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Identification of two new phenathrenones and a saponin as antiprotozoal constituents of *Drypetes gerrardii*

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ABSTRACT

In an *in vitro* screen of 206 extracts from South African plants, the CH_2Cl_2/MeOH (1:1) stem extract of *Drypetes gerrardii* Hutch. var *gerrardii* (Putranjivaceae) inhibited *Plasmodium falciparum* and *Leishmania donovani* (IC_{50}s of 0.50 and 7.31 μg/ml, respectively). In addition, the CH_2Cl_2/MeOH (1:1) extract of the leaves showed activity against *Trypanosoma brucei rhodesiense* (IC_{50} of 12.1 μg/ml). The active constituents were tracked by HPLC-based activity profiling, and isolated by preparative and semi-preparative RP-HPLC chromatography. Their structures were established by HRESIMS, and 1D and 2D NMR (^1H, ^{13}C, COSY, HMBC, HSQC, and NOESY). From the stem extract, a new phenanthrenone derivative, drypetenone D (**1**), and a phenanthrenone heterodimer, drypetenone E (**2**), were isolated. Compound **1** showed potent *in vitro* activity against *P. falciparum* (IC_{50} of 0.9 μM) with a selectivity index (SI) of 71, as calculated from cytotoxicity data for L-6 cells. These data qualified **1** for *in vivo* assessment in the *Plasmodium berghei* mouse model, but the compound turned out to be inactive. Compound **2** also exhibited good *in vitro* antiplasmodial activity (IC_{50} of 2.0 μM) and selectivity (SI 31). From the leaf extract, the saponin putranoside A (**3**) was isolated and identified. Compound **3** showed weak *in vitro* trypanocidal activity, with an IC_{50} of 18.0 μM, and a SI of 4.

1. Introduction

Tropical parasitic diseases have devastating social and economic effects for Third World countries (Renslo and McKerrow, 2006). According to WHO estimations, 207 million cases of malaria infections and 627,000 malaria deaths occurred worldwide in 2012. Malaria is transmitted by mosquitoes of the genus *Anopheles* and caused by five *Plasmodium* species. Artemisinin derivatives are the most recent drugs, but artemisinin-resistant *Plasmodium falciparum* strains have already emerged in Asia (Miller et al., 2013; WHO, 2013a,b). Human African trypanosomiasis (HAT) is confined to sub-Saharan African countries where tsetse flies (genus *Glossina*) transmit the disease. The number of reported cases was 7197 in 2012. *Trypanosoma brucei gambiense* accounts for more than 98% of the reported cases of HAT and *Trypanosoma brucei rhodesiense* for the rest. Drugs in current use show varying degrees of toxicity and efficacy, and all require parenteral administration (WHO, June, 2013; Brun et al., 2011). There are more than 21 species of *Leishmania* sp. that cause various forms of leishmaniasis ranging from the cutaneous form to the lethal, if untreated, visceral form (VL). VL has an estimated global incidence of 200,000–500,000 cases and a prevalence of 5 million cases worldwide. Antileishmanial drugs are available, but suffer from limited efficacy, long treatments, or high costs (Stuart et al., 2008; WHO, 2010). In view of the unsatisfactory therapeutic options currently available, there is an urgent need for new drugs to treat these diseases (Renslo and McKerrow, 2006; Stuart et al., 2008).

Natural products constitute an invaluable source of new drug scaffolds, particularly in the area of infectious diseases (Hannaert, 2011; Renslo, 2013). In the search for new antiprotozoal compounds, we screened a library of South African medicinal

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plants for antiprotozoal activity (Hata et al., 2011, 2013; Mokoka et al., 2011, 2013). A stem extract from *Drypetes gerrardii* Hutch. var. *gerrardii* (Putranjivaceae) showed pronounced antiplasmodial and antileishmanial activities. In addition, a leaf extract of the same plant exhibited trypanocidal properties (Mokoka et al., 2013). There are few reports about the phytochemistry of *D. gerrardii*. So far, triterpenes belonging to the friedelane, hopane and lupane series, and flavone dimers, amentoflavone and drypetdimer A, have been isolated (Ng'ang'a et al., 2008, 2011). The antiplasmodial activity of the dimeric flavonoids was evaluated against the K1 strain of *P. falciparum* (Ng'ang'a et al., 2012). We report here on the identification of the antiprotozoal constituents from stem and leaf extracts, and on the biological assessment of the isolated compounds.

2. Results and discussion

The CH_2Cl_2/MeOH 1:1 extract of the stems of *D. gerrardii* inhibited *P. falciparum* and *L. donovani* with IC_{50} values of 0.50 μg/ml and 7.31 μg/ml, respectively. Activity in the extract was tracked by HPLC-based activity profiling (Fig. 1). The chromatogram exhibited a strong UV-absorbing peak at t_r = 19.0 min which correlated with 71% inhibition of *L. donovani*, and 95% inhibition of *P. falciparum*. The compound was isolated by flash chromatography of the CH_2Cl_2/MeOH (1:1) extract, followed by semi-preparative RP-HPLC. In addition, a structurally related compound **2** was

