/mL in DMSO; injection volume 50 µL; detection at 208 nm] to afford tetrahydrofuroguaiacin B (**1**, 2.2 mg, t_R 22.3 min) and nectandrin B (**2**, 1.9 mg, t_R 29.5 min).

A portion (200 mg) of subfraction C was separated by semipreparative HPLC on a C_{18} column [H_2O (A), MeOH (B); 39→48% B (0-30 min), 48→100% B (30-40 min), 100% B (40−45 min), flow rate 4 mL/min; sample concentration 50 mg/mL in DMSO; injection volume 50 µL; detection at 254 nm], and dihydro-feruloyltyramine (**5**, 2.5 mg, t_R 9.4 min), (*E*)-N-feruloyltyramine (**6**, 10.6 mg, t_R 16.2 min), furoguaiaoxidin (**7**, 1.4 mg, t_R 16.2 min), N,N'-diferuloylputrescine (**8**, 1.4 mg, t_R 23.4 min), 7'-ethoxy-trans-feruloyltyramine (**9**, 1.1 mg, t_R 27.3 min), and 3,3'-dimethoxy-4,4'-dihydroxylignan-9-ol (**10**, 2.5 mg, t_R 36.5 min) were obtained.

For the water fraction, an aliquot (8 g) was re-dissolved in water and separated by preparative HPLC on a Redi*Sep* Rf Gold ® RP-C18, 100 gm cartridge [H_2O (A), CH_3CN (B); 20→65% B (0-120 min), 65→100% B (120−135 min), flow flow rate 20 mL/min]. Fractions with similar TLC patterns were combined to yield 15 subfractions (A-O). HPLC-PDA-MS analysis located the peaks detected in the active time window in subfractions **J** and **K**. Subfraction **J** (17 mg) was submitted to semipreparative HPLC on a C18 column [H_2O (A), CH_3CN (B); 41% B (0−40 min), 41⟶100% B (40−45 min), 100% B (45−50 min), flow rate 4

mL/min; sample concentration 50 mg/mL in DMSO; injection volume 50 µL; detection at 193 nm] to afford compounds **11** and **12** as a mixture (2 mg, t_R 22.3 min).

Subfraction K (53 mg) was submitted to semipreparative HPLC on a C18 column [H_2O (A), CH_3CN (B); 40% B (0−40 min), 40→100% B (40−45 min), 100% B (45−50 min) , flow rate 4 mL/min; sample concentration 50 mg/mL in DMSO; injection volume 50 µL; detection at 193 nm], yielding subfraction K_3. Subfraction K_3 (16 mg) was further purified by semipreparative HPLC on a Nucleodur Hilic column [H_2O (A), CH_3CN (B); 92% B (0−35 min), 92→20% B (35−45 min), flow rate 4 mL/min; sample concentration 50 mg/mL in DMSO; injection volume 50 µL; detection at 193 nm], to afford compounds **11** and **13** as a mixture (4.2 mg, t_R 25.2 min).

Tetrahydrofuroguaiacin (**1**): amorphous solid; $[\alpha]^{25}_D$ -1.9 (*c* 0.2, MeOH); ^{1}H and ^{13}C NMR, see Table S1, Supporting Information; ESI-MS *m/z* 345 $[M+H]^+$.

Nectandrin B (**2**): amorphous solid; $[\alpha]^{25}_D$ -1.2 (*c* 0.2, MeOH); ^{1}H and ^{13}C NMR, see Table S1, Supporting Information; ESI-MS *m/z* 345 $[M+H]^+$.

Fragransin B_2 (**3**): amorphous solid; ESIMS *m/z* 405 $[M + H]^+$.

Fragransin B_1 (**4**): amorphous solid; ESIMS *m/z* 405 $[M + H]^+$.

Dihydro-feruloyltyramine (**5**): amorphous solid; ^{1}H and ^{13}C NMR, see Table S2, Supporting Information; ESIMS *m/z* 316 $[M + H]^+$.

(*E*)-N-*Feruloyltyramine* (**6**): amorphous solid; ^{1}H and ^{13}C NMR, see Table S2,
Supporting Information; ESIMS *m/z* 314 $[M + H]^+$.

Furoguaiaoxidin (**7**): amorphous solid; ^{1}H and ^{13}C NMR, see Table S2,
Supporting Information; ESIMS *m/z* 357 $[M + H]^+$.

N,N'-Diferuloylputrescine (**8**): amorphous solid; ^{1}H and ^{13}C NMR, see Table S2,
Supporting Information; ESIMS *m/z* 441 $[M + H]^+$.

7'-Ethoxy-trans-feruloyltyramine (**9**): amorphous solid; $[\alpha]^{25}_D$ -2.6 (*c* 0.11, MeOH); UV λ_{max} (MeOH) (log ε) 228 (0.23), 288 (0.12), 320 (0.11) nm; ECD (MeOH, *c* 2.0 x 10^{-4} M, 1 mm path length) $\lambda_{max}(\Delta\varepsilon)$ 207 (+4.04), 224 (+2.40), 235 (+1.72) nm; ^{1}H and ^{13}C NMR, see Table S3, Supporting Information; ESIMS *m/z* 358 $[M + H]^+$.

3,3'-Dimethoxy-4,4'-dihydroxylignan-9-ol (**10**): amorphous solid; $[\alpha]^{25}_D$ -4.9 (*c* 0.25, MeOH); UV λ_{max} (MeOH) (log ε) 230 (0.18), 280 (0.08) nm; ECD (MeOH, *c* 1.4 x 10^{-4} M, 1 mm path length) $\lambda_{max}(\Delta\varepsilon)$ 205 (+3.07), 215 (+1.39), 235 (+0.89) nm ;^{1}H and ^{13}C NMR, see Table S3, Supporting Information; ESIMS *m/z* 693 $[2M + H]^+$.

(3S,20S,22R,25S)-Spirost-5-en-3-yl (β-D-xylopyranosyl-(1→3)- β-D-glucopyranosyl-(1→4)[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (**11**): amorphous solid; ^{1}H and ^{13}C NMR, see Table S4, Supporting Information; HRESIMS *m/z* 1039.5083 $[M + Na]^+$ (calcd for $C_{50}H_{80}O_{21}Na^+$ 1039.5085)

(3S,20S,22R,25S)-Spirost-5-en-3-yl (β-D-rhamnopyranosyl-(1→3)- β-D-glucopyranosyl-(1→4)[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (**12**): amorphous solid; ^{1}H and ^{13}C NMR, see Table S4, Supporting Information; HRESIMS *m/z* 1053.5233 $[M + Na]^+$ (calcd for $C_{51}H_{82}O_{21}Na^+$ 1053.5241).

(3S,20S,22R,25R)-Spirost-5-en-3-yl (β-D-xylopyranosyl-(1→3)- β-D-glucopyranosyl-(1→4)[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (**13**)**:** amorphous solid; ^{1}H and ^{13}C NMR, see Table S4, Supporting Information; HRESIMS *m/z* 1039.5072 $[M + Na]^+$ (calcd for $C_{50}H_{80}O_{21}Na^+$ 1039.5085).

Activity against *Leishmania donovani* axenic amastigotes

Amastigotes of *L. donovani* strain MHOM/ET/67/L82 were grown under an atmosphere of 5% CO_2 in air in axenic culture at 37 °C in SM medium [62] at pH 5.4 supplemented with 10% heat-inactivated fetal bovine serum. 50 µL of culture medium was added in the wells of a 96-well plate and serial drug dilutions of eleven 3-fold dilution steps covering a final range from 100 to 0.002 µg/mL were prepared. 50 µL culture medium with $2x10^5$ amastigotes from axenic culture were added to each well. The plates were incubated for 70 h and then inspected under an inverted microscope to assure growth of the controls and sterile conditions. 10 µL of resazurin (12.5 mg resazurin dissolved in 100 mL distilled water) were added to each well of the plates and allowed for additional 2 h incubation. Afterwards, plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analyzed using the software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA). Decrease of fluorescence (= inhibition) was expressed as percentage of the fluorescence of untreated control cultures and plotted against the drug concentrations. From the sigmoidal inhibition curves the IC_{50} values were calculated by linear regression using Microsoft Excel. Miltefosine was used as positive control drug. Assays were performed in two independent replicates at least.

Activity against *Leishmania donovani* intramacrophage amastigotes

Mouse peritoneal macrophages (4 x 10^4 in 100 µL RPMI 1640 medium with 10% heat-inactivated FBS) were seeded into wells of a 96-well plate. After 24 h, 100 µL of 2 x 10^5 amastigote *Leishmania donovani* were added. The amastigotes were taken from an axenic amastigote culture grown at pH 5.4. The medium containing free amastigote forms was removed after 24 h and replaced with fresh medium. The washing step was repeated and afterwards the serial drug dilution was prepared with at least 6 dilution steps. Compound was dissolved in DMSO at 10 mg/mL and further diluted in medium. After incubation for 96 hours at 37 °C under a 5 % CO_2 atmosphere, the medium was removed and cells were fixed by adding 50µl 4% formaldehyde solution followed by a staining with a 5 µM DRAQ5 solution. Plates were imaged in ImageXpress XLS (MD) microscope using a 20x air objective (635 nm excitation: 690/50 emission). 9 images were collected per well. Automated image analysis was performed with a script developed on Meta Xpress Software (MD). Three outputs were provided for each well: i) number of host cell nuclei; ii) numbers of infected and non-infected host cells; iii) number of parasite nuclei per infected host cell. The IC_{50} values were calculated based on the infection rate and the numbers of intracellular amastigotes. Miltefosine was used as control. Assays were performed in two independent replicates at least.

Activity against *Plasmodium falciparum*

In vitro activity against the erythrocytic stages of *P. falciparum* was determined using a ^{3}H-hypoxanthine incorporation assay [63], using the drug-sensitive NF54 strain [64]. Compounds were dissolved in DMSO at 10 mg/mL and further diluted in medium before addition to parasite cultures incubated in RPMI 1640 medium without hypoxanthine, supplemented with HEPES (5.94 g/L), $NaHCO_3$ (2.1 g/L), neomycin (100 U/mL), Albumax^R (5 g/L) and washed human red cells A^+ at 2.5% haematocrit (0.3% parasitaemia). Serial drug dilutions of eleven 3-fold dilution steps covering a range from 100 to 0.002 µg/mL were prepared. The 96-well plates were incubated in a humidified atmosphere at 37 °C; 4% CO_2, 3% O_2, 93% N_2. After 48 h 50 µl of ^{3}H-hypoxanthine (=0.5 µCi) was added to each well of the plate. The plates were incubated for a further 24 h under the same conditions. The plates were then harvested with a Betaplate™ cell harvester (Wallac, Zurich, Switzerland), and the red blood cells transferred onto a glass fibre filter then lysed with distilled water. The dried

filters were inserted into a plastic foil with 10 mL of scintillation fluid, and counted in a Betaplate™ liquid scintillation counter (Wallac, Zurich, Switzerland). IC_{50} values were calculated from sigmoidal inhibition curves by linear regression using Microsoft Excel. Chloroquine (Sigma C6628) was used as positive control. Assays were performed in two independent replicates at least.

Activity against *Trypanosoma brucei rhodesiense*

The stock was originally isolated from a Tanzanian patient and adapted to axenic culture conditions after several mouse passages and cloned. Minimum Essential Medium (50 µL) supplemented with 25 mM HEPES, 1g/L additional glucose, 1% MEM non-essential amino acids (100x), 0.2 mM 2-mercaptoethanol, 1mM Na-pyruvate [65] and 15% heat inactivated horse serum was added to each well of a 96-well microtiter plate. Serial drug dilutions of eleven 3-fold dilution steps covering a range from 100 to 0.002 µg/mL were prepared. Then $4x10^3$ bloodstream forms of *T. b. rhodesiense* STIB 900 in 50 µL were added to each well and the plate incubated at 37 °C under a 5 % CO2 atmosphere for 70 h. 10 µL resazurin solution (resazurin, 12.5 mg in 100 mL double-distilled water) was then added to each well and incubation continued for a further 2–4 h [66]. Then the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analyzed with the graphic programme Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA), which calculated IC_{50} values by linear regression [67], and 4-parameter logistic regression from the sigmoidal dose inhibition curves. Melarsoprol (Arsobal Sanofi-Aventis, received from WHO) was used as control. Assays were performed in two independent replicates at least.

***In vitro* cytotoxicity with L-6 cells**

Assays were performed in 96-well microtiter plates, each well containing 100 µL of RPMI 1640 medium supplemented with 1% L-glutamine (200mM) and 10% fetal bovine serum, and 4000 L-6 cells (a primary cell line derived from rat skeletal myoblasts) [68]. Serial drug dilutions of eleven 3-fold dilution steps covering a range from 100 to 0.002 µg/mL were prepared 24 h post seeding L-6 cells. After 70 hours of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. 10 µL

of resazurin was then added to each well and the plates incubated for another 2 hours. Then the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. The IC_{50} values were calculated by linear regression and 4-parameter logistic regression from the sigmoidal dose inhibition curves using SoftmaxPro software (Molecular Devices Cooperation, Sunnyvale, CA, USA). Podophyllotoxin (Sigma P4405) was used as control. All assays were performed in two independent replicates at least.

Acknowledgments

We wish to thank Monica Cal, Romina Rocchetti and Sonja Keller for help with antiparasitic drug testing, O. Fertig for the sugar analysis, and professors Suad Sulaiman and Marcel Tanner for their mentorship. We gratefully acknowledge financial support by the Amt für Ausbildungsbeiträge Basel and the Emilia Guggenheim-Schnurr Foundation.

Conflict of Interest

The authors declare no conflict of interest.

