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Antiplasmodial and Antitrypanosomal Activity of Pyrethrins and Pyrethroids

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Supporting Information

ABSTRACT: In a screen of 1800 plant and fungal extracts for antiplasmodial, antitrypanosomal, and leishmanicidal activity, the *n*-hexane extract of *Chrysanthemum cinerariifolium* (Trevir.) Vis. flowers showed strong activity against *Plasmodium falciparum*. We isolated the five pyrethrins [i.e., pyrethrin II (**1**), jasmolin II (**2**), cinerin II (**3**), pyrethrin I (**4**), and jasmolin I (**5**)] from this extract. These were tested together with 15 synthetic pyrethroids for their activity against *P. falciparum* and *Trypanosoma brucei rhodesiense* and for cytotoxicity in rat myoblast L6 cells. The natural pyrethrins showed antiplasmodial activity with IC_{50}s between 4 and 12 μM, and antitrypanosomal activity with IC_{50}s from 7 to 31 μM. The pyrethroids exhibited weaker antiplasmodial and antitrypanosomal activity than the pyrethrins. Both pyrethrins and pyrethroids showed moderate cytotoxicity against L6 cells. Pyrethrin II (**1**) was the most selective antiplasmodial compound, with a selectivity index of 24.

KEYWORDS: *Chrysanthemum cinerariifolium*, Asteraceae, pyrethrins, pyrethroids, antiplasmodial, antitrypanosomal, malaria, trypanosomiasis

INTRODUCTION

Pyrethrum (*Chrysanthemum cinerariifolium* (Trevir.) Vis. (synonyms *Tanacetum cinerariifolium* (Trevir.) Schultz Bip. and *Pyrethrum cinerariifolium* (Trevir.)), a herbaceous perennial of the Asteraceae family, is the most widely used botanical insecticide. Currently, it is cultivated in Kenya, Australia, and United States of America, among other countries.[1−3] The secondary metabolites responsible for the insecticidal activity of pyrethrum are six closely related esters of the pyrethric and chrysanthemic acids and the pyrethrolone, cinerolone, or jasomolone alcohols.[1,3,4] Pyrethrins are very potent and selective natural insecticides. However, these compounds are quickly degraded when they are exposed to air and sunlight, which limits their efficacy.[1,5]

To overcome instability issues, pyrethrin-inspired synthetic insecticides, the pyrethroids, were developed. They have increased photostability while maintaining the potent, rapid insecticidal activity, and relatively low acute mammalian toxicity of the pyrethrins.[1,5] Currently, pyrethroids are used in agriculture and as active ingredients of household insecticidal products. They have been applied to control vectors of human diseases such as *Anopheles gambiae*, an important carrier for malaria transmission. As a result, pyrethrins occupy an important place in the world's insecticide market, representing 18% of the U.S. dollar value.[5,6]

Pyrethroids can be classified into the classes type I and type II, the main structural difference being the presence of an α-cyano-3-phenoxybenzylalcohol moiety in type II pyrethroids.[7−9] Pyrethrins and pyrethroids exert their insecticidal activity by modification of the insect's voltage-gated sodium channels by slowing the kinetics of channel gate activation and inactivation. This results in increased anion permeability leading to membrane depolarization and a subsequent rise of neuronal action potential.[5,10,11] Besides sodium channels, pyrethrins and pyrethroids can interact with voltage-gated calcium, potassium, and chloride channels, as well as GABA, glutamate, and acetylcholine receptors.[5,8,10]

Pyrethrins and type I pyrethroids have quantitatively and qualitatively different effects than the type II pyrethroids on ion channels and different visible effects on insects.[7−11]

The biological activity of pyrethrins beyond their insecticidal properties has not been extensively investigated. However, some data on antimycobacterial (*M. avium* and *M. tuberculosis* H37Rv) and antiviral properties (Herpes Simplex Virus, HSV-1, MacIntyre strain ATCC No. VR-539 and HSV-2, MS strain)[4,12] have been reported.