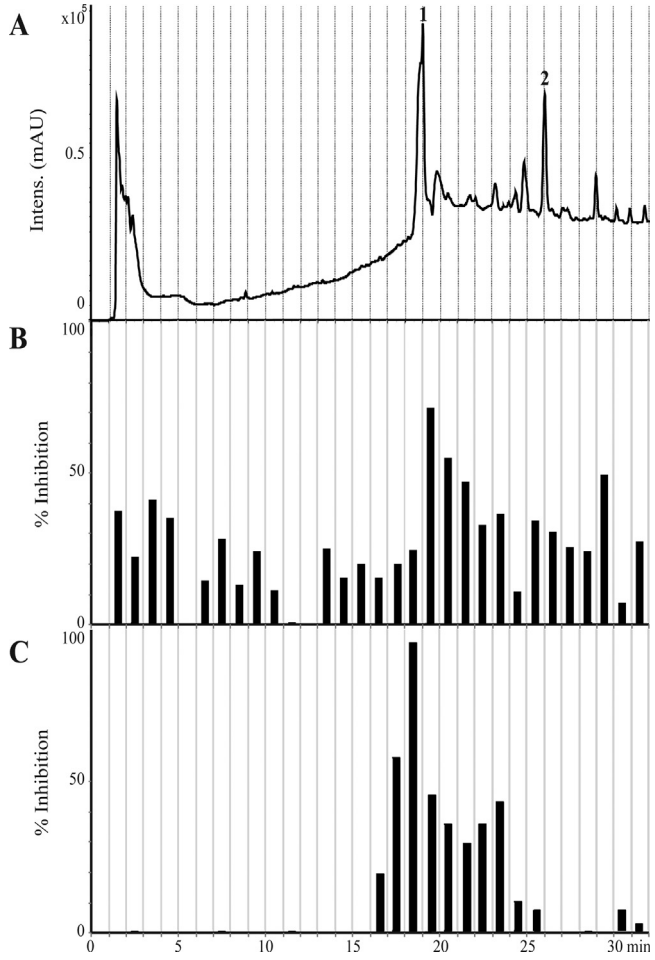

Fig. 1. HPLC-based activity profiling of the CH_2Cl_2/MeOH (1:1) stem extract of *D. gerrardii* for antiprotozoal activity. HPLC-UV chromatogram (210–700 nm) (A), and inhibition (in %) of *L. donovani* (B) and *P. falciparum* (C) by microfractions.

obtained. The structures of **1** and **2** were determined by HRESIMS and NMR analyses (^{1}H NMR, ^{13}C NMR, HMBC, HSQC, and NOESY).

Compound **1** had a molecular formula of $C_{17}H_{16}O_2$ HRESIMS *m/z* = 275.1067 [M+Na]+ (calcd. for $C_{17}H_{16}NaO_2$, 275.1043), thus requiring 10° of indices of hydrogen deficiency. The ^{1}H and ^{13}C NMR spectra displayed resonances in the low field region of the spectrum and were indicative of a highly substituted aromatic ring system. The ^{1}H NMR spectrum in $CDCl_3$ displayed an AX olefinic spin system at δ_H 8.20 (d, *J* = 10.1 Hz) and 6.29 (d, *J* = 10.1 Hz), two *ortho* coupled aromatic protons at δ_H 7.75 (d, *J* = 8.5 Hz) and 7.39 (d, *J* = 8.5 Hz), two aromatic protons as singlets at δ_H 7.59 and 7.53, an aliphatic geminal dimethyl group δ_H 1.51 (6H, s), and an aromatic methyl group at δ_H 2.42. The ^{13}C NMR spectrum displayed seventeen signals consistent with three methyls, six methines, eight quaternary carbons including a carbonyl (δ_C 205.5), one oxygenated aromatic carbon (δ_C 154.7) and a quaternary carbon bearing the *gem*-dimethyl group (δ_C 48.1). A careful analysis of HMBC and NOESY correlations (Fig. 4) suggested that **1** possessed a phenanthrenone skeleton similar to trigohowilol A (**4**) (Tang et al., 2012) except for the absence of the hydroxyl group at C-9 and of the *O*-methyl substituent at C-6 in **1**. The location of the scalar coupled doublets (δ_H 7.75 and 7.39, *J* = 8.5 Hz) at positions C-9 and C-10, respectively, was inferred from HMBC correlations from H-9 to C-8, C-4b and C-10a, and from H-10 to C-4a and C-8a. This structural assignment was corroborated by a NOESY correlation between H-9 and H-8, and between H-10 and the *gem*-dimethyl group at C-1. Thus, the structure of **1** was established as shown in Fig. 3 and the compound was named drypetenone D.

The molecular formula of compound **2** was determined as $C_{34}H_{30}O_4$ HRESIMS *m/z* = 525.2063 [M+Na]+ (calcd. for $C_{34}H_{30}NaO_4$, 525.2036), with 20 indices of hydrogen deficiency. The ^{1}H and ^{13}C spectra showed the presence of two sets of signals resembling those found for compound **1**, thus suggesting a heterodimeric structure for **2**. The ^{1}H NMR spectrum displayed the resonances of: (1) two *gem*-dimethyl groups, Me-1 (δ_H 1.43, s) and Me-1′ (δ_H 1.45, s); (2) two aromatic methyl groups CH_3-7 (δ_H 2.76, s), CH_3-7′ (δ_H 2.51, s); (3) two sets of AX olefinic doublets H-3 (δ_H 6.03) and H-3′ (δ_H 5.96), H-4 (δ_H 7.40) and H-4′ (δ_H 8.70); (4) two sets of *ortho* coupled aromatic protons H-9 (δ_H 7.78) and H-9′ (δ_H 7.88), H-10 (δ_H 7.43) and H-10′ (δ_H 7.49); (5) three uncoupled aromatic protons H-5 (δ_H 6.93), H-8 (δ_H 7.77) and H-8′ (δ_H 7.66).