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4.6 Supporting Information:

Lignans, Amides, and Saponins from *Haplophyllum tuberculatum* and their Antiprotozoal Activity

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Table S 1. ^{1}H and ^{13}C NMR Spectroscopic Data for Compound **1** and **2** (DMSO-*d6*; 500.13 MHz for ^{1}H and 125.77 for ^{13}C NMR; δ in ppm)

	1		**2**	
Position	δ_C [a]	δ_H (mult *J* in Hz)	δ_C [a]	δ_H (mult *J* in Hz)
2	81.9, CH	4.98, d (6.4)	86.5, CH	4.34, d (5.8)
3	40.7, CH	2.62, m	43.8, CH	2.20, m
4	40.7, CH	2.62, m	43.8, CH	2.20, m
5	81.9, CH	4.98, d (6.4)	86.5, CH	4.34, d (5.8)
1', 1"	131.4, C		133.1, C	
2', 2"	110.7, CH	6.94, s	110.8, CH	6.95, s
3', 3"	147.2, C		147.5, C	
4', 4"	145.3, C		146.1, C	
5', 5"	115.1, CH	6.79 [b]	115.3, CH	6.78 [b]
6', 6"	118.7, CH	6.79 [b]	118.8, CH	6.81 [b]
3-Me	11.5, CH_3	0.51, d (6.4)	12.6, CH_3	0.94, d (6.1)
4-Me	11.5, CH_3	0.51, d (6.4)	12.6, CH_3	0.94, d (6.1)
3'-OMe, 3"-OMe	55.6, CH_3	3.76, s	55.6, CH_3	3.75, s

[a] ^{13}C NMR data extracted from HSQC and HMBC spectra, [b] Overlapping signals.

Table S2. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds **5-8** (DMSO-*d6*; 500.13 MHz for ^{1}H and 125.77 for ^{13}C NMR; δ in ppm)

	5		**6**		**7**		**8**	
Position	δ_C [a]	δ_H (mult *J* in Hz)	δ_C [a]	δ_H (mult *J* in Hz)	δ_C [a]	δ_H (mult *J* in Hz)	δ_C [a]	δ_H (mult *J* in Hz)
1	132.2, C		126.8, C		[c]		[c]	
2	112.7, CH	6.76, d (1.5)	111.3, CH	7.14, br s	112.0, CH	7.01, d (1.5)	111.0, CH	7.11, br s
3	147.4, C		148.1, C		[c]		[c]	
4	144.8, C		148.6, C		[c]		[c]	
5	115.2, CH	6.70 [b]	116.1, CH	6.87, d (8.2)	114.8, CH	6.76, d (8.2)	115.9, CH	6.82, d (8.2)
6	120.3, CH	6.59, dd (7.8, 1.4)	121.9, CH	7.03 [b]	124.5, CH	7.14, dd (8.2, 1.5)	121.5, CH	6.98, br d (8.2)
7	30.8, CH_2	2.72, t (7.6)	139.6, CH	7.46, d (15.6)	[c]		138.8, CH	7.32, d (15.9)
8	37.4, CH_2	2.34, t (7.8)	119.3, CH	6.56, d (15.6)	[c]		119.3, CH	6.46, d (15.9)
9	171.6, C		166.2, C		16.9, CH_3	2.03, s	[c]	
10							38.3, CH_2	3.20 [b]
11							26.9, CH_2	1.50, m
1'	132.2, C		129.8, C		[c]		[c]	
2'	129.3, CH	6.95, d (8.4)	129.7, CH	7.05 [b]	112.0, CH	7.01, d (1.5)	111.0, CH	7.11, br s
3'	115.2, CH	6.70 [b]	115.5, CH	6.76, d (8.2)	[c]		[c]	
4'	155.7, C		155.9, C		[c]		[c]	
5'	115.2, CH	6.70 [b]	115.5, CH	6.76, d (8.2)	114.8, CH	6.76, d (8.2)	115.9, CH	6.82, d (8.2)
6'	129.3, CH	6.95, d (8.4)	129.7, CH	7.05 [b]	124.5, CH	7.14, dd (8.2, 1.5)	121.5, CH	6.98, br d (8.2)
7'	34.4, CH_2	2.58, t (7.3)	34.7, CH_2	2.73, t (7.0)	[c]		138.8, CH	7.32, d (15.9)
8'	40.5, CH_2	3.22, dt (5.2, 6.7)	40.9, CH_2	3.45, dt (5.5, 5.5)	[c]		119.3, CH	6.46, d (15.9)
9'					16.9, CH_3	2.03, s	[c]	
10'							38.3, CH_2	3.20 [b]
11'							26.9, CH_2	1.50, m
3-OMe	55.7, CH_3	3.75, s	55.9, CH_3	3.81, s				
3'-OMe					55.5, CH_3	3.70, s	55.5, CH_3	3.81, s
NH		7.77, t (5.2)		7.97, t (5.5)				7.93, t (4.9)

[a] ^{13}C NMR data extracted from HSQC and HMBC spectra, [b] Overlapping signals, [c] Signal not visible due to low amount of compound.

Table S3. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds **9-10** (DMSO-*d6*; 500.13 MHz for ^{1}H and 125.77 for ^{13}C NMR; δ in ppm)

	9		**10**	
Position	δ_C [a]	δ_H (mult *J* in Hz)	δ_C [a]	δ_H (mult *J* in Hz)
1	126.3, C		132.3, C	
2	111.1, CH	7.11 [b]	113.1, CH	6.68 [b]
3	147.8, C		147.2, C	
4	147.6, C		144.2, C	
5	115.7, CH	6.82, d (7.9)	115.0, CH	6.70 [b]
6	121.7, CH	6.97, dd (7.9, 1.5)	121.0, CH	6.57, dt (7.8, 2.5)
7	139.1, CH	7.31, d (15.6)	32.0, CH_2	2.56, dd (13.7, 5.5) 2.32 [b]
8	119.0, CH	6.52, d (15.9)	45.7, CH	1.67, m
9	165.5, C		61.0, CH_2	3.34, dd (12.5, 7.0)
1'	130.5, C		132.3, C	
2'	127.7, CH	7.11 [b]	113.1, CH	6.68 [b]
3'	115.1, CH	6.77, d (8.2)	147.2, C	
4'	157.2, C		144.2, C	
5'	115.1, CH	6.77, d (8.2)	115.0, CH	6.70 [b]
6'	127.7, CH	7.11 [b]	121.0, CH	6.57, dt (7.8, 2.5, 2.5)
7'	79.5, CH	4.28, dd (7.5, 5.0)	39.1, CH_2	2.64, dd (13.7, 6.4) 2.32 [b]
8'	45.3, CH_2	3.34, d	33.6, CH	2.04, m
9'			15.1, CH_3	0.82, d (6.7)

1"	63.3, CH_2	3.31, q (7.0)		
2"	15.1, CH_3	1.10, t (6.9)		
3-OMe	55.7, CH_3	3.81, s	55.4, CH_3	3.73, s
3'-OMe			55.4, CH_3	3.73, s
NH		7.86, t (5.5)		

[a] ^{13}C NMR data extracted from HSQC and HMBC spectra, [b] Overlapping signals.

Table S4. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds **11-13** (DMSO-*d6*; 600.18 Hz for ^{1}H and 150.92 for ^{13}C NMR; δ in ppm)

	11		**12**		**13**	
Position	δ_C [a]	δ_H (mult *J* in Hz)	δ_C [a]	δ_H (mult *J* in Hz)	δ_C [a]	δ_H (mult *J* in Hz)
1	37.2, CH_2	1.78 [b] 0.96 [b]	37.2, CH_2	1.78 [b] 0.96 [b]	37.1, CH_2	1.76 [b] 0.96 [b]
2	29.4, CH_2	1.77 [b] 1.44 [b]	29.4, CH_2	1.77 [b] 1.44 [b]	29.4, CH_2	1.78 [b] 1.43 [b]
3	76.9, CH	3.46 [b]	76.9, CH	3.46 [b]	76.9, CH	3.46 [b]
4	38.1, CH_2	2.39, br d (11.4) 2.15, dd (11.4, 11.4)	38.1, CH_2	2.39, br d (11.4) 2.15, dd (11.4, 11.4)	38.0, CH_2	2.38 [c] 2.15 [c]
5	140.7, C		140.7, C		140.6, C	
6	121.8, CH	5.33, m	121.8, CH	5.33, m	121.5, CH	5.30 [c]
7	31.9, CH_2	1.91 [b] 1.49 [b]	31.9, CH_2	1.91 [b] 1.49 [b]	31.8, CH_2	1.92 [b] 1.47 [b]
8	31.4, CH	1.54 [b]	31.4, CH	1.54 [b]	31.3, CH	1.53 [b]
9	50.0, CH	0.88 [b]	50.0, CH	0.88 [b]	50.0, CH	0.87 [b]
10	36.8, C		36.8, C		36.8, C	
11	20.8, CH_2	1.47 [b] 1.38 [b]	20.8, CH_2	1.47 [b] 1.38 [b]	20.7, CH_2	1.46 [b] 1.37 [b]
12	39.5, CH_2	1.67 [b] 1.12 [b]	39.5, CH_2	1.67 [b] 1.12 [b]	39.5, CH_2	1.67 [b] 1.11 [b]
13	40.2, C		40.2, C		39.8, C	
14	56.2, CH	1.07 [b]	56.2, CH	1.07 [b]	56.2, CH	1.05 [b]
15	31.8, CH_2	1.88 [b] 1.17 [b]	31.8, CH_2	1.88 [b] 1.17 [b]	31.4, CH_2	1.86 [b] 1.17 [b]
16	80.7, CH	4.27 [b]	80.7, CH	4.27 [b]	80.5, CH	4.26 [b]
17	62.0, CH	1.65 [b]	62.0, CH	1.65 [b]	61.8, CH	1.64 [b]

18	16.4, CH_3	0.72, s	16.4, CH_3	0.72, s	16.3, CH_3	0.71 [b]
19	19.4, CH_3	0.94, s	19.4, CH_3	0.94, s	19.2, CH_3	0.93, s
20	42.0, CH	1.75 [b]	42.0, CH	1.75 [b]	41.4, CH	1.78 [b]
21	14.9, CH_3	0.92, d (5.6)	14.9, CH_3	0.92, d (5.6)	14.6, CH_3	0.88 [c]
22	109.4, C		109.4, C		108.5, C	
23	25.9, CH_2	1.80 [b] 1.26 [b]	25.9, CH_2	1.80 [b] 1.26 [b]	30.9, CH_2	1.60 [b] 1.45 [b]
24	25.8, CH_2	1.87 [b] 1.33 [b]	25.8, CH_2	1.87 [b] 1.33 [b]	28.5, CH_2	1.54 [b] 1.29 [b]
25	26.8, CH	1.63 [b]	26.8, CH	1.63 [b]	30.1, CH	1.50 [b]
26	64.7, CH_2	3.78, br d (10.0) 3.21 [b]	64.7, CH_2	3.78, br d (10.0) 3.21 [b]	65.9, CH_2	3.38 [b] 3.17 [b]
27	16.3, CH_3	0.99, d (6.0)	16.3, CH_3	0.99, d (6.0)	17.4, CH_3	0.71 [b]
1- Glc1	98.4, CH	4.43, d (7.5)	98.4, CH	4.43, d (7.5)	98.4, CH	4.41 [b]
2- Glc1	76.4, CH	3.22 [b]	76.4, CH	3.22 [b]	76.0, CH	3.23 [b]
3- Glc1	76.4, CH	3.50 [b]	76.4, CH	3.50 [b]	76.3, CH	3.51 [b]
4- Glc1	81.1, CH	3.32 [b]	81.1, CH	3.32 [b]	81.0, CH	3.34 [b]
5- Glc1	74.9, CH	3.28 [b]	74.9, CH	3.28 [b]	74.7, CH	3.27 [b]
6- Glc1	60.5, CH_2	3.69 [b] 3.58 [b]	60.5, CH_2	3.69 [b] 3.58 [b]	60.5, CH_2	3.70 [b] 3.59 [b]

[a] ^{13}C NMR data extracted from HSQC and HMBC spectra, [b] Overlapping signals, [c] broad signal due to concentrated sample.

Table S4. (continued)

	11		**12**		**13**	
Position	δ_C [a]	δ_H (mult *J* in Hz)	δ_C [a]	δ_H (mult *J* in Hz)	δ_C [a]	δ_H (mult *J* in Hz)
1- Rha1	100.4, CH	5.04, br s	100.4, CH	5.04, br s		
2- Rha1	70.8, CH	3.63 [b]	70.8, CH	3.63 [b]	70.7, CH	3.64 [b]
3- Rha1	71.0, CH	3.40 [b]	71.0, CH	3.40 [b]	70.9, CH	3.40 [b]
4- Rha1	72.3, CH	3.18 [b]	72.3, CH	3.18 [b]	72.2, CH	3.19 [b]
5- Rha1	68.3, CH	3.97, m	68.3, CH	3.97, m	68.2, CH	3.97 [c]
6- Rha1	18.2, CH_3	1.07 [b]	18.2, CH_3	1.07 [b]	18.0, CH_3	1.08 [b]
1- Glc2	103.0, CH	4.34, d (7.5)	103.2, CH	4.28 [b]	102.9, CH	4.34 [b]
2- Glc2	73.1, CH	3.18 [b]	74.4, CH	3.09 [b]	72.9, CH	3.20 [b]
3- Glc2	86.4, CH	3.40 [b]	81.3, CH	3.38 [b]	86.2, CH	3.41 [b]
4- Glc2	68.4, CH	3.16 [b]	68.7, CH	3.11 [b]	68.3, CH	3.16 [b]
5- Glc2	76.9, CH	3.24 [b]	77.0, CH	3.24 [b]	76.4, CH	3.26 [b]
6- Glc2	61.1, CH_2	3.66 [b] 3.42 [b]	61.1, CH_2	3.66 [b] 3.42 [b]	60.9, CH_2	3.68 [b] 3.41 [b]
1- Xyl	105.0, CH	4.38, d (7.1)	-	-	104.7, CH	4.38 [b]
2- Xyl	74.1, CH	3.05 [b]	-	-	73.9, CH	3.06 [b]
3- Xyl	76.6, CH	3.13 [b]	-	-	76.1, CH	3.14 [b]

4- Xyl	69.8, CH	3.29 [b]	-	-	69.6, CH	3.30 [b]
5- Xyl	66.2, CH_2	3.72 [b] 3.06 [b]	-	-	66.0, CH_2	3.73 [b] 3.08 [b]
1- Rha2	-	-	101.0, CH	5.01, br s		
2- Rha2	-	-	70.9, CH	3.69 [b]		
3- Rha2	-	-	70.9, CH	3.47 [b]		
4- Rha2	-	-	72.4, CH	3.17 [b]		
5- Rha2	-	-	68.5, CH	3.87, m		
6- Rha2	-	-	18.2, CH_3	1.07 [b]		

[a] ^{13}C NMR data extracted from HSQC and HMBC spectra, [b] Overlapping signals, [c] broad signal due to concentrated sample.