Our interest in pyrethrum was raised when, in a project for natural product based lead discovery, we performed an antiprotozoal screen of 1800 plant and fungal extracts for effects against the parasites *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Plasmodium falciparum*, and *Leishmania donovani*. These protozoal parasites are the causal agents of human African trypanosomiasis, Chagas disease, malaria, and leishmaniasis, respectively. The extract library included an *n*-hexane extract of pyrethrum flowers that exhibited potent activity against *P. falciparum* and *T. brucei rhodesiense* at a test concentration of 4.8 μg/mL; the inhibition was 87% and 99%, respectively. To identify the compounds responsible for the activity, a series of pyrethrins

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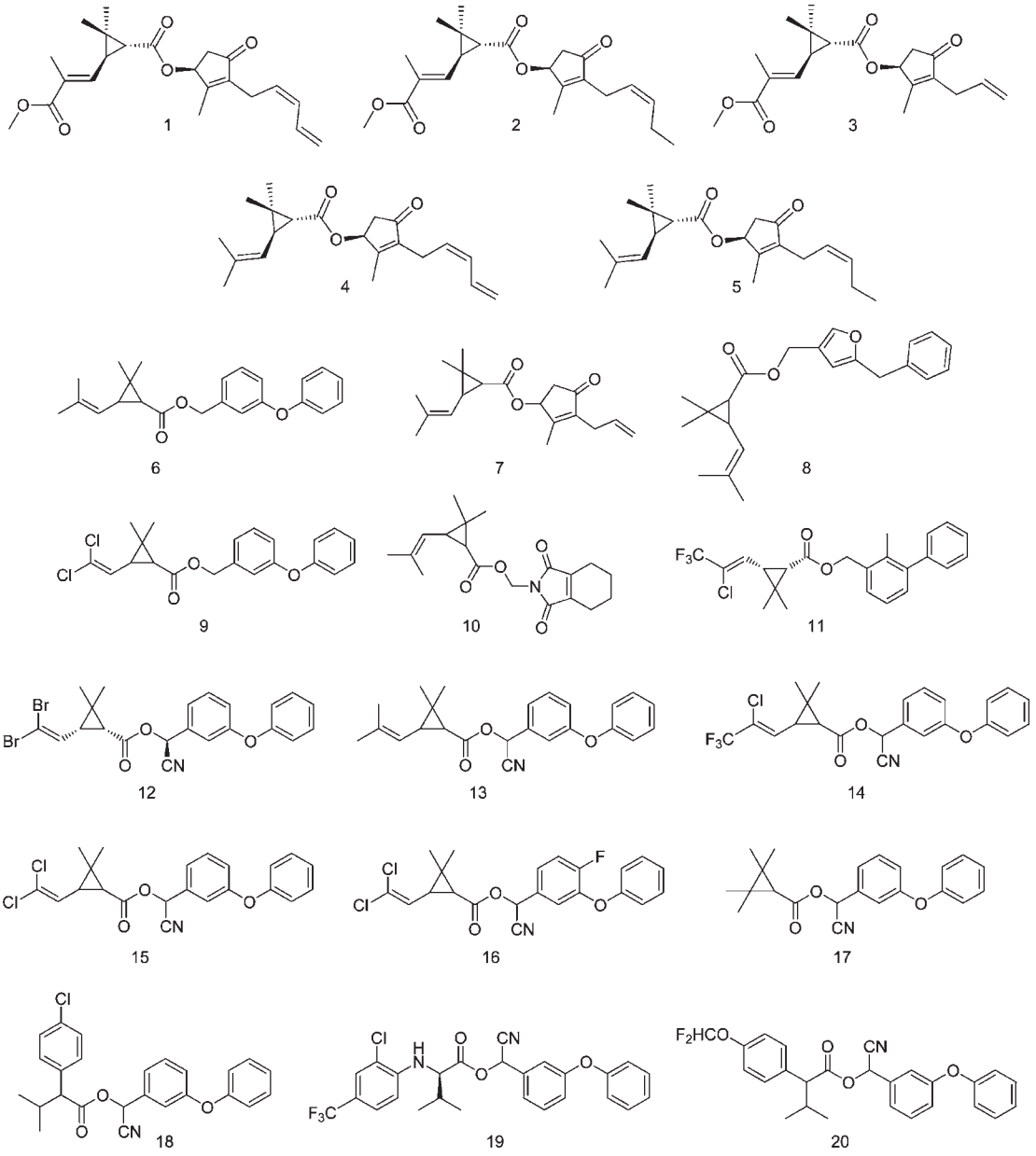

Figure 1. Structures of the pyrethrins **1**−**5**, type I pyrethroids **6**−**11**, and type II pyrethroids **12**−**20**. The comercial pyrethroids (**6**−**20**) are diasteromeric mixtures, with the exception of **11** (*Z*)-(1*S*,3*S*) and **12** (*S*)-(1*R*,3*R*).