The ^{13}C NMR spectrum showed the signals for the aforementioned protonated carbons (see Table 1) and additionally: (1) two carbonyl C-atoms resonating at δ_C 204.5 (C-2) and δ_C 203.9 (C-2′); (2) three oxygen bearing aromatic carbons δ_C 154.6 (C-6), δ_C 147.7 (C-6′) and δ_C 134.2 (C-5′); (3) two aliphatic quaternary carbons δ_C 48.0 (C-1), δ_C 48.6 (C-1′); (4) ten aromatic quaternary carbons δ_C 122.2 (C-4a) and δ_C 121.7 (C-4a′), 130.3 (C-4b) and δ_C 123.5 (C-4b′), 127.0 (C-7) and δ_C 127.6 (C-7′), 129.0 (C-8a) and δ_C 128.7 (C-8a′), 147.5 (C-10a) and δ_C 149.2 (C-10a′). Based on HMBC and NOESY correlations the two substructures A and B (Fig. 5) were identified. Substructure A corresponded to compound **1**, while in substructure B position C-5′ was hydroxylated. In substructure B, the substitution of the aromatic C ring unit was established on the basis of the NOESY correlations between H-8′ and H-9′ and between H-8′ and CH_3-7′. The positions C-6′ (δ_C 147.7) and C-5′ (134.2) of ring C were oxygenated; C-6′ chemical shift was assigned to be δ_C 147.7 by the $^3J_{H-C}$ HMBC correlation from 7′-CH_3 while the remaining upfield shifted ^{13}C resonance was assigned to position C-5′. Considering the substituents of the substructures A and B it became obvious that they were connected through an ether linkage between position C-6 of substructure A and positions C-5′ or C-6′ of substructure B. Unfortunately, no cross peaks were detected in the HMBC spectrum from H-5 to substructure B. In a further effort to discriminate between the two possible regioisomers, a long-range optimized (4 Hz) HMBC spectrum was

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Table 1
^{1}H and ^{13}C NMR spectroscopic data of compounds **1** and **2** in $CDCl_3$.

Position	**1**		**2**[a]		HMBC (H → C)
	δ_H (J in Hz)	δ_C[b]	δ_H (J in Hz)	δ_C[c]	
1	–	48.1	–	48.0	
2	–	205.5 5	–	204.5	
3	6.29 (d, 10.1)	123.6 6	6.03 (d, 10.4 Hz)	124.0	C-1, C-4a
4	8.20 (d, 10.1)	139.9	7.40 (d, 10.4 Hz)	138.8	C-2, C-4b, C-10a
5	7.53 (s)	104.22	6.93 (s)	103.0	C-6, C-7, C-4a, C-8a
6	–	154.7	–	154.6	
7	–	126.8	–	127.0	
8	7.59 (s)	130.2	7.77 (s)	131.3	C-6
9	7.75 (d, 8.5)	130.2	7.78 (d, 8.7 Hz) 13,139.0	130.0	C-4b, C-10a
10	7.39 (d, 8.5)	121.8	7.43 (d, 8.7 Hz)	123.0	C-1, C-4a, C-8a
4a	–	121.6	–	122.2	
4b	–	131.0	–	130.3	
8a	–	127.9	–	129.0	
10a	–	147.1	–	147.5	
1-Me × 2	1.51 (s)	28.0	1.43	27.8	C-1, C-2, C-10a
7-Me	2.42 (s)	16.4	2.76	17.2	C-6, C-7, C-8
1′			–	48.6	
2′			–	203.9	
3′			5.96 (d, 10.6 Hz)	123.0 CC-1′	C-1′, C-4a′
4′			8.70 (d, 10.6 Hz)	141.8	C-2′, C-4b′, C-10a′
5′			–	134.2	
6′			–	147.7	
7′			–	127.6	
8′			7.66 (s)	128.0	C-6′, C-9′, C-4b′
9′			7.88 (d, 8.6 Hz) 13	131.0	C-4b′, C-10a′
10′			7.49 (d, 8.6 Hz)	122.9	C-1, C4a′, C-8a′
4a′			–	121.7	
4b′			–	123.5	
8a′			–	128.7	
10a′			–	149.2	
1′-Me × 2			1.45	27.8	C-1′, C-2′, C-10a′
7′-Me			2.51	16.5	C-6′, C-7′, C-8′

[a] The numbering does not follow the IUPAC nomenclature.
[b] 125.77 MHz.
[c] 150.95 MHz.

measured. No correlations between the C rings of the two substructures were detected. However, position C-5′ could be unequivocally assigned by the $^{4}J_{H-C}$ HMBC observed with H-9′ and H-8′. It was identified as the point of attachment of the ether linkage on substructure B considering the diagnostic upfield shift of δ_C C-5′ (δ_C 134.2), with respect to C-6′ (δ_C 147.7). A further support to this structure assignment came from the NOESY spectrum that showed a strong dipolar coupling between CH_3-7 (δ_H 2.76) and H-4′ (δ_H 8.70). On the basis of these data the structure of **2** was assigned as reported in Fig. 3 and the new compound named drypetenone E.

A conformational analysis of **2** was performed, followed by geometrical optimization for the conformers found within 1 kcal/mol from the corresponding global minimum energy. Two isoenergetic conformers were found (Fig. 6). DFT-optimized structures showed that the two substructures A and B were almost perpendicular and the distance between CH_3-7 and H-4′ was 3.45 Å for both conformers, thus matching the observed dipolar coupling. The tridimensional rearrangement of drypetenone E (**2**) could also provide an explanation of the significant differences in the ^{1}H chemical shift between drypetenone D (**1**) and substructure A of **2**.

There are few reports of naturally occurring phenanthrenones. Such compounds have been isolated from a marine bacterium, *Pseudomonas stutzeri* (Uzair et al., 2008), and from terrestrial plants of the genera *Strophioblachia* (Seephonkai et al., 2013a,b; Seephonkai et al., 2009) and *Trigonostemon* (Hu et al., 2009; Kokpol et al., 1990; Tang et al., 2012; Zhu et al., 2010), which belong to the Euphorbiaceae family. Interestingly, the genus *Drypetes* was formerly included in this family.

Compound **1** showed low *in vitro* activity against *L. donovani* (Table 2). In contrast, **1** remarkably inhibited *P. falciparum* (IC_{50} of

Table 2
In vitro antiprotozoal activities of **1**–**3** against *P. falciparum*, *T. b. rhodesiense*, *L. donovani*, and cytotoxic activity against L-6 cells.