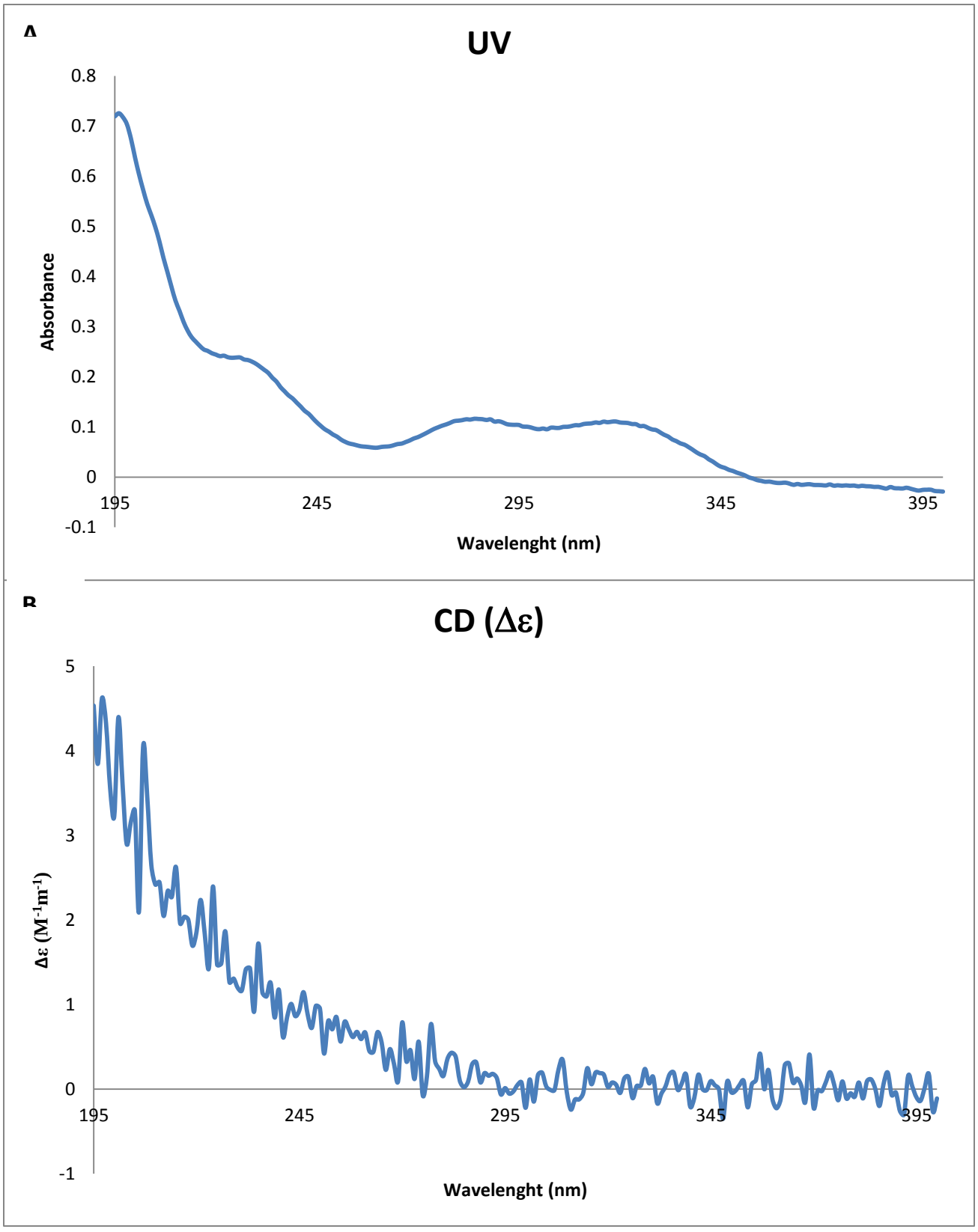

Figure S5. UV (**A**) and ECD (**B**) spectra for compound **9** in MeOH (0.07 mg/mL).

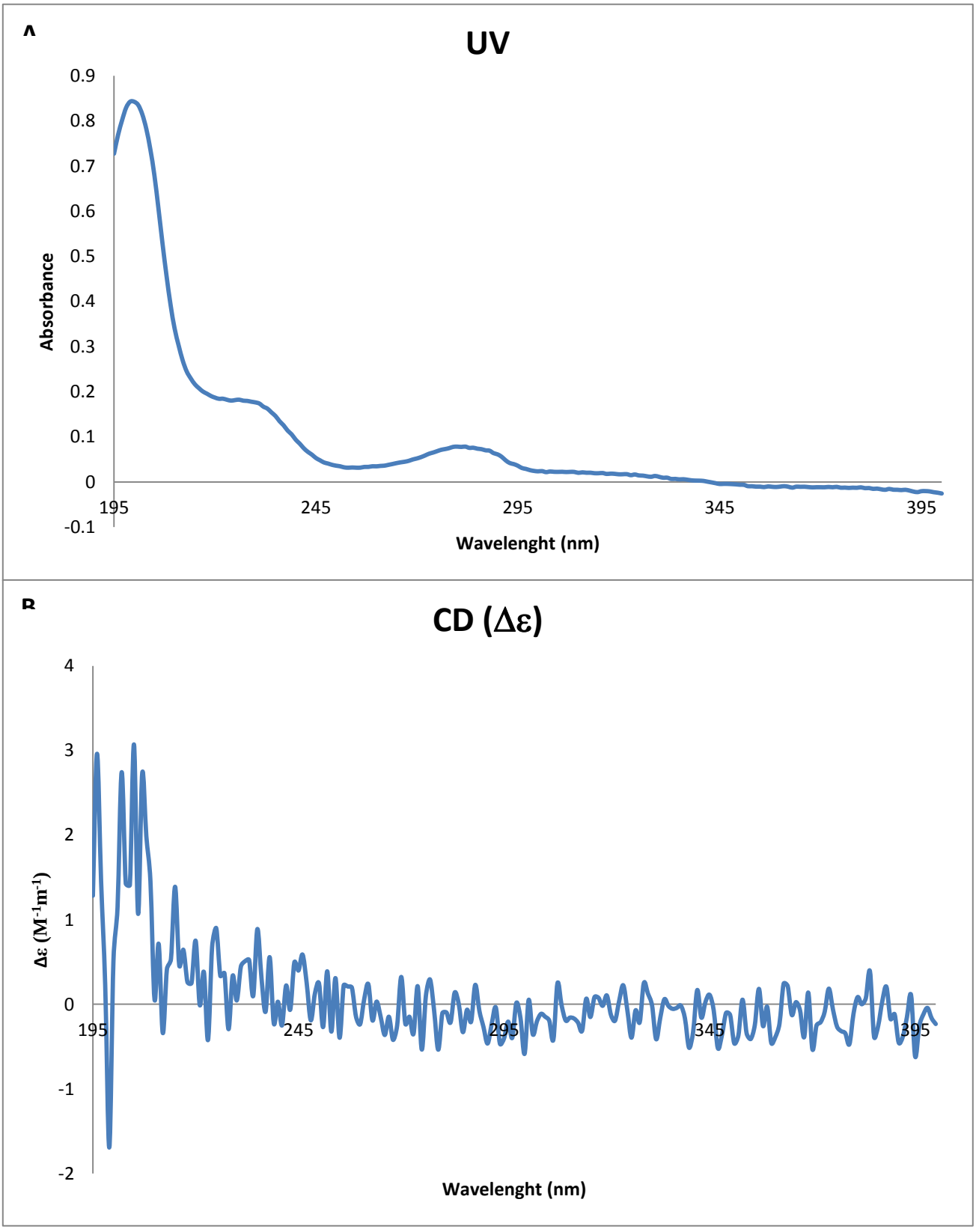

Figure S2. UV (**A**) and ECD (**B**) spectra for compound **10** in MeOH (0.05 mg/mL).

5. Natural Products Against *Madurella mycetomatis*

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5.1 Abstract

Eumycetoma is a chronic, debilitating, inflammatory fungal infection caused mainly by *Madurella mycetomatis* and recently enlisted by the WHO among the neglected tropical diseases (NTDs). Current therapies include long-term treatment with itraconazole and surgical intervention in most of the cases. Given the limited efficacy, frequent side effects, and unaffordable price of treatment, there is an urgent need for new antimycetomal drugs. The chloroform and the water fractions of the ethanolic extract of *Haplophyllum tuberculatum* roots (Forssk.) A. Juss. (Rutaceae) were selected from a subset of extracts from a repository of Sudanese medicinal plants traditionally used as anti-infectives. Isolated compounds **1**-**13**, obtained by HPLC-activity profiling, were screened for *in vitro* activity against *M. mycetomatis* employing a resazurin-based viability assay. Additional natural compounds isolated from other plant species, and which had been reported for their antifungal and anti-infective activities, were also screened for their antimycetomal activity. Of these compounds; eudesmane sesquiterpenes from *Verbesina lanata* B. L. Rob. & Greenm. (Asteraceae) (**14**-**27**), the neolignans honokiol (**28**) and magnolol (**29**), as well as the diterpenes serratol (**30**), and 1*S*,3*E*,7*R*,8*R*,11*E*-7,8-epoxy-cembra-3,11-dien-1-ol (**31**), isolated from *Boswellia serrata* (Burseraceae). The MICs against one or more *M. mycetomatis* strains, along with the cytotoxicity against L-6 rat skeletal cells, were determined. The eudesmane sesquiterpenes (**14**, **15**, and **22**) possessed MIC values within a range of 20 to 40 µM. Of them, 6β-Cinnamoyloxy-1β,2α-dihydroxyeudesm-4(15)-ene (**22)** exhibited the highest antifungal activity (MIC of 20.8 µM), and selectivity (SI 1.3) against *M. mycetomatis*. The lignans, honokiol (**28**) and magnolol (**29**), showed similar activity profiles against the SO1 strain (MIC value 30.1 µM). However, magnolol exhibited 2-fold higher selectivity indices compared to honokiol. Moreover, magnolol (**29**) possessed the highest activity (MIC 15 µM) and selectivity (SI 4.9) against the CBS131320 strain among all tested compounds.

5.2 Introduction

Mycetoma is a chronic, progressively destructive, morbid inflammatory disease acquired by traumatic inoculation of certain fungi (Eumycetoma) or bacteria (Actinomycetoma) into the subcutaneous tissue [1]. Usually the foot is the most affected part but any part of the body can be involved [2]. It is one of the most neglected diseases at all levels and was recently included in the WHO list of neglected tropical diseases (NTDs) [3]. The disease is geographically distributed through what is called “the Mycetoma belt”, which includes India, Yemen, Somalia, Sudan, Senegal, Mexico, Venezuela, Colombia, and Argentina [4]. Sudan is among the most affected countries with more than 6000 cases, of which 64% are under the age of 30 [5]. The bacterial type of mycetoma, actinomycetoma, is readily cured by antibiotic combination therapy [6]. In contrast, management of the fungal type (Eumycetoma), which accounts for 70% of the cases in Sudan [5], is much more challenging. Treatment usually involves surgical excision combined with prolonged antifungal therapy, which has limited efficacy, toxic adverse effects, is expensive, and has high percentage of treatment failures [7]. Late chronic stages of the disease result in destruction, deformity, loss of function, and may often lead to amputation. Hence, there is a dire need to find new therapeutic agents for eumycetoma that are efficient, affordable, safe, and reduce the treatment period and surgical interventions [8].

Fungi are important plant pathogens. Many natural products derived from plant secondary metabolites with a wide variety of scaffolds, namely alkaloids, terpenoids, saponins, and phenolic compounds have antifungal activity and could serve as potential hits also against human pathogenic fungi [9,10]. However, very limited reports [11-14] are available on the antimycetomal activity of natural products.

In an ongoing search for promising hits against eumycetoma, several Sudanese medicinal plant extracts were screened for their *in vitro* activity against *Madurella mycetomatis* using the resazurin viability assay [15]. Of these extracts, the chloroform

and the water fractions of the ethanolic extract of *Haplophyllum tuberculatum* roots (Forssk.) A. Juss. (Rutaceae) revealed inhibitory activity towards eumycetoma (MIC >80% at 10 µg/mL). Compounds isolated from these fractions by HPLC- activity profiling approach were screened for their antimycetomal activity.

Moreover, we persued an "educated-guess" approach to find additional antimycetomal hits of natural origin. A set of natural compounds of different classes, belonging to different plant species, that have been previously reported for their antifungal and anti-infective activities were also screened for their activity against *M. mycetomatis.*

5.3 Results and Discussion

In a screening of Sudanese medicinal plant extracts for antimycetomal activity, the ethanolic extract of *Haplophyllum tuberculatum* roots (Forssk.) A. Juss. (Rutaceae), was found active (MIC >80% at 10 µg/mL). HPLC-based activity profiling allocated the activity to the chloroform and the water fractions. Combination of preparative and semi-preparative chromatography yielded compounds **1**-**10** from the chloroform fraction (Figure 1). The water fraction yielded subfractions **J** (containing a mixture of compounds **11** and **12**) and subfraction **K** (containing a mixture of compounds **11** and **13**) (Figure 2) [16].