(Figure 1) were isolated from the *n*-hexane extract and evaluated for their in vitro antiprotozoal activity. Since the natural products showed activity against the two protozoan parasites, we included six type I pyrethroids and nine type II pyrethroids (Figure 1) to evaluate their effects on the parasites too. The idea was that these commercial compounds, if exerting their antiprotozoal activity possibly on the same targets as the active natural products, might be good leads due to their favorable physicochemical properties, potency, and stability. Besides the antiprotozoal activity, cytotoxicity in rat L6-cells was determined to assess the selectivity of an inhibitory effect on the parasites rather than mammalian cells.

MATERIALS AND METHODS

General Experimental Procedures. Analytical grade solvents for extraction and HPLC grade solvents for chromatography were purchased from Scharlau (Barcelona, Spain). HPLC grade water was obtained by an EASY-pure II from a Barnstead water purification system (Dubuque, Iowa). Formic acid (98.0−100.0%) was from Sigma-Aldrich (Buchs, Switzerland). Artemisinin was obtained from Sigma Aldrich (Buchs, Switzerland) and melarsoprol (Arsobal) from Sanofi-Aventis, (Geneva, Switzerland). The pyrethroids tetramethrin (**10**), bifenthrin (**11**), τ-fluvalinat (**19**), and flucythrinate (**20**) were purchased from Sigma-Aldrich (Buchs, Switzerland). Dr. Ehrenstorfer GmbH (Augsburg, Germany) provided phenothrin (**6**), allethrin (**7**), resmethrin (**8**), permethrin (**9**), deltamethrin (**12**), cyphenothrin (**13**), λ-cyhalothrin (**14**), cypermethrin (**15**), cyfluthrin (**16**), fenpropathrin (**17**), and fenvalerate (**18**).

Preparative HPLC was done on a LC-8A preparative system consisting of two Model LC-8A pumps, a Model SPD-M10A VP PDA detector, and VP2 software (all from Shimadzu, Tokyo, Japan). Medium pressure liquid chromatography (MPLC) was performed using a Büchi

Sepacore system (Büchi, Flawil, Switzerland), consisting of a control unit C-620, a fraction collector C-660, a UV photometer C-635, and two pump modules C-605, on a prepacked silica gel (40−63 μm) polypropylene cartridge (40 × 150 mm, Büchi). TLC was conducted on precoated Kieselgel 60 F_{254}, 0.25 mm plates (Merck, Darmstadt, Germany). NMR data were acquired at a target temperature of 18 °C on an Avance III 500 MHz spectrometer (Bruker, Fällanden, Switzerland) operating at 500.13 MHz for ^{1}H and 125.77 MHz for ^{13}C. A 1 mm TXI-microprobe with a z-gradient was used for ^{1}H-detected experiments. For processing and evaluation Topspin 2.1 (Bruker) software was used. NMR experiments were done as previously described.[13]

Plant Material. Dried flowers of *Chrysanthemum cinerariifolium* (Trevir.) Vis. (Asteraceae) were purchased from Dixa AG (St. Gallen, Switzerland). A voucher specimen (NL 769) is deposited at Herbarium of the Division of Pharmaceutical Biology, University of Basel, Switzerland.

Extraction and Isolation. One kilogram of finely ground plant material was extracted three times with three liters of *n*-hexane for two hours each, at room temperature. Extracts were combined, and the solvent was evaporated at reduced pressure to yield 16.2 g of crude extract. The pyrethrins were isolated from this extract by preparative chromatography in two steps. The first isolation step consisted of MPLC. A portion (10.9 g) of extract was separated on a silica gel prepacked cartridge (40 × 150 mm) by gradient elution with 0−25% ethyl acetate in *n*-hexane over 4 h. The flow rate was 20 mL/min. Fractions were collected every 60 s and compared by TLC [mobile phase: ethyl acetate/*n*-hexane (25:75 v/v)]. Pyrethrins were detected as dark blue spots after spraying with vanillin sulfuric acid reagent (solvent A, vanillin 1% in ethanol; and solvent B, ethanolic solution of sulfuric acid 10%) and heating at 110 °C.[14] Similar fractions were pooled to give two pyrethrin-enriched fractions 3 (570 mg) and 6 (118 mg). Preparative HPLC was used for the isolation of pyrethrins from these fractions. The column employed was a SunFire RP-18 column (5 μm, 30 mm × 150 mm; Waters, Wexford, Ireland). The mobile system was water (solvent A) and methanol (solvent B) using the following gradient: 50% B to 100% B in 30 min, then 100% B for 5 min. The flow rate was 20 mL/min. Sixty milligrams of the fraction in 300 μL of tetrahydrofuran was injected in each run. The UV absorption was monitored at 230 nm.