Compound	*P. falciparum*		*T. b. rhodesiense*		*L. donovani*		Cytotoxicity
	IC_{50} (μM)[a]	SI	IC_{50} (μM)[a]	SI	IC_{50} (μM)[a]	SI	IC_{50} (μM)[a]
1	0.96 ± 0.25	71.4	6.0 ± 2.7	11.5	14.0	4.9	68.4 ± 4.6
2	2.04 ± 0.15	31.4	–	–	–	–	64.0 ± 22.4
3	inactive[b]		18.0 ± 2.6	3.8	7.8	8.8	68.2 ± 3.9
Chloroquine[c]	0.004 ± 0.001	–	–	–	–	–	–
Melarsoprol[c]	–	–	0.003 ± 0.001	–	–	–	–
Miltefosine[c]	–	–	–	–	0.552 ± 0.051	–	–
Podophyllotoxin[c]	–	–	–	–	–	-	0.019 ± 0.006

SI (selectivity index): Quotient of IC_{50} for L-6 cells and IC_{50} against parasite.
[a] Values are expressed as mean ± standard error of the mean.
[b] No activity observed at the highest test concentration of 90 μg/ml, which corresponds to a molar test concentration of 115.7 μM.
[c] Reference drugs.

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0.96 μM) and displayed low cytotoxicity for the L-6 cell line (IC_{50} of 68 μM). The selectivity index (SI) calculated as the quotient of IC_{50} for L-6 cells and IC_{50} against parasites gave a value of 71. Compound **2** displayed good *in vitro* activity against *P. falciparum* (IC_{50} of 2.0 μM), even though the selectivity (SI = 31) was not as high as the one of **1**. The low yield of the compound probably explains that its activity was not detected in the HPLC-based activity profiling. These results granted *in vivo* assessment of the antimalarial efficacy of **1** in a mouse model. The full suppressive four-day test was performed on female NMRI mice infected with a GFP-transfected *Plasmodium berghei* strain ANKA. Animals treated with compound **1** showed no parasitemia reduction on day 4 compared to untreated control mice. The absence of activity in this model can be due either to absorption problems or unfavourable PK.

Molecular properties associated with drug-likeness and toxicity are essential in early compound selection. Descriptors, such us Lipinski's "rule of 5" (molecular weight, log P, sum of H-donors/acceptors), rotatable bonds, and polar surface area (PSA) can predict drug-likeness. Compound **1** had a molecular weight of 252 Da, log P of 3.96, sum of H-donors/acceptors of 2, 0 rotatable bonds and a PSA of 37.3 $Å^2$, as calculated by Molinspiration Cheminformatics software (Cheminformatics, 2014). All these values fulfil established drug-likeness criteria (Lipinski and Hopkins, 2004; Pajouhesh and Lenz, 2005). Accordingly, oral absorption and blood-brain barrier permeability can be expected for compound **1**. Oral availability is a desired characteristic for an ideal antimalarial drug (Fidock et al., 2004) while blood-brain barrier permeability is prerequisite for an advanced HAT infection (Brun et al., 2011).

An early *in silico* toxicity prediction can help to anticipate potential "off-target" activity.

There are a series of proteins involved in development of adverse effects. When the ability of a compound to bind to these proteins is simulated and quantified *in silico*, it is possible to obtain an estimated toxic potential (TP). By using the virtual technology "Virtual ToxLab" we estimated a TP of endocrine and/or metabolic disruption, carcinogenicity, and cardiotoxicity (Vedani et al., 2012). Binding affinities of **1** towards 16 target proteins, were calculated to obtain a TP of 0.52. This TP value categorizes **1** as a class I compound (in a scale of 0 to IV, being IV the most toxic). The binding affinity to critical proteins such as hERG, a potassium ion channel related with cardiotoxicity (IC_{50} of 40.2 μM), was low. Nevertheless, **1** bound the progesterone receptor with high affinity (IC_{50} of 46.8 nM). In case the compound progresses through the drug discovery pipeline, this aspect would require further attention since this receptor has a fundamental role during pregnancy, and since antimalarial drugs are expected to be administered also to pregnant women (Fidock et al., 2004).

The second active extract, the CH_2Cl_2/MeOH 1:1 leaf extract, showed inhibition against *T. b. rhodesiense* with an IC_{50} of 12.1 μg/ml. HPLC-based activity profiling revealed strong inhibition in the time window of 19 – 20 min which matched with an intense peak in the HPLC-ELSD chromatogram (Fig. 2). Targeted isolation of this compound by a combination of flash chromatography and open column chromatography afforded **3**. Acid hydrolysis afforded two sugar moieties which were identified by GC–MS analysis after derivatization with cysteine methyl ester and silylation (Severi et al., 2010) as L-rhamnose and D-glucuronic acid. The compound was identified by means of HRESIMS, extensive NMR analyses (^1H NMR, ^{13}C NMR, HMBC, HSQC, and NOESY) and comparison with literature data as 3-*O*-[α-L-rhamnopyranosyl-(1 → 3)-β-D-glucuropyranosyl] oleanolic acid, known as putranoside A (Fig. 3) (Borel and Hostettmann, 1987; Hariharan, 1973, 1974; Melek et al., 2003). Compound **3** showed weak *in vitro* trypanocidal (IC_{50} of 18.0 μM) and antileishmanial activities (IC_{50} of 7.8 μM).

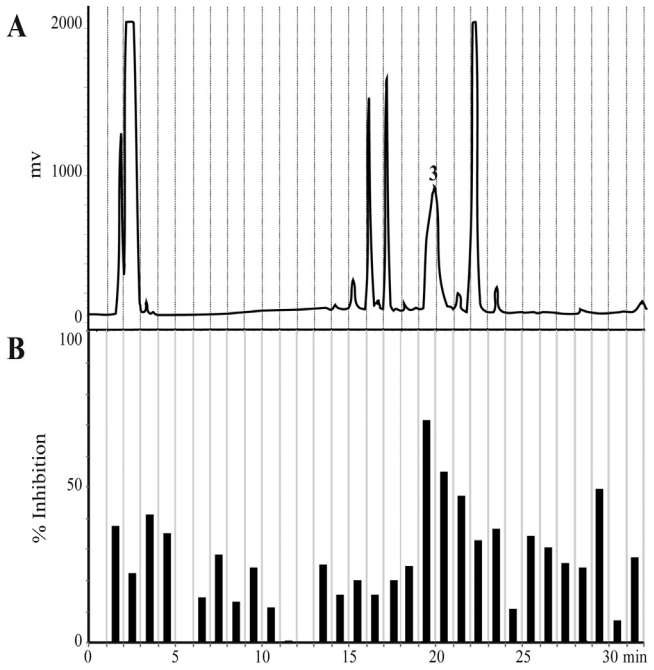

Fig. 2. HPLC-based activity profiling of the CH_2Cl_2/MeOH (1:1) leaf extract of *D. gerrardii* for antitrypanosomal activity. HPLC-ELSD chromatogram (A) and inhibition (in %) of *T. b. rhodesiense* (B) by microfractions.