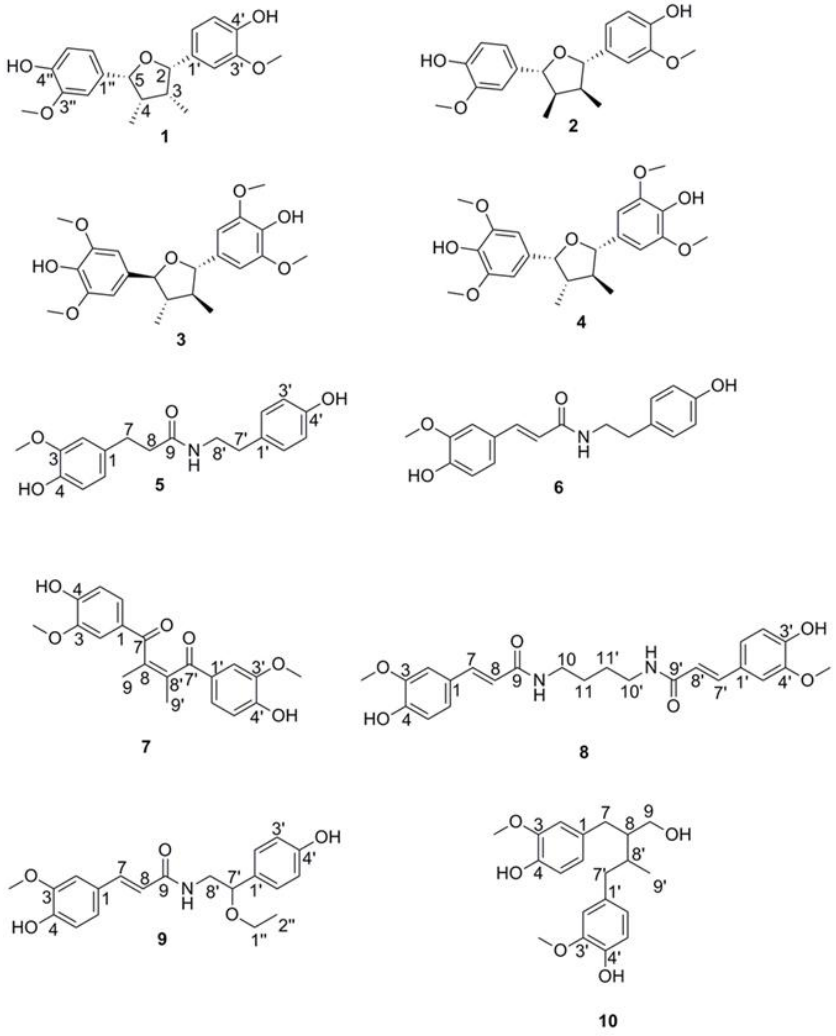

Figure 1. Structures of compounds **1**-**10** from *Haplophyllum tuberculatum*.

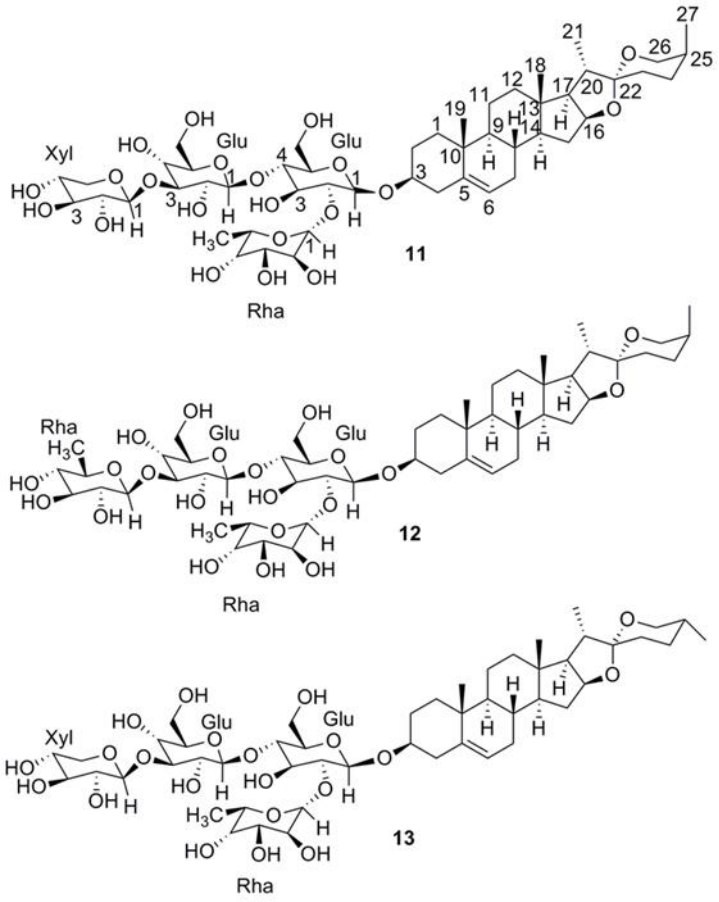

Figure 2. Structures of saponins **11**-**13** from *Haplophyllum tuberculatum*.

These compounds were screened in a 96-well microtitre plates for *in vitro* activity against *M. mycetomatis* employing resazurin viability assay [15] (Table 1). The Minimal inhibitory concentration (MIC) was defined as the lowest concentration of the compound that exhibited ≥ 80% growth inhibition. The MICs against one or more of *M. mycetomatis* strains, along with the cytotoxicity against L-6 rat skeletal cells, were determined.

The compounds isolated from *Haplophyllum tuberculatum* **1**-**10** as well as the **J** and **K** fractions (mixtures of compounds **11**-**13**), were found inactive below 100 µM (MIC >256 µg/mL for fractions **J** and **K**) against CBS131320. Therefore, an educated-guess approach for further selection of natural products was followed.

Table 1: *In vitro* minimal inhibitory concentrations (MIC_{80}) and selectivity (SI) of Compounds **1**–**10**, isolated from the chloroform and water fractions of the ethanolic extracts of the roots of *Haplophyllum tuberculatum* against *Madurella mycetomatis* (CBS131320 strain) and Cytotoxicity in L6 Cells.

Compound	Name	*M. mycetomatis* (CBS131320)		L6 cells
No.		MIC[a] (μM)	SI[b]	
1	Tetrahydrofuroguaiacin B	743.3	0.1	95.2 ± 9.3
2	Nectandrin B	1486.6	0.1	115.0 ± 2.6
3	Fragransin B2	1266.7	0.1	170.5 ± 40.1
4	Fragransin B1	1266.7	0.1	138.9 ± 3.8
5	Dihydroferuloyltyramine	158.7	n.d	>317.5
6	feruloyltyramine	319.5	0.8	246.8 ± 3.7
7	furoguaiaoxidin	280.9	n.d	>280.9
8	diferuloylputrescine	227.3	0.6	146.5 ± 18.8
9	7'-ethoxy trans feruloylT	280.1	0.7	207.8 ± 21.6
10	3,3′-Dimethoxy-4,4′-dihydroxylignan-9-ol	289.0	0.4	127.6 ± 3.9
Positive control		0.4[c]		0.03[d]

[a] The MICs are mean values from at least two independent replicates.
[b] Selectivity index (SI): IC_{50} in L6 cells divided by MIC in the titled parasitic strain.
[c] Itraconazole, [d] Podophyllotoxin.
n.d: Not determined

A set of natural compounds of different chemical classes and obtained from different plant species, and that had been reported for their antifungal and anti-infective activities, were screened for their activity against *M. mycetomatis.* Of these compounds (Figure 3); eudesmane sesquiterpenes from *Verbesina lanata* B. L. Rob. & Greenm. (Asteraceae) (**14**-**27**) [17], the neolignans honokiol (**28**) and magnolol (**29**), as well as the diterpenes serratol (**30**), and 1*S*,3*E*,7*R*,8*R*,11*E*-7,8-epoxy-cembra-3,11-dien-1-ol (**31**), previously isolated from *Boswellia serrata* (Burseraceae) [18]. The antimycetomal activity displayed by these compounds was followed up by testing against an additional *M. mycetomatis* strain (SO1) (Table 2). The eudesmane sesquiterpenes from *V. lanata* (**14**, **15**, and **22**) possessed MIC values within a range of 20 to 40 μM. Compound **22** exhibited the highest antifungal activity (MIC of 20.8 μM), and selectivity (SI 1.3) against the two tested strains of *M. mycetomatis,* consistently. Copmound **21** was inactive. Since compounds **22** and **21** are positional isomers, this indicates that isomerism has

considerable impact on both the activity and selectivity of these compounds. Compound **14** showed consistent activity and selectivity against the two strains (MIC 20.7 µM, SI 1.5). The vast majority of the eudesmane sesquiterpenes showed low selectivity indices (SI <1) and no interesting activity.

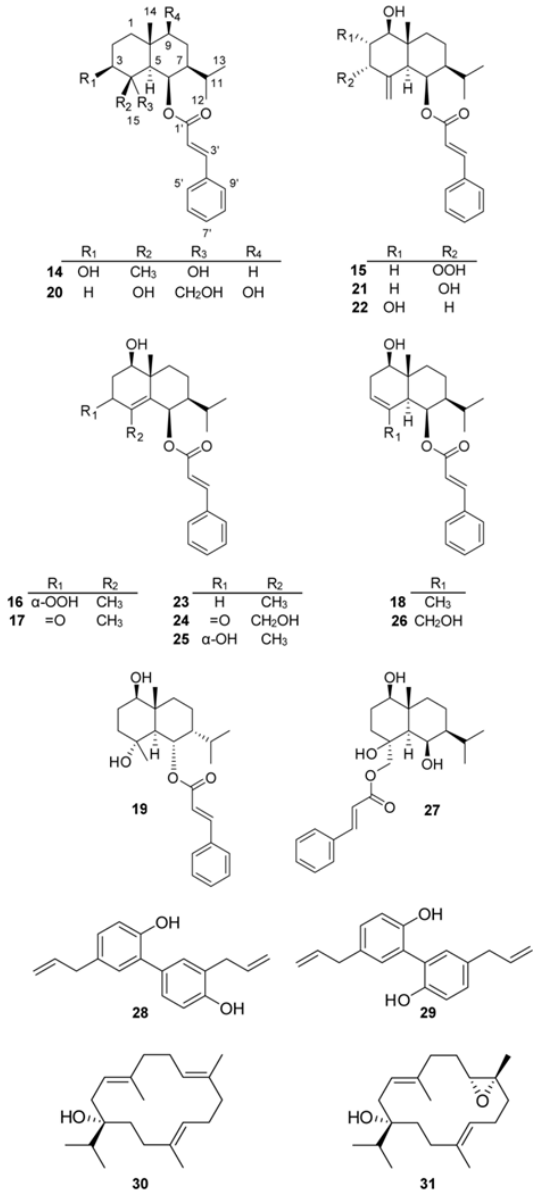

Figure 3. Chemical structures of natural compounds **14**-**31** screened for their *in vitro* antimycetomal activity.

Table 2: *In vitro* minimal inhibitory concentrations (MIC) and selectivity (SI) of compounds **14**–**31** against different strains of *M. Mycetomatis*.

		MIC (µM)		
NO.	**Compound**	**CBS131320**	**SO1**	**L6 Cytotox**
14	6β-Cinnamoyloxy-3β,4α-dihydroxyeudesmane	20.7 (1.5) [b]	20.7 (1.5) [b]	30.5
15	6β-Cinnamoyloxy-3α-hydroperoxy-1β-hydroxyeudesm-4(15)-ene	20.0 (0.4)	80.0 (0.1)	8.4
16	6β-Cinnamoyloxy-3α-hydroperoxy-1β-hydroxyeudesm-4-ene	40.0 (0.8)	159.9 (0.2)	33.8
17	6β-Cinnamoyloxy-1β-hydroxyeudesm-4-en-3-one	334.9 (0.1)	334.9 (0.1)	30.2
18	6β-Cinnamoyloxy-1β-hydroxyeudesm-3-ene	86.9 (0.5)	347.6 (0.1)	43.4
19	7-epi-6α-Cinnamoyloxy-1β,4α-dihydroxyeudesmane	662.8 (0.1)	662.8 (0.1)	80.3
20	6β-Cinnamoyloxy-4β,9β,15-trihydroxyeudesmane	318.2 (0.3)	318.2 (0.3)	98.4
21	6β-Cinnamoyloxy-1β,3α-dihydroxyeudesm-4(15)-ene	666.3 (0.1)	666.3 (0.1)	34.2
22	6β-Cinnamoyloxy-1β,2α-dihydroxyeudesm-4(15)-ene	20.8 (1.3)	20.8 (1.3)	27.7
23	6β-Cinnamoyloxy-1β-hydroxyeudesm-4-ene	695.2 (0.1)	695.2 (0.1)	44.0
24	6β-Cinnamoyloxy-1β,15-dihydroxyeudesm-4-en-3-one	160.7 (0.1)	160.7 (0.1)	21.6
25	6β-Cinnamoyloxy-1β,3α-dihydroxyeudesm-4-ene	41.6 (1.0)	166.6 (0.2)	40.1
26	6β-Cinnamoyloxy-1β,15-dihydroxyeudesm-3-ene	83.3 (0.5)	333.1 (0.1)	44.0
27	15-Cinnamoyloxy-1β,4β,6β-trihydroxyeudesmane	636.5 (0.1)	636.5 (0.1)	42.3
28	Honokiol	30.0 (1.3)	30.0 (1.3)	38.5
29	Magnolol	15.0 (4.9)	30.0 (2.4)	73.3
30	Serratol	110.2 (1.8)	220.3 (0.9)	203.1
31	1S,3E,7R,8R,11E-7,8-epoxy-cembra-3,11-dien-1-ol	417.9 (0.3)	417.9 (0.3)	111.0
	Itraconazole	0.2	0.1	
	Podophyllotoxin			0.03

[a] The MICs are mean values from at least two independent replicates.