From fraction number 3, we isolated pyrethrin I (**4**) (10.4 mg), jasmolin I (**5**) (0.8 mg), and jasmolin II (**2**) (0.7 mg). Fraction 6 afforded pyrethrin II (**1**) (9.4 mg) and cinerin II (**3**) (0.6 mg). Extract, fractions, isolated pyrethrins, as well all the commercial pyrethroids were stored protected from light and oxygen, at 2−8 °C under an argon atmosphere.

The structures of pyrethrins were established by 1D and 2D NMR (COSY, HMBC, HSQC, and NOESY). The data are provided as Supporting Information and are in good accordance with the literature.[3,4,15] Purity of pyrethrins was >95% as determined by the integration of ^{1}H NMR spectra.

Biological Tests. The *n*-hexane extract, pyrethrins, and the synthetic pyrethroids were dissolved in DMSO to obtain a final stock solution concentration of 10 mg/mL. The samples were stored at −20 °C until they were used. Further dilutions were done in media so that the DMSO concentration in the highest test concentration before serial dilution was <1%. The screening of the extract library against *P. falciparum* and *T. brucei rhodesiense* was performed in 96 well plates (Costar, Kennebunk, ME), at concentrations of 4.81 and 0.81 μg/mL.[16] Tests were done in triplicate and repeated twice. IC_{50} values against both parasites as well as rat myoblasts (L6-cells) were determined by serial dilution in duplicate and repeated three times.

Testing against P. falciparum K1 Strain. A modification of the [^{3}H]-hypoxanthine incorporation assay was used to determine intraerythrocytic inhibition of parasite growth. Infected erythrocytes (final parasitemia and hematocrit were 0.3% and 1.25%, respectively) in RPMI 1640 medium were exposed to 2-fold serial drug dilution in 96 well plates (Costar, Kennebunk, ME), which covered a range from 10 μg/mL (19.8 to 33.1 μM) to 0.156 μg/mL (0.31 to 0.52 μM). After 48 h of incubation, 50 μL of [^{3}H]-hypoxanthine (0.5 μCi) in the medium was added, and plates were incubated for an additional 24 h. Parasites were harvested onto glass-fiber filters, and radioactivity was counted using a Betaplate liquid scintillation counter (Wallac, Zürich, Switzerland). The results were recorded as counts per minute (cpm) per well at each drug concentration and expressed as a percentage of untreated controls.[13,16]

Testing against T. brucei rhodesiense STIB 900. Minimum essential medium (MEM) supplemented with 2-mercaptoethanol and 15% heat-inactivated horse serum was used for determining the inhibition of parasite growth. Serial 3-fold drug dilution, which covered a range of 90 μg/mL (178 to 298 μM) to 0.123 μg/mL (0.24 to 0.41 μM), was prepared in 96 well plates. Bloodstream forms of *T. brucei rhodesiense* in 50 μL of medium were added to each well except of the background. The plate was incubated under humidified 5% CO_2 atmosphere at 37 °C for 68 h. Resazurin (Sigma-Aldrich, Zürich, Switzerland) solution (12.5 mg of resazurin dissolved in 100 mL of distilled water; 10 μL) was then added to all wells and the incubation continued for a further 2 to 4 h. The plate was read in a Spectramax Gemini XS micro plate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Fluorescence development was measured and expressed as a percentage of the control.[16,17]

Cytotoxicity Test Using L6 Cells. The rat skeletal myoblast cell line (L-6 cells) was used to assess cytotoxicity. The cells were seeded in RPMI 1640 medium supplemented with 1% L-glutamine (200 nM) and 10% fetal bovine serum under humidified 5% CO_2 at 37 °C. Assays were performed in 96 well microtiter plates, with each well receiving 100 mL of culture medium with 4×10^4 cells. After 24 h, the medium was removed from all wells, and serial 3-fold drug dilutions were prepared covering a range from 90 (178.1 to 297.9 μM) to 0.123 μg/mL (0.24 to 0.41 μM). After 68 h of incubation, the plates were inspected under an inverted microscope to ensure the growth of the controls and sterile conditions. Then, 10 μL of resazurin solution (12.5 mg of resazurin dissolved in 100 mL of distilled water) was added to each well, and the plates were incubated for another 2 h. Then, the plates were read with a Spectramax Gemini XS Microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. IC_{50} values were determined using the microplate reader software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA).[18]