It is noteworthy that the antiplasmodial triterpenoids which were previously reported from the leaves and stems of *D. gerradii*, including the most active lupane-type derivative resinone (IC_{50} of 0.09 mg/ml, SI 942) (Ng'ang'a et al., 2011, 2012), were not detected by HPLC-MS analysis in the course of our study.

3. Materials and methods

3.1. General experimental procedures

HPLC-grade methanol (MeOH), acetonitrile (MeCN) (Scharlau Chemie S.A.), and water (obtained by an EASY-pure II from Barnstead water purification system, Dubuque) were used for HPLC separations. HPLC solvents contained 0.1% HCO_2H (Sigma) for analytical separations. $CDCl_3$ (100 atom% D), DMSO-d_6 (100 atom% D), and pyridine-d_5 (100 atom% D) for NMR were purchased from Armar Chemicals (Switzerland). Solvents used for extraction were of analytical grade (Romil Pure Chemistry). Reference drugs for bioassays were chloroquine (>98%, Sigma–Aldrich), melarsoprol (purity > 95%, Sanofi-Aventis), miltefosine (purity > 95%, VWR), and podophyllotoxin (purity > 95%, Sigma–Aldrich). HPLC-PDA-MS analyses were performed on an Agilent 1100 system consisting of a degasser, a quaternary pump, a column oven, a PDA detector and to an Esquire 3000 plus ion trap mass spectrometer (Bruker Daltonics). Data acquisition and processing were performed using HyStar 3.0 software (Bruker Daltonics). HPLC-PDA-ELSD analyses were carried out on an Alliance 2695 HPLC system (Waters) equipped with a 996 PDA (Waters) and an Alltech 2000ES ELSD. ELSD parameters were set as follows: N_2 flow = 3.2 l/min, temp. = 115 °C, gain = 8, impactor = off. Empower Pro software (Waters) was used to acquire and process data. Sugar analysis of compound **3** was performed with a HP 5890 Series II gas chromatograph equipped with a HP 5971 mass detector. He was used as a carrier gas. Flash chromatography was carried out on a chromatography system PuriFlash® 4100 (Interchim), controlled with InterSoft V5.0 software. Semi-preparative HPLC was performed on an Agilent 1100 series instrument equipped with a PDA

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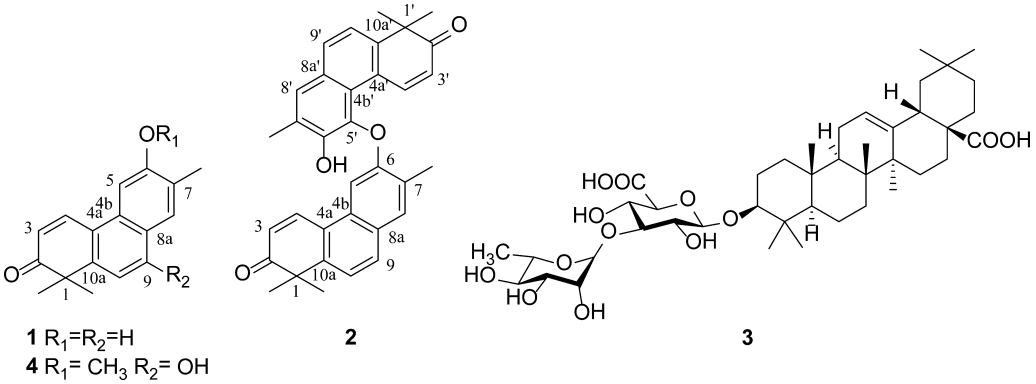

Fig. 3. Structures of compounds **1**–**4**.

detector. Data acquisition and processing were carried out using HyStar 3.2 software (Bruker Daltonics). For HRESIMS a micrOTOF ESI-MS system (Bruker Daltonics) was used. Mass calibration was performed with a solution of formic acid 0.1% in 2-PrOH/H_2O (1:1) containing 5 mM NaOH. Mass spectra were recorded in the range of *m*/*z* 150–1500 in positive ion mode with the aid of micrOTOF control software 1.1 (Bruker Daltonics). NMR spectra were recorded at 18 °C on a Bruker AVANCE III^{TM} 500 MHz spectrometer operating at 500.13 (^{1}H) and 125.77 MHz equipped with a 1 mm TXI microprobe (^{1}H and 2D NMR) or a 5 mm BBO probe (^{13}C NMR) (Bruker BioSpin). The ^{13}C spectrum of **2** was recorded on a Bruker Ascend^{TM} III 600 MHz spectrometer equipped with a QCI cryoprobe. Chemical shifts are reported as δ values (ppm) with the residual solvent signal as internal reference, *J* in Hz. Standard pulse sequences from Topspin 3.0 software package were used.

3.2. Plant material

Samples of the stems of *D. gerrardii* were collected at Hlatikulu Forest on top of the Lebombo mountains in Gwalewemi, KwaZulu Natal Province in South Africa in April 2012 by a botanist, Mr. Hans Vahrmeijer. A plant specimen was deposited at the South African National Biodiversity Institute (SANBI), and the plant identified as *D. gerrardii* Hutch. var. *gerrardii* (Putranjivaceae) (voucher specimen number PRE 45954).