[b] Selectivity index (SI): IC_{50} in L6 cells divided by MIC in the titled parasitic strain, given in parentheses.

Eudesmane sesquiterpenes had been isolated from other plants, mostly Asteraceae, and have shown activity against different fungal species [19,20]. However, this is the first report on their antimycetomal activity.

The lignans honokiol (**28**) and magnolol (**29**) showed similar activity profiles against SO1 with MIC 30 µM. However, magnolol exhibited a 2-fold higher selectivity index compared to honokiol. Moreover, magnolol (**29**) possessed the highest activity (MIC 15 µM) and selectivity (SI 4.9) against the CBS131320 strain among all tested compounds.

Honokiol (**28**) and magnolol (**29**), previously isolated from *Magnolia obovata*, have been reported for their activity against various human pathogenic fungi [21]. Honkiol has been reported to increase the antioxidant enzymatic activity of *Candida albicans* by inducing reactive oxygen species-mediated apoptosis through mitochondrial dysfunction [22]. This is the first report on the activity of the aforementioned compounds against *M. mycetomatis*.

4.4 Materials and Methods

Preparation of plant extracts

Dried plant material of *Haplphyllum tuberculatum* was milled to coarse powder in a hammer mill. 100-500 g of powdered material was extracted for 4 h with 500 ml of 70% ethanol in water bath. Extracts were filtered through Whatman no. 1 filter paper and concentrated by solvent removal in a rotary vacuum evaporator. The crude extract were was suspended in water and partitioned consecutively with petroleum ether, chloroform, ethyl acetate, and n-butanol. Crude extract and the 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.

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 ionisation 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 was carried out by analytical RP-HPLC on the same instrument (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 sample were collected into the corresponding wells of a 96-deepwell plate. Plates were then dried in a Genevac EZ-2 evaporator prior testing [23,24].

Exrtaction and isolation of compounds

Extraction, isolation and structure elucidation of the compounds **1**-**13** from the chloroform and water fractions of the ethanolic extract of *Haplophyllum tuberculatum* roots (Forsskal) A. Juss. (Rutaceae) was achieved by HPLC- based activity profiling approach. Detailed preprative and semipreparative procedures, along with NMR data were as previously described [16].

The eudesmane sesquiterpenes **14**-**27** were isolated as described in a previous project of *Verbesina lanata* B. L. Rob. & Greenm. (Asteraceae) for their inhibitory activity against grapevine downy mildew caused by *Plasmopara viticola* (Berk. & M. A. Curtis) Berl. & de Toni [17].

The lignans, honokiol (**28**) and magnolol (**29**), were purchased from Sigma-Aldrich. Serratol (**30**) and 1*S*,3*E*,7*R*,8*R*,11*E*-7,8-epoxy-cembra-3,11-dien-1-ol (**31**) has been previously isolated from *Boswellia serrata* (Burseraceae) [18].

In vitro* susceptibility assay against *Madurella Mycetomatis

Clinical isolates of *M. mycetomatis* strains (CBS131320 and SO1) from different geographical origins identified to the species level by sequencing the rRNA ITS region were used for susceptibility assays. Each strain was independently inoculated in RPMI 1640 medium supplemented with 0.35 g/L L-glutamine and 1.98 mM 4-morpholinepropane sulfonic acid. The mixture was sonicated for 10 sec at 28 um (QSONICA Q55), and incubated for 7 days at 37°C. The mycelia were harvested by another sonication step and 5-min centrifugation (Andres Hettich GMBH, EBA20). The pellets were washed and resuspended in fresh RPMI 1640 medium to obtain a fungal suspension of 68-72% transmission range (JENWAY 6305 UV/Vis Spectrophotometer). A 1:2 serial drug dilution covering a range from 256 to 0.063 µg/mL was prepared. 100 µL

of adjusted fungal suspension was added to each well of a 96-well microtiter plate. 20 µL resazurin solution (at final concentration of 0.15 mg/mL) was then added to each well. The plates were incubated for 7 days at 37°C and afterwards, plates were inspected for visual and spectrometrical endpoints. Absorbance was measured at 570 nm using a microplate reader (Themo Scientific Multiskan Spectrum, Thermo Fisher Scientific, Finland). The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the compound with ≥ 80% growth inhibition. Itraconazole was used as control. Assays were performed in two independent replicates at least [15].

***In vitro* cytotoxicity with L-6 cells**

Assays were performed in 96-well microtiter plates, each well containing 100 µL of RPMI 1640 medium supplemented with 1% L-glutamine (200 mM) and 10% fetal bovine serum, and 4000 L-6 cells (a primary cell line derived from rat skeletal myoblasts) [25]. Serial drug dilutions of eleven 3-fold dilution steps covering a range from 100 to 0.002 µg/mL were prepared. The plates were incubated for 70 h and inspected under an inverted microscope to assure growth of the controls and sterile conditions. 10 µL of resazurin was then added to each well and the plates incubated for another 2 hours. Then the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. The IC_{50} values were calculated by linear regression and 4-parameter logistic regression from the sigmoidal dose-inhibition curves using SoftmaxPro (Molecular Devices Cooperation, Sunnyvale, CA, USA). Podophyllotoxin (Sigma P4405) was used as control. Assays were performed in two independent replicates at least.

Acknowledgments

We wish to thank Dr. Ramseyer for the eudesmane sesquiterpenes, Professor Thomas J. Schmidt and Dr. Greve for the *Boswellia* compounds. We gratefully acknowledge financial support by the Amt für Ausbildungsbeiträge Basel and the Emilia Guggenheim-Schnurr Foundation.

Conflict of Interest

The authors declare no conflict of interest.

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6. *In vitro* testing of redox-active parasiticides identifies niclosamide as a hit for *Madurella mycetomatis* and *Actinomadura* spp.

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Running title: *In vitro activity of niclosamide against causative agents of mycetoma*

Submitted for publication

I have performed compounds preliminary antimycetomal activity testing. Compounds were selected by Marcel Kaiser. Shereen Abd Algaffar performed further confirmative tests and the testing for the salicylanilides.

Abstract

Redox-active prodrugs are the mainstay of parasite chemotherapy. To assess their repurposing potential for eumycetoma, we have tested a set of nitroheterocycles and peroxides against *Madurella mycetomatis*. All the compounds were inactive except for niclosamide. The analog MMV665807 and niclosamide ethanolamine were active as well. The three compounds also exhibited good activity against *Actinomadura* spp., causative agents of actinomycetoma. We therefore propose to further evaluate salicylanilides, in particular niclosamide, as repurposing candidates for mycetoma.

Keywords

Madurella mycetomatis, Actinomadura, Mycetoma, Drug repurposing,
Nitroheterocycles, Niclosamide, MMV665807, Salicylanilide

Eumycetoma is endemic from India over the Middle East and across the Sahel, with the highest prevalence in Sudan [1,2] . It is a chronic subcutaneous mycosis that slowly spreads, starting from an initial lesion at the site of inoculation, into the skin and deeper tissues, ultimately destroying muscles, tendons, and bones. The leg and foot are most often affected, likely due to inoculation via thorn pricks. While eumycetoma can be caused by various fungi [3], the majority of cases in Sudan are due to *Madurella mycetomatis* [2]. Eumycetoma is a debilitating, disfiguring, and stigmatizing disease. It is also an enigmatic disease in the light of the many open questions regarding its epidemiology, pathogenesis, and the biology of the causative agents [2,4].

There is no satisfactory treatment for eumycetoma. The current therapy consists of a combination of surgery and long-term chemotherapy with antifungal azoles such as itraconazole [2]. However, the cure rates are low and amputation of the affected limb may be the only measure to stop the flesh-eating fungus [5]. Given the urgent need for better drugs and the fact that eumycetoma is a neglected disease affecting neglected patients, drug repurposing suggests itself as a fast and cost-effective way towards new antimycetomal agents [6,7]. Here we pursue this strategy by testing a small set of redox-active parasiticides and antibiotics for their *in vitro* activity against *M. mycetomatis*.

Redox-active molecules are the mainstay of current parasite chemotherapy [8, 9]. Since saprophytic fungi can dwell in hypoxic environments and may possess reducing agents of low redox potential, we speculated that *Madurella*, too, might be susceptible to prodrugs that are activated by electron transfer. A selection of nitroheterocycles and peroxides was evaluated for their *in vitro* activity against two isolates of *M. mycetomatis*, SO1 and CBS131320 (mycetoma collection of the Erasmus Medical Centre, Rotterdam, the Netherlands). The mycelia were grown at 37 °C in RPMI 1640 medium supplemented with 0.35 g/L L-glutamine and 1.98 mM 4-morpholinepropane sulfonic acid in 96-well microtiter plates in serial dilution of test compounds; resazurin was added (0.15 mg/mL) for spectrometric read-out (absorbance at 570 nm) [10]. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of test

compound that, after 7 days of incubation, had inhibited the growth by at least 80% compared to untreated cultures.

The tested peroxides were inactive, which is in agreement with the reported lack of activity of artemisinin [11]. The nitroimidazoles and nitrofurans were all inactive as well. The one notable exception was niclosamide, which had a MIC around 1 µg/ml (Table 1). This interesting finding was followed up by testing niclosamide and two related compounds also against *Actinomadura* spp., causative agents of actinomycetoma. *A. madurae* SAK-A05 and *A. syzygii* SAK-A08 were originally isolated from Sudanese patients and cultured in the pharmaceutical research laboratory, University of Science and Technology repository (Omdurman, Sudan). The bacteria were grown for 5 days at 35 °C in Mueller Hinton II broth (CAMHB) medium in 96-well microtiter plates with serial dilution of test compounds and 0.15 mg/mL resazurin.

Niclosamide, niclosamide ethanolamine, and MMV665807 exhibited good activity against *Madurella* as well as *Actinomadura*, with MIC values somewhat higher than the reference drug itraconazole for *M. mycetomatis* and considerably lower than the reference drug cotrimoxazole for *Actinomadura* (Table 2). MMV665807 is a salicylanilide from the Medicines for Malaria Venture's malaria box [12] that has shown antibacterial [13], antiprotozoal [14,15], and anticestodal [16,17] activity. Niclosamide ethanolamine (NEN, also called niclosamide olamine or clonitralide) has a better water-solubility and bioavailability than niclosamide [18,19], and it is being considered for different (re)purposes [20-24].

Niclosamide itself is an old drug of many uses [25,26]. It was developed by Bayer in the 1950s as a molluscicide for schistosomiasis control. Since 1982, when it was approved by the FDA for human use, its primary indication has been as a broad-spectrum anthelmintic for tapeworms (*Taenia* spp., *Diphyllobothrium latum*) and intestinal fluke (*Fasciolopsis buski*) [27]. Niclosamide was shown to have promising antibacterial [28,29] as well as antifungal [30] activity. What restricted its use to intestinal pathogens was the poor oral bioavailability, i.e. the fact that niclosamide is not significantly absorbed from

the gastrointestinal tract [19,31] . Different carriers or formulations have been employed to overcome this issue; see e.g. [32-34].

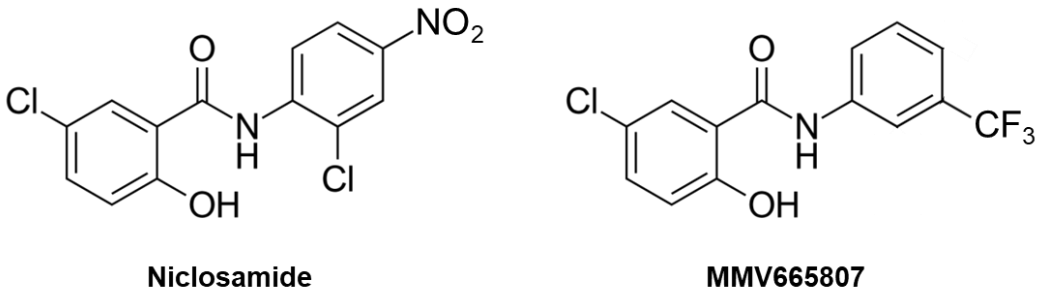
In conclusion, we have found a promising hit even though our hypothesis that the metabolism of the fungus would activate redox-active prodrugs turned out to be wrong. Given the lack of activity of the tested nitrofurans and nitroimidazoles, the observed activity of niclosamide is likely not due to its nitro group but due to the salicylanilide moiety. This is supported by the good activity of MMV665807 a salicylanilide that lacks a nitro group (Figure 1). The finding that a drug like niclosamide, which is on the WHO's list of Essential Medicines, exhibits *in vitro* activity against both *Madurella mycetomatis* and *Actinomadura* spp. warrants the testing of further salicylanilides against these pathogens and the consideration of niclosamide or its ethanolamine salt as repurposing candidates for mycetoma.

Table 1. Selected redox-active agents and their *in vitro* activity against the two *M. mycetomatis* isolates SO1 and CBS131320. For the experimental compounds Ro 15-6547 [35], RJ-55, RJ-164 [36], and OZ78 [37,38], the envisaged indication is in parentheses (HAT, human African trypanosomiasis). All assays were performed in at least two independent replicates.

Compound	**Class**	**Primary indication**	**MIC [µg/ml]**	
			SO1	**CBS131320**
Niclosamide	Salicylanilide	Tapeworms	0.78	1.6
Secnidazole	Nitroimidazole	Bacterial vaginosis	>256	>256
Metronidazole	Nitroimidazole	Broad spectrum antimicrobial	>256	>256
Fexinidazole	Nitroimidazole	HAT	>256	>256
RJ-164	Nitroimidazole	(HAT)	>256	>256
RJ-55	Nitroimidazole	(HAT)	>256	>256
Ro 15-6547	Nitroimidazole	(HAT)	>256	>256
Nifurtimox	Nitrofuran	Chagas' disease, HAT	>256	>256
Nifuroxazide	Nitrofuran	Colitis and diarrhea	>256	>256
Nitrofurantoine	Nitrofuran	Urinary tract infections	>256	>256
OZ 78	Peroxide	(Malaria, trematodes)	>256	>256
Artemisinin	Peroxide	Malaria	16	16
Dihydroartemisinin	Peroxide	Malaria	>256	>256
Artesunate	Peroxide	Malaria	>256	>256
Artemether	Peroxide	Malaria	64	64
Itraconazole	Triazole	Antifungal	0.13	0.25

Table 2. Niclosamide and related compounds tested against two *M. mycetomatis* isolates (SO1 and CBS131320) and two *Actinomadura* species, *A. madurae* (SAK-A05) and *A. syzygii* (SAK-A08); EN, ethanolamine. All assays were performed in at least two independent replicates.