RESULTS AND DISCUSSION

A medium throughput screen showed that the *n*-hexane extract of *C. cinerariifolium* flowers had significant activity against *P. falciparum* (86% inhibition at a test concentration of 4.8 μg/mL) and against *T. brucei rhodesiense* (99% inhibition at the same concentration). Since highly lipophilic pyrethrins are the characteristic secondary metabolites in an *n*-hexane extract of pyrethrum flowers, we carried out a targeted isolation of pyrethrins. Among the compounds extracted were three esters of pyrethric acid, [pyrethrin II (**1**), jasmolin II (**2**), and cinerin II (**3**)], and two esters of chrysantemic acid, [pyrethrin I (**4**) and jasmolin I (**5**)] (Figure 1). When tested against *P. falciparum*, *T. brucei rhodesiense*, and L-6 cells, most of these exhibited antiprotozoal activity and low cytotoxicity (Table 1). The most active compound in the antiplasmodial assay was **1** with an IC_{50} of 4.0 ± 1.1 μM, followed by **2** and **3** with IC_{50} values of 5.0 ± 0.4 and 5.8 ± 0.4 μM, respectively. Compounds **4** and **5** were somewhat less active (IC_{50} values of 11.7 ± 1.5 and 9.3 ± 1.2 μM, respectively). Accordingly, the three derivatives of pyrethric acid showed the most potent antiplasmodial effect.

Table 1. In Vitro Antiprotozoal Activities of Isolated Natural Pyrethrins 1−5 and Synthetic Pyrethroids 6−20 against *Plasmodium falciparum* and *Trypanosoma brucei rhodesiense*, Cytotoxic Activity in L-6 Cells, and Selectivity Indices (IC_{50} L-6 cells/IC_{50} parasite)[a]

	IC_{50} (μM) ± SEM			selectivity index	
compound	*P. falciparum*	*T.b. rhodesiense*	L-6 cells	*P. falciparum*	*T.b. rhodesiense*
pyrethrin II (**1**)	4.0 ± 1.1	10.6 ± 0.4	95.1 ± 2.5	23.6	9.0
jasmolin II (**2**)	5.0 ± 0.4	12.0 ± 0.2	31.5 ± 2.3	6.3	2.6
cinerin II (**3**)	5.8 ± 0.4	12.2 ± 0.0	28.0 ± 7.8	4.9	2.3
pyrethrin I (**4**)	11.7 ± 1.5	6.9 ± 1.1	146.6 ± 34.5	12.6	21.2
jasmolin I (**5**)	9.3 ± 1.2	30.9 ± 1.4	86.6 ± 12.9	9.3	2.8
phenothrin (**6**)	inactive[b]	36.4 ± 2.4	57.4 ± 3.1		1.6
allethrin (**7**)	18.0 ± 1.5	26.4 ± 3.4	21.2 ± 1.7	1.2	0.8
resmethrin (**8**)	26.1 ± 2.1	25.5 ± 1.1	51.6 ± 1.4	2.0	2.0
permethrin (**9**)	22.4 ± 0.7	24.5 ± 2.3	66.7 ± 2.2	3.0	2.7
tetramethrin (**10**)	inactive[b]	12.9 ± 2.2	15.5 ± 1.32		1.2
bifenthrin (**11**)	8.9 ± 2.0	24.1 ± 2.0	61.1 ± 6.3	6.9	2.5
deltamethrin (**12**)	inactive[b]	51.4 ± 2.1	142.0 ± 10.5		2.8
cyphenothrin (**13**)	inactive[b]	29.8 ± 0.1	93.1 ± 2.8		3.1
λ-cyhalothrin (**14**)	inactive[b]	inactive[c]	181.2 ± 15.9		
cypermethrin (**15**)	inactive[b]	49.9 ± 0.5	150.7 ± 6.7		3.0
cyfluthrin (**16**)	inactive[b]	27.4 ± 2.6	119.1 ± 14.6		4.4
fenpropathrin (**17**)	inactive	26.2 ± 4.4	21.1 ± 1.9		0.8
fenvalerate (**18**)	14.8 ± 0.9	57.8 ± 6.2	111.0 ± 6.4	7.5	1.9
τ-fluvalinate (**19**)	inactive[b]	26.2 ± 2.6	47.7 ± 2.3		1.8
flucythrinate (**20**)	17.6 ± 0.8	33.4 ± 1.9	111.1 ± 5.5	6.3	3.3
artesunate[d]	0.01 ± 0.006	n.d.	n.d.		
chloroquine[d]	0.40 ± 0.252	n.d.	n.d.		
melarsoprol[e]	n.d.	0.01 ± 0.001	n.d.		
podophyllotoxin[f]	n.d.	n.d.	0.02 ± 0.004		