3.3. Extraction

Stems and leaves were collected, separated and dried separately in an oven at 30–60 °C. Dried plant material was ground to a coarse powder using a hammer mill, and stored at ambient temp. prior to extraction. 800 g of dried, ground stems were extracted with 4 l of a mixture of CH_2Cl_2 and MeOH (1:1) at room temperature for 1 h with occasional stirring. The extract was filtered, and the residual plant material further extracted overnight with 2 l CH_2Cl_2/MeOH (1:1), followed by filtration. Finally, a third extraction of the pulp was carried out with 2 l solvent for 1 h with filtration. The filtrates were combined and concentrated using a rotary vacuum evaporator at a temp. below 45 °C, and the residue dried *in vacuo* at room temperature for 24 h. The dried extract of 35.0 g (4.4%, w/w) was stored at −20 °C.

The dried, ground leaves (115 g) of the plant were extracted in a similar way as described above to yield 6.31 g (5.5%, w/w) of extract.

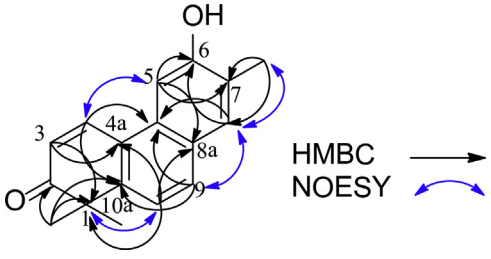

Fig. 4. Key HMBC and NOESY correlations of compound **1**.

3.4. Microfractionation and activity profiling

HPLC-based activity profiling was performed on a SunFire^{TM} C18 column (3.5 μm, 3.0 mm × 150 mm, Waters) equipped with a precolumn (3 mm × 10 mm) as previously described (Adams et al., 2009). H_2O (solvent A) and MeCN (solvent B) were used as solvents, and the following gradient was applied: 10% → 100% B in 30 min, then 100% B for 1 min. The flow rate was 0.5 ml/min. Aliquots (35 μl) of extracts (10 mg/ml in DMSO) were injected, and 32 one-minute fractions collected into a 96 deep well plate (Eppendorf) with the aid of a F204 fraction collector (Gilson). After solvent removal in an Evaporex 96 channel N_2-evaporator (Apricot Designs), the dried fractions were re-dissolved in methanol (100 μl) and transferred to 96 v-well plates (Thermo Scientific), dried again, and stored in a refrigerator (2–8 °C) until use for bioassay.

3.5. Preparative isolation

The CH_2Cl_2/MeOH (1:1) stem extract of *D. gerrardii* (3.4 g) was fractionated by flash chromatography on a silica gel 60 column (15–40 μM, 35 mm × 430 mm, 180 g) with a gradient of 100–0% hexane in EtOAc in 3 h, using a flow rate of 40 ml/min. Fractions (22 ml each) were collected and compared by TLC analysis [silica gel; hexane/EtOAc (9:1 and 7:3); anisaldehyde/sulphuric acid spraying reagent, heated at 110 °C]. Fractions 10 (121.4 mg) and 11 (34.8 mg) were found to contain phenanthrenones. They were purified by semi-preparative RP-HPLC using a SunFire^{TM} C18 column (3.5 μM, 10 mm × 150 mm; Waters) equipped with a precolumn (10 mm × 10 mm). H_2O (Solvent A) and MeCN (Solvent B) were used as solvents and the following gradient was applied: 40% → 60% B in 5 min, then 60% → 70% B in 25 min. The flow rate was 4 ml/min. The fractions were dissolved in DMSO (Fr.10: 10 mg/ml; Fr. 11: 20 mg/ml) and injected in aliquots of 300 μl.

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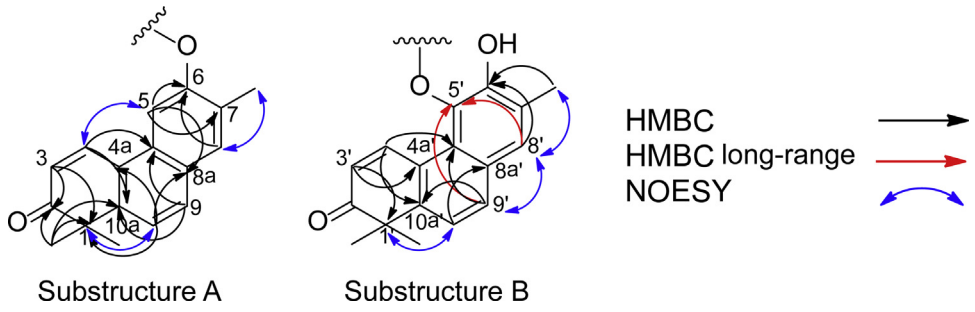

Fig. 5. Key HMBC and NOESY correlations for substructures A and B of compound **2**.

Compound **1** (24.8 mg, t_R 7.7 min) was isolated from Fr. 10 and Fr. 11. Compound **2** (1.4 mg, t_R 24.0 min) was obtained from Fr. 10.

The CH_2Cl_2/MeOH (1:1) leaf extract (355 mg) was separated by flash chromatography on a Sepacore® system (Büchi) by using a pre-packed silica gel 60 cartridge (40–63 μm, 40 mm × 150 mm) with a gradient of 0–60% EtOAc in MeOH, over 2 h. The flow rate was 15 ml/min. Fractions (20 ml; each) were combined based on TLC analysis [silica gel; $CHCl_3$/MeOH/H_2O (65:30:5); vanillin/sulphuric acid reagent, heated at 110 °C]. Similar fractions were pooled into four fractions (Fr. A–D). Fr. B (148.7 mg) was further purified by CC on silica gel (40–63 μm, 8 mm × 160 mm, 12.1 g) using a step gradient of $CHCl_3$ and MeOH (100:0, 95:5, 92.5:7.5, 90:10 87.5:12.5, 85:15, 80:20, 70:30, 60:40, 50:50; 50 ml each) to yield 9 sub-fractions (Fr. B_1–B_9). Subfraction Fr. B_4 eluted with $CHCl_3$/MeOH (80:20) afforded pure compound **3** (31.3 mg).