	MIC [µg/ml]			
	SO1	**CBS131320**	**SAK-A05**	**SAK-A08**
Niclosamide	0.78	1.6	0.39	0.39
Niclosamide-EN	0.78	1.6	0.19	0.39
MMV665807	1.6	1. 6	0.39	0.39
Itraconazole	0.13	0.25	n.d.	n.d.
Cotrimoxazole	n.d.	n.d.	20	10



Niclosamide **MMV665807**

Figure 1. Chemical structures of niclosamide and MMV665807.

Acknowledgments

We thank DNDi for the nitroheterocyclic drugs, Jonathan Vennerstrom for the peroxides, and Britta Lundström-Stadelmann and Reto Rufener for MMV665807 and helpful advice. We are most grateful to Marcel Tanner and Suad Sulaiman for their continuous support.

Funding

This work was supported by fellowships to A.B.M of the Amt für Ausbildungsbeiträge of the Canton Basel-Stadt and the Emilia Guggenheim-Schnurr Foundation. The sponsors had no role in study design; in the collection, analysis and interpretation of data; in the writing of the report; nor in the decision to submit the article for publication.

Competing interests

None.

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7. GENERAL DISCUSSION

7.1 Overview of the research outcomes

Tropical plants of the arid zones are a treasure chest for the discovery of bioactive secondary metabolites. Sudan has a highly diverse flora, coupled with a very rich ethnomedical heritage that remains a promising untapped reservoir for the discovery of diverse bioactive natural products (NPs). Subjecting Sudanese medical practice to the currently available modern scientific tools can be considered as the first step in the direction to validate its scientific merits. In this study, we compiled a review of plants that are used in Sudanese traditional medicine, with a focus on malaria and neglected tropical diseases caused by protozoa. On the basis of this survey, a total of 62 plant species belonging to 35 different families were assembled. The plant materials were extracted in 70% ethanol and further fractionated consecutively by liquid-liquid partitioning using solvents of increasing polarity. This resulted in a library of 235 fractions. Phenotypic screening was pursued. The library was tested *in vitro* against panel of protozoan parasites: *Plasmodium falciparum* (proliferative erythrocytic stages), *Trypanosoma brucei rhodesiense* (bloodstream forms), *Trypanosoma cruzi* (intracellular amastigotes), and *Leishmania donovani* (axenic amastigotes). Extracts with antiparasitic activity were also tested for cytotoxicity. This was done against rat L6 skeletal myoblast cells. The most susceptible parasite was *P. falciparum*, followed by *T. b. rhodesiense*, *L. donovani*; the least susceptible was *T. cruzi* (Chapter 2).

A dereplication approach was performed for active extracts to enable prioritization for follow-up selection. This was achieved by HPLC activity profiling in combination with on-line spectroscopy, which enabled a rapid identification of known active compounds, i.e. guieranone A from *Guiera senegalensis* J.F.Gmel*.,* pseudosemiglabrin from *Tephrosia apollinea* (Delile) DC*,* in addition to other ubiquitous plant secondary metabolites. Plants that displayed interesting activities, namely *Croton gratissimus*, *Cuscuta hyalina*, and *Haplophyllum tuberculatum* were further pursued for a follow-up HPLC-activity profiling. Preparative isolation of active compounds to perform structure elucidation and *in vitro*

testing was achieved. Nine compounds were isolated from the chloroform fraction of the ethanolic extract of *Croton gratissimus* (Chapter 3). Of these, six flavonoids were identified as quercetin-3,3',4'-trimethylether (**1**), ayanin (**2**), retusin (**3**), naringenin (**4**), quercetin-3,4'-dimethyl ether (**5**), quercetin-3,7-dimethylether (**6**) , along with 3-methoxy-4-hydroxybehzoic acid (7), and two benzyltetrahydroisoquinoline alkaloids; laudanine (**8**) and laudanosine (**9**). All of them are being described for the first time from *C. gratissimus.* Quercetin-3,7-dimethylether (**6**) was the most active against the trypanosomatids (50% inhibitory concentration (IC_{50}) <5 μM; selectivity index (SI) >10) followed by ayanin (**2**). The antiprotozoal activities of (**2**) and (**6**) are being described for the first time. The structure-activity relationships (SAR) among the bioactive compounds were discussed in relation to the three parasites. The chloroform fraction of the ethanolic extract of *Cuscuta hyalina* yielded four secondary metabolites, including the furofuran lignan, pinoresinol (**10**), and the flavonoids, isorhamnetin (**11**), (-)-pseudosemiglabrin (**12**)**,** and kaempferol (**13**). Pseudosemiglabrin (**12**) is being described for the first time from *Cuscuta* species (Chapter 3).

In addition, the chloroform and water fractions of *Haplophyllum tuberculatum* were investigated (Chapter 4). In total, 13 compounds were identified. HPLC-based activity profiling led to the isolation of eight compounds from the chloroform fraction, including four lignans; tetrahydrofuroguaiacin B (**1**)**,** nectandrin B (**2**), furoguaiaoxidin (**7**) and 3,3'-dimethoxy-4,4'-dihydroxylignan-9-ol (**10**), in addition to four cinnamoylphenethyl amides; dihydro-feruloyltyramine (**5**), N-trans-feruloyltyramine (**6**), N,N'-diferuloylputrescine (**8**), and (*E*)-7'-ethoxy-feruloyltyramine (**9**). The water fraction yielded a mixture of steroidal saponins, specifically the tetraglycosidic spirostenes; (*3*S,*20*S,*22R*,*25*S)-spirost-5-en-3-yl-(β-D-xylopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (**11**), (*3*S,*20*S,*22R*,*25*S)-spirost-5-en-3-yl-(β-D-rhamnopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)[α-L rhamnopyranosyl -(1→2)]-β-D-glucopyranoside (**12**), and (*3*S,*20*S,*22*R,*25*R)-spirost-5-en-3-yl-(β-D-xylopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (**13**). The isolation of compounds **1**, **2**, and **5**-**13** is being reported for

the first time from *Haplophyllum* species and the family Rutaceae. Nectandrin B (**2**) exhibited the highest activity against *L. donovani* (IC_{50} 4.5 µM) with a promising selectivity index (SI) of 25.5. The lignan 3,3′-dimethoxy-4,4′-dihydroxylignan-9-ol (**10**) was the most active against *P. falciparum* (IC_{50} 9.3 µM; SI 13.7), while the steroidal saponins were the least selective.

Regarding the fungal disease mycetoma, two drug discovery approaches were tackled: a natural products approach and a drug repurposing (repositioning) approach. For the natural products (Chapter 5), *Haplophyllum tuberculatum* was found active during preliminary screening, hence the previously isolated compounds were screened for their *in vitro* antimycetomal activity. However, the compounds were not active. Therefore, we proposed the educated guess strategy. Pure natural compounds of different classes from different plant species, that had been previously reported for their antifungal and anti-infective activities, were screened for their *in vitro* activity against *Madurella mycetomatis*, the main causative agent of fungal mycetoma. These compounds were eudesmane sesquiterpenes from *Verbesina lanata* B. L. Rob. & Greenm. (Asteraceae) (**14**-**27**), the neolignans honokiol (**28**) and magnolol (**29**), in addition to the diterpenes serratol (**30**), and 1*S*,3*E*,7*R*,8*R*,11*E*-7,8-epoxy-cembra-3,11-dien-1-ol (**31**), previously isolated from *Boswellia serrata* (Burseraceae). The Minimal inhibitory concentration (MIC) was set as the lowest concentration of antifungal activity producing ≥ 80% growth inhibition. The MICs against one or more of *M. mycetomatis* strains, along with the cytotoxicity against L-6 rat skeletal cells, were determined. The eudesmane sesquiterpenes (**14**, **15**, and **22**), isolated from *V. lanata* has possessed MIC_{80} values within a range of 20 to 40 µM. Of them, 6β-Cinnamoyloxy-1β,2α-dihydroxyeudesm-4(15)-ene (**22)** exhibited the highest activity (MIC of 20.8 µM), and selectivity (SI 1.3) across the three tested strains of *M. mycetomatis*. The lignans, honokiol (**28**) and magnolol (**29**), showed similar activity profiles against SO1 strain with MIC (30.1 µM). However, magnolol exhibited 2-fold higher selectivity indices compared to honokiol against the aforementioned strains. Moreover, magnolol (**29**) possessed the highest

activity (MIC 15 µM) and selectivity (SI 4.9) against CBS131320 strain among all tested compounds.

Since the natural products approach did not yield very promising candidates, we pursued a repurposing approach for eumycetoma in parallel. A series of nitroimidazole compounds were screened *in vitro* against *M. mycetomatis*. From this screening, niclosamide showed interesting activity at <5 µM. Furthermore, additional niclosamide analogues were tested for the proof of concept. The tested compounds showed similar activity against both fungal and bacterial mycetoma compared to niclosamide.

Overall, it can be concluded that, this assessment provides a comprehensive overview of Sudanese medicinal plants and supports the notion that they are a potential source of bioactive molecules against parasitic infections. Moreover, it also emphasizes the potentialities of the repurposing approach for the fungal disease eumycetoma. In the following, I shall briefly discuss the approaches and methodology used in this research and highlight provisions of improvements in the context of this work.

7.2 Why phenotypic screening?

The advent of modern molecular biology methods and the knowledge of the human genome have dramatically changed the drug discovery strategy in the pharmaceutical industry into a hypothesis-driven target-based approach [1]. Yet the contribution of the classical phenotypic approach remains of great value for drug discovery, in particular for diseases of poverty. This arises from the fact that it does not require a prior knowledge of validated molecular target or specific mechanism of action, which is the case for most of the antiparasitic drugs that are available today [2,3]. For many years, drug discovery has been driven by chemocentric approaches, i.e., approaches based on a specific compound or compound class which served as starting point for further optimization. These chemotypes were either discovered through ethnobotanical knowledge or derived from natural ligands and substances. The unique case of the discovery of the antimalarial quinine represented a milestone in the history of drug discovery where the

drug (quinine) was discovered before the differentiation and elaboration of the disease itself [4]. Furthermore, phenotypic screening has given other non-systematic approaches, e.g. serendipity, the consideration of being a source of successful molecules of unexpected bioactivity. Well-known examples are the anticancer compounds vinblastine and taxol [5].

7.3 Why an ethnobotanical approach?

Ethnomedicine represents one of the primary sources of health care in Africa and many developing countries [6]. This widespread use is justified by the limited availability and/or accessibility of conventional medicine-based health services. In contrary, traditional medicine is being present on the ground and readily affordable. Since medicinal plants are the 'backbone' of traditional medicine, this reflects the considerable utilization of medicinal plants by the vast majority of the population in the less developed countries, including Sudan. Through the ethnobotanical knowledge, the African continent has made considerable contribution to the field of drug discovery. For example, the cardiac glycosides strophanthin and ouabin from the Mozambican seeds *Strophanthus gratus*, and the parasympathomimetic alkaloid, physostigmine, from the West African Calabar bean (*Physostigma venenosum*) [4]. Thus, plants must not be ignored also in rational drug development.

Data analyzed from drug discovery screening campaigns have shown that molecules from ethnomedically used plants have a higher hit rate of bioactivity than randomly selected molecules [7]. In our antiprotozoal drug discovery screening we have confirmed a high hit rate: 125 extracts out of 235 (53%) showed growth inhibitory activity >80% at 10 µg/ml, and >50% at 2 µg/ml against at least one of the tested protozoan parasites. Moreover, regarding the antiplasmodial activity, the plants that were documented as antimalarial remedies (n=17) were slightly more active against *P. falciparum in vitro* than the other plants, 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).

7.4 Caveats

Unlike the traditional Chinese medicine or Ayurvedic medicine, which involves a high degree of authenticated medical practice and well written documented history, the African traditional medicine is usually of no formal educational component. In such a system, whereby information is passed on from person to person, father to son, guru to disciple, the information is considered highly secretive and is not in manuscript format [8]. Sudan is not an exception. There is a lack of well documented and scientifically sound ethnomedical surveys that could serve as platforms for further phytochemical investigations. Thus, qualitative analyses of the local remedies and practices and their rationalization (i.e. valorization) is desperately needed. A key factor to achieve this is through a multidisciplinary integrative approach that includes traditional plant specialists, anthropologists, pharmacologists, medical doctors, and phytochemists. Concurrently, issues of accessibility, fair and equitable sharing of the benefits arising from the usage of these resources [9] should be adequately addressed.

Though the aforementioned points are beyond the context of this thesis, yet, in this regard, Chapter 2 in this work could set a starting point of a collective approach for further in-depth pre-clinical assessment and clinical evaluation of the efficacy and safety of Sudanese medicinal plants used as antiparasitics.