[a] n.d. not determined. SEM: standard error of the mean. Selectivity index: quotient of the activity of the compounds on the parasites and the mammalian cells. [b] No activity observed at the highest test concentration of 10 μg/mL, which corresponds to molar test concentrations of 19.8 to 33.1 μM. [c] No activity observed at the highest test concentration of 90 μg/mL, which corresponds to molar test concentrations of 178 to 298 μM. [d] Reference drug *P. falciparum* assay. [e] Reference drug *T. b. rhodesiense* assay. [f] Reference drug cytotoxicity assay.

The antitrypanosomal activity of the isolated compounds was less pronounced (Table 1). Pyrethrin I (**4**) showed an IC_{50} of 6.9 ± 1.1 μM. The IC_{50} values of compounds **1**, **2**, **3**, and **5** were above 10 μM (10.6 ± 0.4, 12.1 ± 0.2, 12.2 ± 0.02, and 30.9 ± 1.4 μM, respectively).

The compound with the lowest cytotoxicity against L6 cells was **4** followed by **1** (Table 1). With a SI of 23.6, pyrethrin II (**1**) was the most selective compound against *P. falciparum*. The other four pyrethrins showed selectivity indices (SI) between 4.9 and 12.6 (Table 1). Pyrethrin I (**4**) showed the highest SI (21.2) against *T. brucei rhodesiense*, while the other four pyrethrins exhibited SI between 2.3 and 9.0.

We then tested a series of 6 type I (**6**−**11**) and 9 type II pyrethroids (**12**−**20**). Type I pyrethroids were more active against *P. falciparum* than type II compounds but less active than the pyrethrins (**1** − **5**), with the exception of **11**, which showed an IC_{50} of 8.9 ± 2.0 μM. Six (**12**−**16** and **19**) of the nine type II pyrethroids did not show antiplasmodial activity at the highest tested concentration of 10 μg/mL. This corresponds to the molar test concentrations ranging from 19.8 to 33.1 μM for the various substances (Table 1).

Both, type I and type II pyrethroids displayed low activity against *T. brucei rhodesiense* (Table 1). Their IC_{50} values were in the range of 12.9 ± 2.2 μM for **10** (a type I pyrethroid) and 57.8 ± 6.2 μM for **18** (a type II pyrethroid). Selectivity toward the protozoa was low.

In conclusion, pyrethrins were the compounds responsible for the antiplasmodial activity of the lipophilic pyrethrum extract. These compounds had reasonably selective antiplasmodial properties and weaker trypanocidal activity. Among them, pyrethrin II (**1**) was the most active and selective compound. In contrast, all 15 pyrethroids showed a weaker and less selective inhibition on *P. falciparum* and *T. brucei rhodesiense*.

As mentioned above, the main target for pyrethrins and pyrethroids in insects are voltage-gated sodium channels. Neither in *P. falciparum* nor in *T. brucei rhodesiense* has the presence of such channels been reported.[19−21] The antiprotozoal effects reported here may involve some of the other channels or structurally related ones with which pyrethrins and pyrethroids can interact. For instance *T. brucei rhodesiense* has potassium channels,[19] and *P. falciparum* has channels such as aquaporin PfAQP, which could possibly be targets for these compounds.[20,21]

■ ASSOCIATED CONTENT

Supporting Information. ^{1}H NMR spectroscopic data (500 MHz, $CDCl_3$) for compounds (**1**−**5**). This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

GABA, γ-aminobutyric acid; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; NMR, nuclear magnetic resonance; MPLC, medium pressure liquid chromatography; COSY, correlation spectroscopy; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum correlation; NOESY, nuclear Overhauser effect spectroscopy; RPMI 1640, Roswell Park Memorial Institute medium; MEM, minimum essential medium; SI, selectivity index.

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