Purity of compounds **1** and **3** was >95%, and that of **2** >90%, as determined by ^{1}H NMR (Figs. 1S, 5S, 9S, and 15S of Supplementary content).

6-Hydroxy-1,1,7-trimethylphenanthren-2(1*H*)-one (drypetenone D, **1**): Yellow amorphous substance; ^{1}H and ^{13}C NMR data in $CDCl_3$ and DMSO-d_6, see Table 1; HRESIMS: m/z = 275.1067 [M+Na] + (calcd. for $C_{17}H_{16}Na\ O_2$, 275.1043).

6-Hydroxy-1,1,7-trimethyl-5-((2,8,8-trimethyl-7-oxo-7,8-dihydrophenanthren-3-yl)oxy)phenanthren-2(1*H*)-one (drypetenone E, **2**): Yellow amorphous substance; ^{1}H and ^{13}C NMR data in $CDCl_3$, see Table 1; HRESIMS: m/z = 525.2063 [M+Na] + (calcd. for $C_{34}H_{30}NaO_4$, 525.2036).

3-*O*-[α-L-Rhamnopyranosyl-(1 → 3)-β-D-glucuropyranosyl] oleanolic acid (putranoside A, **3**) [37–40]: White amorphous substance; ^{1}H and ^{13}C NMR data see Table 1S Supplementary content; HRESIMS: m/z = 801.4396 [M+Na] + (calcd. for $C_{42}H_{66}NaO_{13}$, 801.4396).

3.6. Conformational analysis and geometrical optimization

Conformational analysis of compound **2** was accomplished with Schrödinger MacroModel 9.1 software employing the OPLS 2005 (optimized potential for liquid simulations) force field in H_2O. Conformers within a 1 kcal/mol energy window from the global minimum were picked for geometrical optimization and energy calculation applying DFT with the B3LYP functional using the 6-31G (d,p) basis set in the gas phase with the Gaussian 09 programme package (Frisch et al., 2009). Vibrational evaluation was done at the same level to confirm minima.

3.7. Bioassays

Extracts were tested *in vitro* against *P. falciparum* (NF54 strain), *T. b. rhodesiense* (STIB 900 strain), and *Leishmania donovani* (strain MHOM/ET/67/L82), in 96-well microtiter plates at concentrations of 10.0 μg/ml and 2.0 μg/ml. IC_{50}s were subsequently determined for active extracts. HPLC microfractions from the CH_2Cl_2/MeOH (1:1) stem extract of *D. gerrardii* were assayed against *P. falciparum* and *L. donovani*. Those from the CH_2Cl_2/MeOH (1:1) leaf extract

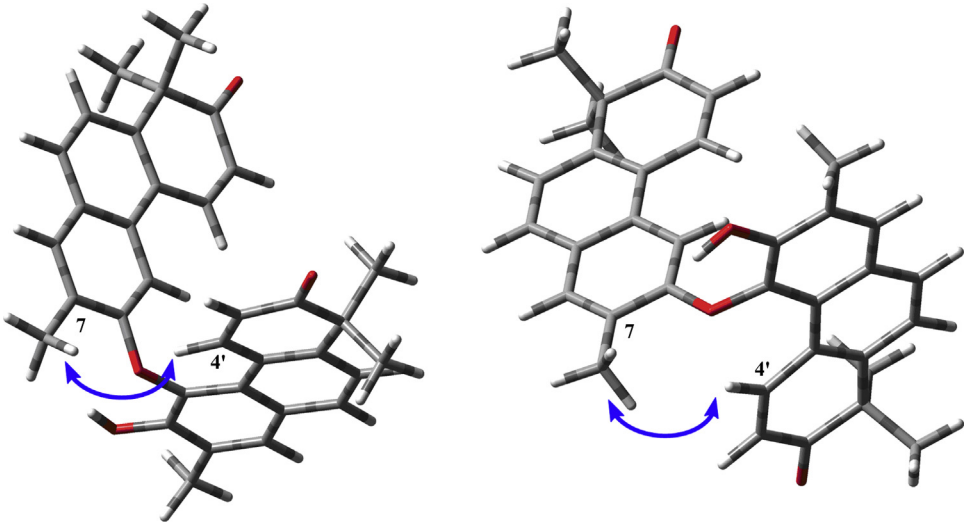

Fig. 6. DFT optimized conformers of compound **2**. Atoms CH_3-7 and H-4′ showing a key NOESY correlation between the 2 substructures. Intramolecular distance between CH_3-7 and H-4′ was 3.45 Å in both conformers.

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were tested on *T. b. rhodesiense*. Compounds **1** and **3** were evaluated for their antiprotozoal activity against the three parasites. Due to the scarcity of isolated material, compound **2** was tested only against *P. falciparum*. Additionally, for pure compounds, cytotoxicity was determined by using a rat skeletal myoblast cell line (L-6 cells). For the assays DMSO stock solutions (10 mg/ml) of extracts and purified compounds were freshly diluted in medium (final DMSO concentration in assay <1%). Assays were performed in two independent replicates. *In vivo* antimalarial activity was assessed as previously described (Peters, 1987) and conducted according to the rules and regulations for the protection of animal rights ('Tierschutzverordnung') of the Swiss 'Bundesamt für Veterinärwesen'. They were approved by the veterinary office of the Canton Basel-Stadt, Switzerland. The animal experiment approval number for *in vivo* testing against *P. berghei* is 1731. Details on assay protocols are provided as Supplementary content.

Author's contributions

Yoshie Hata, HPLC microfractionation of the extracts of *D. gerrardii*, preparative isolation of compounds, recording and interpretation of analytical data for structure elucidation (HPLC-PDA-ESI-MS, TOF-MS, 1D and 2D NMR), preparation of manuscript draft.

Maria De Mieri, measurement and interpretation of NMR data, manuscript preparation.

Samad Nejad Ebrahimi, contributed to isolation, and manuscript preparation.