7.5 Extraction procedures

Extraction is the first step in the drug discovery process from plants. Several general procedures like solid-liquid extraction techniques and water maceration and/or decoction represent the first choice, since traditional healers commonly use water as solvent [10]. Further sequential extraction with liquid-liquid partitioning using solvents of increasing polarity, such as petroleum ether, chloroform, and ethyl acetate, are necessary for a preliminary separation based on the hydro-/lipophilic properties of the biologically active compounds as well as to maximize the chances of discovering new bioactive NPs from complex mixtures [11]. Such enrichment procedures may improve

biological and chemical profiling screening steps. A vivid example from the preliminary screening performed (Chapter 2; Supplementary table 1) is given by *Cassia occidentalis* L. (Leguminosae). The crude ethanolic extract of this plant showed only weak antiplasmodial inhibition (< 30% at 10 µg/mL). However, the petroleum ether fraction of the crude extract showed strong inhibition of the same parasite (91.6% at 10 µg/mL). This indicates that the displayed activity is a result of the enrichment procedure for the bioactive compounds in the apolar fraction. In addition, the fractionation of extracts prior to bioassays can mask potential synergistic or antagonistic effects observed within crude extracts. This step also offers an indisputable advantage by increasing the constituent concentration in each fraction. It is also common that chemical transformation occurs during the extraction procedures leading to artifacts. For example, ethyl gallate detected in *Acacia nilotica* could be possibly formed from gallate due to extraction with ethanol. Therefore, this issue should be considered during extraction process. Moreover, variations between extraction methods used in traditional medicine and those exhaustive ones used for phytochemical investigations and their potential effects on the overall quality of extract should not be overlooked.

7.6 Bioassays and screening procedures

A number of pivotal quality standards need to be set at the level of primary evaluation in bioassay screening models to guarantee sound selection of extracts or molecules with relevant pharmacological action and worthy of follow-up. Some special considerations to limit the number of leads for follow-up evaluation include a high selectivity, a high sensitivity (to detect low concentrations of active compounds) as well as adaptability to poorly soluble compounds and chemically complex materials [12]. A review by Cos et al has discussed a number of considerations and provided recommendations that enable to define a more sound 'proof-of-concept' for antiparasitic potential in natural products [13]. On the light of this review, the major following points will be addressed:

I. *In vitro models* using the whole target organism should be used whenever possible. In addition, activity should be discriminated from unspecific cell

toxicity, thus compelling the inclusion of a parallel evaluation in host cell lines (cytotoxicity evaluation): On this regard, the bioassays performed in this work for antiparasitic screening were based on *in vitro* whole organism. In parallel, extracts with interesting activity were pursued further for cytotoxicity testing against rat L6 skeletal myoblast cells. Concentration-response curves allowed the calculation of both 50% and 90% inhibitory concentrations (IC_{50} and IC_{90}; Table 3, Chapter 2). 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.

II. Use of sensitive endpoint reading techniques: Bioassay interference with natural products cannot be excluded in fluorescence and UV/visible read-out assays [14]. These include two counteractant phenomena; i) binding to proteins in the aqueous media that are predominantly used in bioassays (such as bovine serum albumine) or precipitation as major cause for false negatives, and ii) light scattering in UV/visible read-out assays, and membrane disruption leading to false positives. The latter can be excluded in our case since we have employed viability assays with fluorescence read-out for activity determination. However, a compound could inhibit reduction of resazurin (false positive hit) or be fluorescent itself (false negative). As these noisy effects cannot be omitted at the level of extracts, they should be considered at compound level. Assay-interfering compounds were designated as pan-assay interference compounds (PAINS) [15]. Some of these compounds that had manifold ascribed bioactivities were listed by occurrence, activity, and distinct activity, and were labelled as invalid metabolic panaceas (IMPs) [16]. Luckily, some of these compounds were rapidly dereplicated at the preliminary screening level, as will be discussed in more detail in the next section (Section 1.6).

III. Determination of optimal criteria for efficacy: the biological activity levels as inclusion criteria of extracts or purified compounds should be clearly defined. Many articles on natural products claim so-called "exciting" anti-infective activities, despite major flaws in the used methodologies [17]; among these is the lack of sound criteria for activity. In this regard, activity criteria were set from the preliminary screening performed. 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 were considered active. Of the 235 extracts in our library, 125 (53%) fulfilled these activity criteria. However, concerns regarding concentration of crude extracts to be tested and cut-off values for activity criteria are still argumentative in regards to validation of herbal medicines [18]. Therefore, there is a dire need for establishing standard criteria for the evaluation of plant extract activities to enable the comparison of different studies [19]. One way to make different assays comparable is the inclusion of reference drugs as was done herein: chloroquine for *P. falciparum*, melarsoprol for *T. brucei*, miltefosine for *L. donovani*, and podophyllotoxin for L6 cells. This provides a point of reference to which to compare the recorded activities of extracts, fractions, and purified molecules.

IV. Whenever possible, activities discovered at one particular screening level should be confirmed using a model in the next higher evaluation level: We have considered this particularly for plant extracts of antileishmanial activity. Extracts that have displayed potent and selective activity against *L. donovani* axenic amastigotes in the preliminary screening were further investigated for their activity in intramacrophage amastigotes. Based on the latter high content image screening, the chloroform fractions of the ethanolic extracts of *Croton gratissimus* and *Cuscuta hyalina* were selected for further HPLC-activity profiling (Chapter 3). Compounds isolated from these two plants were tested for their activity in both axenic and intracellular activity models. Some compounds like quercetin-3,7-dimethylether and ayanin have shown IC_{50}s of <10 µM and

selectivity indices >10 in the axenic amastigotes screening, these compounds showed considerably higher IC_{50}s against intracellular parasites. This discrepancies could be justified by different factors; I) the compound is not reaching the parasite, either because it has to cross several membranes or due to protein binding in the host cell cytosol; II) the compound is exposed to hydrolysis or metabolism in the host cell phagolysosome [20]. Nevertheless, compounds that were not active in the axenic assays were also inactive intracellularly. Compounds that are active against axenic amastigotes but not against intracellular amastigotes do not fulfill lead criteria for further development. However, if their selectivity for axenic amastigotes over mammalian cells is high, such compounds are still of interest to identify novel, parasite-specific drug targets.

7.7 Dereplication

Developing advanced analytical procedures for the rapid identification of known natural products (NPs) in crude plant extracts (dereplication) is indispensable to avoid their unnecessary re-isolation when their bioactivity has already been described. Dereplication strategies for the early identification of NPs in complex mixtures have evolved considerably over the last decade [16]. Hyphenated techniques incorporating online (e.g. MS and UV) and offline (NMR) spectroscopic data linked to bioactivity results acquired in screening campaigns, along with previously reported pharmacological data, allows interpretation of screening results from a novel and holistic perspective. It also allows for the prioritization of NPs and for the targeted isolation of new bioactive molecules of interest.

Dereplication is particularly useful to detect “frequent hitters”, or the so called pan-assay interference compounds (PAINS) [17]. These are compounds exhibiting multiple behaviors that could interfere in assay readouts, such as metal chelation, redox cycling, and protein reactivity and hence, falsely reported as actives in bioassays.

In this regard, a relevant example for the successful use of HPLC-activity profiling approach in prioritization of extracts for further follow-up from preliminary screening results is the rapid dereplication of the ubiquitous compounds (also PAIN); ellagic acid and quercetin from *Anogeissus leiocarpus* (DC.) Guill. & Perr., and catechin, ethyl gallate, and epicatechin gallate from *Acacia nilotica* (L.) Delile. Extracts containing such compounds, though active, were excluded from further investigations, since the activity was most likely due to presence of the tannins. Hence, their follow up isolation was prevented in first place.

However, incorporation of comprehensive natural product databases with relevant spectroscopic data and linking it to the setting format would have further improved the high performance dereplication workflows and the prioritization process consequently.

7.8 HPLC-based activity profiling

The efficient tracking of bioactive molecules remains a major challenge in the search for new lead compounds in plant extracts. The classical bioactivity-guided isolation strategy involving several iterative steps of purification and biological testing is time consuming and requires large amounts of plant starting material. It frequently leads to the isolation of ubiquitous metabolites and may ultimately lead to loss of the originally observed activity [21]. A number of more efficient alternatives and innovative approaches involving the recent advances in chromatography and spectroscopy have been described for tracking bioactivity in complex matrices; among them is the HPLC-based activity profiling approach [22].

HPLC-based activity profiling in combination with on-line and off-line spectroscopic data allowed complete characterization of bioactive compounds and chemical structure determination from a minimal amount of starting material as well as performing target preparative isolation of the active principles present in the active fraction, enabling rapid dereplication of known compounds [23,24]. This platform has been successfully applied to the prioritization and subsequent characterization of hits using different

bioassay formats such as whole organism assays, cell-based functional assays, and target-based screens. These include parasitic diseases where HPLC-based activity profiling protocols have been validated and applied for the follow-up of extracts with antiprotozoal activities [25]. This approach has enabled the identification of some compound classes that had not been previously reported from the plant species or the family. The isolation of the steroid saponins from *Haplophyllum tuberculatum* (Chapter 4) is a relevant example. Moreover, many compounds of different classes have been reported from the respected plant species for the first time (Chapters 3 and 4).

7.8.1 Considerations in the HPLC-activity profiling approach

Efficient isolation of pure compounds directly from crude extracts at the preparative chromatographic scale while maintaining similar analytical scale chromatographic selectivity and resolution levels is still challenging. Solving this issue would prevent tedious multiple chromatographic steps applied on large-scale extraction. Moreover, efficient chromatographic gradient transfers from analytical to semi-preparative HPLC (i.e. method optimization) is another critical step that should be optimized. Fractions are often diluted and injected in large volumes of organic solvents when introduced to semi-preparative HPLC, hence solubility issues, notably with reverse-phase separation, could significantly compromise the resolution. Also, extrapolation of pure compound yield from early separation steps is rather difficult. This is important especially in case of minor compounds, whose low yield may not permit to perform successive chemical measurements and biological testing procedures.

Innovative approaches are emerging for the rapid identification of NPs in mixtures and prioritization for target isolation based on novelty and/or bioactivity [26]. An example is the bioactivity-based, multi-informative molecular network approach which involves spectra annotations through interrogation of public and private databases, combined with bioactivity and taxonomy which can be mapped on the molecular network for extract prioritization and targeted isolation [27]. Nevertheless, a limiting factor could be

the quality and completeness of MS/MS databases that are still incomplete compared to the total number of NPs described to date.

7.9 The empirical antiparasitic drug discovery approach applying biological screening and activity-oriented separation of medicinal plants: Could it be improved?

Optimal screening strategies, assay standardization, data analyses, and optimization of hit selection criteria are subjects of continuing discussions. There are certain gaps or bottlenecks that are encountered in the classical drug discovery strategies of natural products used in traditional medicine (Figure 1). These bottlenecks can be summarized in two major points:

(a) The approach itself that restricts the activity/cytotoxicity of a whole plant to one or a few active compounds. This is rather reductionistic and disregards the potential synergistic / antagonistic interactions of other, "inactive" phytoconstituents which could be lost during fractionation. This is illustrated by the case of berberine, whose antimicrobial activity was enhanced by more than 100-fold in combination with an inactive component, 5'-methoxyhydnocarpin, isolated from the same plant [28]. This will lead to a further challenging question, how to identify and prove synergism or antagonism? Which compounds should be tested? Answering these questions is particularly challenging if other compounds from the investigated plant are still unknown! However, combining activity and metabolomics data can reveal some answers. In parallel, screening efforts should be carried out using a multiple screening approach on different mammalian cells and potential target systems.

(b) *In vitro* / *in vivo* correlation (IVIVC): While in vitro antiparasitic activity does not necessarily indicate clinical efficacy, clinical efficacy vice versa is not always detectable in *in vitro* systems. Prodrugs, i.e. compounds which have to be metabolized in order to exhibit activity, cannot be detectable in *in vitro* systems where no host metabolism is present [29]. Neither are compounds that have indirect activity by stimulating the immune system, or by alleviation or reduction of

clinical symptoms (palliative properties). The latter point might not be applicable for fatal parasitic diseases, especially in trypanosomiasis since the parasite has to be killed; however, this could be more relevant to other infections (i.e. bacterial, viral, or fungal infections).

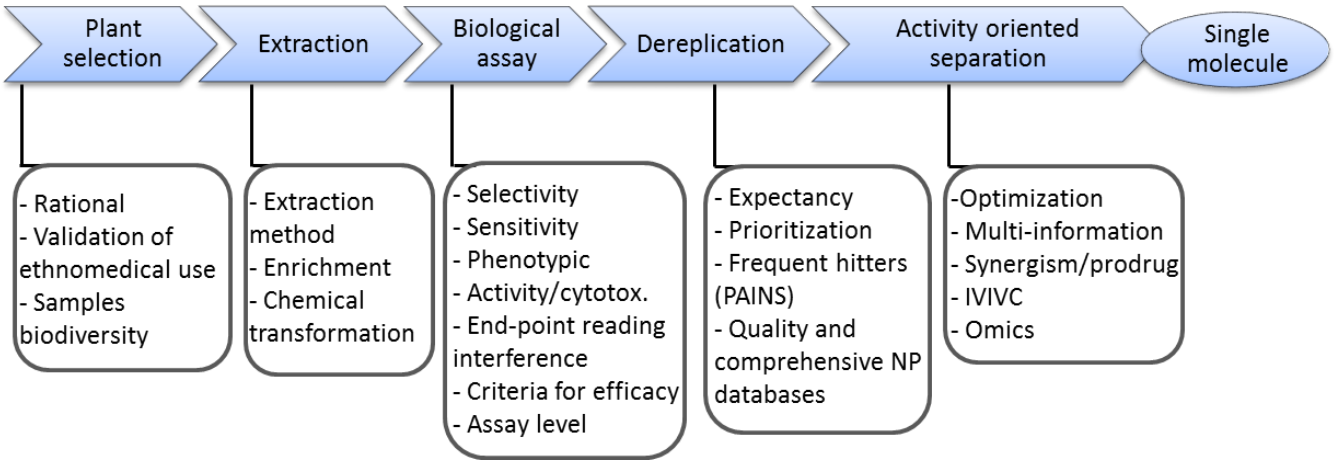

Figure 1: Empirical drug discovery approach from medicinal plants and considerations for improvements

7.10 Other options for exploring traditional medicine: lessons from history

Considering all these challenging and critical aspects, the paradigm of "single compound – single target" will not help much to understand the potentially complex bioactivity of mixtures. Other approaches could be revitalized. For instance, pharmacological evaluation of extracts from medicinal plants may lead to the establishment of standardized extracts that serve as a good starting point [12]. Other option is the "bedside–bench–bedside" approach, also known as "Reverse pharmacology [30]", which relies on evidence-based traditional medicine. This has been very successful in traditional Chinese medicine [31]. Development of artemisinin-based antimalarials represents one of the great victories in drug discovery, combining a holistic traditional approach with a modern, evidence-based approach [32]. Crucial

prerequisites for the aforementioned approaches are the quality control and quality assessment as well as safety and efficacy of botanical extracts for drug development.

7.11 Mycetoma Drug Discovery

Despite the advances made in the research of mycetoma and advocacy, thanks to the recent recognition of the disease by WHO as an NTD, many gaps and challenges are still present. In particular, finding effective medicines and, consequentially, optimizing therapeutic approaches [33]. The treatment of mycetoma depends mainly on its etiological agent and the extent of the disease. Knowing the mycetoma type (*actinomycetoma* or *eumycetoma*) is vital for the correct medical management [34]. *Actinomycetoma* is curable by different antibiotic combination therapies depending on the severity, dissemination, and location of the granulomas [35]. In contrast, the management of *Madurella mycetomatis*, the most common fugal type of *eumycetoma*, is more complicated. Current treatment usually implicates surgical excision combined with the long-term use of azole antifungals. However, late chronic stages of the disease result in destruction, deformity, and loss of function and may often lead to amputation [36].

There have been several studies regarding the *in vit*ro susceptibility of *M. mycetomatis* against known antifungals [37,38]. Nevertheless, there is relatively few information on their clinical efficacy. The poor response to antifungal therapy observed in clinical practice may be due to the fact that *M. mycetomatis* produces melanin, which is thought to protect the fungus from the host immune system and from antifungal agents [39]. Local administration of antifungals showed inconsistent results with a high rate of failure and complications [40]. The first randomized clinical trial for eumycetoma has started early 2017, supported by DNDi, to assess the clinical efficacy and safety of fosravuconazole, which had been found to be active in a previous *in vitro* screen [41]. However, fosravuconazole has the same mechanism of action as other azole antifungals, which rises the threat of cross-resistance. Hence, there is a pressing need for novel therapeutic alternatives based on new chemotypes.

7.11.1 Natural products against Eumycetoma

Although traditional remedies have been investigated in various infectious diseases, very limited studies have been conducted on the activity of medicinal plants against eumycetoma. Furthermore, it was reported that 42.2% of the Sudanese myctoma patients at the Mycetoma Center had used herbal medicine at some stage of their illness, with a complication rate of 29.3% [42]. Tea tree oil was shown to inhibit the growth of *M. mycetomatis* at concentrations below 0.25% (v/v) [43]. Another study tested the *in vitro* antimycetomal activity of some Sudanese medicinal plants, and has shown that stigmatriene from *Boswellia papyrifera* was active (MIC_{50} of 32 µg/ml) [44]. However, these reports lacked a full characterization of the active compounds and cytotoxicity profiling.

We used the so called "educated guess" for the search of an antimycetomal lead. Natural compounds of known antifungal and anti-infective activities were tested *in vitro* against *M. mycetomatis* employing viability assays with a resazurin read-out (Chapter 5). The cytotoxicity of these compounds against L-6 rat skeletal cells was also determined. Of all tested natural compounds, magnolol possessed the highest activity (MIC of 15 µM) and selectivity (SI of 4.9). It is recommended that confirmatory tests should be performed with a larger set of *Madurella* strains or with eumycetoma causative agents other than *Madurella* spp. Moreover, the effect of melanin on the MIC should be considered in secondary assays.

7.11.2 Repurposing approach

"The most fruitful basis for the discovery of a new drug is to start with an old one", a quote from the pharmacologist and Nobel laureate 1988 James Black, seems quite relevant specially in the area of neglected tropical diseases, where an enormous unmet need for therapies still remains [45]. This strategy has the benefits of lowering the costs and timeline for drug development, as well as profiting from the availability of clinical data from pre-existing programs [46].

Drug repurposing has been considered in eumycteoma drug screening campaigns. Indeed, the frontrunner clinical candidate fosravuconazole was repurposed from Chagas disease [41]. Drug-like molecules from The Pathogen and Stasis boxes were screened for *in vitro* and *in vivo* activity against eumycetoma, and fenarimols were identified as potential hits [47]. Fenarimols are fungicides that act by inhibiting ergosterol biosynthesis of fungi [48]. However, these compounds are known for their endocrinal [49] and neurological toxicities [50], rendering them rather less favorable options, particularly, for long-term use which is the case for eumycetoma. Niclosamide, which was identified as a potential antimycetomal candidate in Chapter 6, could be a safer option. While niclosamide by itself is not absorbed from the gastrointestinal tract, the ethanolamine salt of niclosamide (NEN) has a better solubility in water and is systemically absorbed. NEN is presently being studied for different diseases [51–57] and might be repurposable for eumycetoma, too. Yet, further investigations concerning the accessibility of the drug into the fungal grain and its *in vivo* activity will need to be performed to evaluate the potential of niclosamide-ethanolamide as an antimycetomal agent.

7.12 Final conclusion

This work afforded a comprehensive overview of Sudanese medicinal plants, and it demonstrated that these plants are a promising source of bioactive molecules against protozoan parasites. Systematic evaluation of their antiparasitic and cytotoxicity profiles was achieved. A dereplication strategy was accomplished for a number of active extracts and allowed prioritization for follow-up selection. Three plants, namely *Croton gratissimus*, *Cuscuta hyalina*, and *Haplophyllum tuberculatum*, were pursued for HPLC-based activity profiling. Preparative isolation of active compounds to perform structure elucidation and *in vitro* test was performed. Thanks to HPLC-hyphenated techniques, the isolation of some of these compounds from the investigated plants as well as their antiprotozoal activities have been reported for the first time. The neglected disease mycetoma has received special consideration, and different approaches were tackled to

ultimately identify potential hits. With regard to antimycetomal natural products, several compounds were selected based on an educated-guess and were assessed accordingly. Furthermore, this work explored the potential of drug repurposing as a promising strategy for mycetoma drug development.

All in all, this thesis is a contribution to the scientific validation of Sudanese medicinal plants and to the discovery of new molecules against protozoa and mycetoma. The encouraging outcomes of this research have clearly demonstrated that natural products represent an unparalleled reservoir of molecular diversity for drug discovery and development, and contribute to a better understanding of the natural products–based drug discovery approach.

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Curriculum Vitae

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PROFESSIONAL EXPERIENCE: (National and International)

03.2016 – 06.2020 **PhD in Microbiology- entitled "Mining Sudanese Medicinal plants for Natural Compounds against Malaria and Neglected Tropical Diseases"**

Parasite Chemotherapy Unit, Swiss Tropical and Public Health Institute, University of Basel, Switzerland

- Natural products-based drug discovery of antiprotozoal and antifungal agents using the following procedures: extraction, chromatographic techniques (HPLC, LC/MS/MS, UV, ELSD) and isolation of bioactive compounds.
- Performed drug screening protocols for antiparasitic activity and cytotoxicity that encompass different cell viability assays (Alamar blue, radioactive [^{3}H]hypoxanthine, fluorometry, and high content imaging).
- Visiting scientist at Pharmaceutical Biology division, Department of Pharmaceutical Sciences, University of Basel, Switzerland.
- Co-ordinated research projects in a multi-national collaboration network and multi-cultural environment.
- Achievements: 3 publications and 2 submitted manuscripts, oral and poster presentations in six different international conferences and prize awardee.

08.2010- Present **Lecturer**

Department of Pharmaceutics, Faculty of Pharmacy, University of Khartoum.

- Tutoring and supervising students in practical pharmaceutics laboratory.
- Preparing, leading discussions and practical classes in laboratories of pharmaceutics for theoretical lectures.
- Holding office hours, invigilating tutorial tests and exams and recording students' grades.

09.2009 – 0.2.2016 **Production Line manager Pharmacist**

Production Department, Humavet Drugs Industry, Sudan.

- *Developing and reviewing SOP's, ensuring manufacturing compliance under GMP guidelines and related regulatory standards.*
- Oversight responsibilities of staffing, training and management of more than 120 employees in production department.
- *Method development and validation for In-process quality control procedures (i.e, fragility, disintegration, and dissolution tests for solid dosage forms).*
- Planning and monitoring manufacturing processes, issuing and documentation on batch manufacturing record (BMR).
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12.2008 – 09.2009 **Community Pharmacist**

Hiba Pharmacy, Khartoum, Sudan.

- Implementing Good Pharmacy Practices and good Dispensing Practices.
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EDUCATION AND QUALIFICATIONS

2016-2020 **PhD in Microbiology**

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2009 – 2012	**Master Degree in Clinical Pharmacy** *Faculty of Pharmacy, University of Khartoum, Sudan.* Thesis title: Implementation of Bioequivalence studies in Sudan: Regulatory perspective.
2003 – 2008	**Bachelor Degree in Pharmacy** *Faculty of Pharmacy, University of Khartoum, Sudan.*

EXPERIMENTAL AND ANALYTICAL SKILLS

Chromatographic and Separation techniques	RP, NP, analytical, preparative/semi-preparative), HPLC-UV/MS (ESI, APCI, single quad, and triple quad) and ELSD.
Qualitative analyses	Friability, disintegration, and dissolution tests for solid dosage forms.
Biological assays	In vitro cell culture techniques, cell viability assays (Resazurin, radioactive [^{3}H]hypoxanthine, fluorometry), MTS and IC50.
Computer skills	▪ Chemistry-related software: LabSolutions, ACD-labs, ChemStation, ChemDraw, ChemSketch, SoftMax Pro. ▪ Graphic/Application software: MS Office package, Adobe illustrator.

LANGUAGE SKILLS

English:	Professional working proficiency
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AWARDS

2018	Prize for the **Best Student Presentation**, Swiss Society of Tropical Medicine and Parasitology.
2017	Award of **Best Poster** prize, International Conference on Science and Society 2017: Phytomedicine and Biopiracy (ICSS-2017)", Mainz, Germany.

SCHOLARSHIPS AND GRANTS

2016-2019	Scholarship commission for junior staff from developing countries, Amt für Ausbildungsbeiträge – Kanton Basel-Stadt, Switzerland.

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PEER-REVIEWED PUBLICATIONS

- **Mahmoud, A.B.**; Mäser, P.; Kaiser, M.; Hamburger, M.; Khalid, S. Mining Sudanese Medicinal Plants for Antiprotozoal Agents. *Front. Pharmacol*. **2020**, 11, 865.

- **Mahmoud, A.B.**; Danton, O.; Kaiser, M.; et al. Lignans, Amides, and Saponins from *Haplophyllum tuberculatum* and Their Antiprotozoal Activity. *Molecules*. 2020;25(12):E2825.

- **Mahmoud, A.B.**; Danton, O.; Kaiser M.; et al. HPLC-Based Activity Profiling for Antiprotozoal Compounds in *Croton gratissimus* and *Cuscuta hyalina*. *Front. Pharmacol*. 2020;11:1246.

- **Mahmoud, A.B.**; Abd Algaffar, S.; De Sande, W.; et al. In vitro testing of redox-active parasiticides identifies niclosamide as a hit for *Madurella mycetomatis* and *Actinomadura* spp. (submitted).

CONFERENCE PARTICIPATIONS

September 2019 Poster entitled "HPLC-based activity profiling of *Haplophyllum tuberculatum* In vitro activity against *Madurella mycetomatis*" presented at "The 67th Annual Meeting of the Society for Medicinal Plant and Natural Product Research" Innsbruck, Austria.

February 2019 Poster presentation entitled "Repurposing the anthelmintic drug niclosamide inhibited *Madurella mycetomatis* – one of the most neglected diseases" at "The mycetoma sixth international conference", Khartoum, Sudan.

November 2018 Oral presentation entitled "Antiparasitic activity of some Sudanese medicinal plants" at "The Annual meeting of Swiss Society of Tropical Medicine and Parasitology", Sigriswill, Switzerland.

September 2017 Poster entitled "Screening of Selected Sudanese Medicinal Plants for in vitro Activity against Protozoan Neglected Tropical Diseases" presented at "The 65th Annual Meeting of the Society for Medicinal Plant and Natural Product Research" Basel, Switzerland.

July 2017 Poster Presentation entitled "Dereplication in drug discovery against neglected tropical diseases: *Acacia nilotica* (L.) Willd.ex Del. As an example". At the "International Conference on Science and Society 2017: Phytomedicine and Biopiracy (ICSS-2017)", Mainz, Germany.

September 2016 Poster presentation entitled "Mining Sudanese medicinal plants for natural compounds against neglected tropical diseases" presented at "The joint annual meeting of the Swiss Society of Tropical Medicine and Parasitology", Montreux, Switzerland.

February 2015 Oral symposium session entitled "BCS-based Biowaiver applicability: Regulatory and industrial perspective" – 6th medical and health sciences studies conference-Khartoum-Sudan.

March 2013 Poster and oral presentation entitled (Implementation of Bioequivalence Studies in Sudan) in Dubai Pharmaceutical and Technology Conference (DUPHAT 2013), Dubai, United Arab Emirates.

PROFESSIONAL DEVELOPMENT COURSES: (total 40 ECT)

Courses accomplished at University of Basel, Switzerland:

2019 Project Management- Toolbox for Scientists.

2017- 2018 Biostatistics and Experiment Planning.
Practical Exercises in Medical Parasitology.
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Pharmacogenomics.
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2016- 2017	Essentials of Drug Developments and Clinical Trials. Drug Discovery and Development of Parasitic Diseases. Computer Modeling of Adverse Effects. Drug Metabolism and Pharmacokinetics. Drug Delivery and Targeting. Industrial Pharmacy.

EXTRACURRICULAR ACTIVITIES AND INTERESTS

Extracurricular activities

2008-2010	Volunteer member of Sudan Aid Charity Organization- which provides financial and psychological support for leukemia children, Sudan.
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Interests

Music	Oud (Andalusia Guitar)
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