Tsholofelo Mokoka, processing of plant material, extraction.

Gerda Fouche, manuscript preparation, responsible for the project at the CSIR.

Marcel Kaiser, performed *in vitro* and *in vivo* assays.

Reto Brun, writing manuscript, responsible for the project at the Swiss TPH.

Olivier Potterat, data analysis and interpretation, manuscript preparation.

Matthias Hamburger, analysis and interpretation of data, manuscript preparation, and overall responsibility for the project.

All authors have approved the final version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2014.05.005.

References

Adams, M., Zimmermann, S., Kaiser, M., Brun, R., Hamburger, M., 2009. A protocol for HPLC-based activity profiling for natural products with activities against tropical parasites. Nat. Prod. Commun. 4, 1377–1381.

Borel, C., Hostettmann, K., 1987. Molluscicidal saponins from *Swartzia madagascariensis* Desvaux. Helv. Chim. Acta 70, 570–576.

Brun, R., Don, R., Jacobs, R.T., Wang, M.Z., Barrett, M.P., 2011. Development of novel drugs for human African trypanosomiasis. Future Microbiol. 6, 677–691.

Cheminformatics, M., 2014. Molinspiration. http://molinspiration.com/, Slovak Republic (accessed January 2014).

Fidock, D.A., Rosenthal, P.J., Croft, S.L., Brun, R., Nwaka, S., 2004. Antimalarial drug discovery: efficacy models for compound screening. Nat. Rev. Drug Discov. 3, 509–520.

Frisch, M., Trucks, G.W., Schlegel, H.B., Scuseria, G.E., Robb, M.A., Cheeseman, J.R., Scalmani, G., Barone, V., Mennucci, B., Petersson, G.A., Nakatsuji, H., Caricato, M., Li, X., Hratchian, H.P., Izmaylov, A.F., Bloino, J., Zheng, G., Sonnenberg, J.L., Hada, M., Ehara, M., Toyota, K., Fukuda, R., Hasegawa, J., Ishida, M., Nakajima, T., Honda, Y., Kitao, O., Nakai, H., Vreven, T., Montgomery, J.A., Peralta, J.E., Ogliaro, F., Bearpark, M., Heyd, J.J., Brothers, E., Kudin, K.N., Staroverov, V.N., Kobayashi, R., Normand, J., Raghavachari, K., Rendell, A., Burant, J.C., Iyengar, S.S., Tomasi, J., Cossi, M., Rega, N., Millam, J.M., Klene, M., Knox, J.E., Cross, J.B., Bakken, V., Adamo, C., Jaramillo, J., Gomperts, R., Stratmann, R.E., Yazyev, O., Austin, A.J., Cammi, R., Pomelli, C., Ochterski, J.W., Martin, R.L., Morokuma, K., Zakrzewski, V.G., Voth, G.A., Salvador, P., Dannenberg, J.J., Dapprich, S., Daniels, A.D., Farkas, O., Foresman, J.B., Ortiz, J.V., Cioslowski, J., Fox, D.J., 2009. Gaussian 09, Revision A02 ed. Gaussian Inc., Wallingford, CT.

Hannaert, V., 2011. Sleeping sickness pathogen (*Trypanosoma brucei*) and natural products: therapeutic targets and screening systems. Planta Med. 77, 586–597.

Hariharan, V., 1973. Saponins of seed-coats of *Putranjiva roxburghii*. Indian J. Chem. 11, 830–831.

Hariharan, V., 1974. Structures of putranosides from the seed coats of *Putranjiva roxburghii*. Indian J. Chem. 12, 447–449.

Hata, Y., Raith, M., Ebrahimi, S.N., Zimmermann, S., Mokoka, T., Naidoo, D., Fouche, G., Maharaj, V., Kaiser, M., Brun, R., Hamburger, M., 2013. Antiprotozoal isoflavan quinones from *Abrus precatorius* ssp. *africanus*. Planta Med. 79, 492–498.

Hata, Y., Zimmermann, S., Quitschau, M., Kaiser, M., Hamburger, M., Adams, M., 2011. Antiplasmodial and antitrypanosomal activity of pyrethrins and pyrethroids. J. Agric. Food Chem. 59, 9172–9176.

Hu, X.-J., Wang, Y.-H., Kong, L.-Y., He, H.-P., Gao, S., Liu, H.-Y., Ding, J., Xie, H., Di, Y.-T., Hao, X.-J., 2009. New phenanthrenes from *Trigonostemon lii* Y.T. Chang. Tetrahedron Lett. 50, 2917–2919.

Kokpol, U., Thebpatiphat, S., Boonyaratavej, S., Chedchuskulcai, V., Ni, C.Z., Clardy, J., Chaichantipyuth, C., Chittawong, V., Miles, D.H., 1990. Structure of trigonostemone, a new phenanthrenone from the Thai plant *Trigonostemon reidioides*. J. Nat. Prod. 53, 1148–1151.

Lipinski, C., Hopkins, A., 2004. Navigating chemical space for biology and medicine. Nature 432, 855–861.

Melek, F.R., Miyase, T., Khalik, S.M.A., El-Gindi, M.R., 2003. Triterpenoid saponins from *Schefflera arboricola*. Phytochemistry 63, 401–407.

Miller, L.H., Ackerman, H.C., Su, X.-z., Wellems, T.E., 2013. Malaria biology and disease pathogenesis: insights for new treatments. Nat. Med. 19, 156–167.

Mokoka, T.A., Zimmermann, S., Julianti, T., Hata, Y., Moodley, N., Cal, M., Adams, M., Kaiser, M., Brun, R., Koorbanally, N., Hamburger, M., 2011. In vitro screening of traditional South African malaria remedies against *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Leishmania donovani*, and *Plasmodium falciparum*. Planta Med. 77, 1663–1667.

Mokoka, T.A., Peter, X.K., Fouche, G., Moodley, N., Adams, M., Hamburger, M., Kaiser, M., Brun, R., Maharaj, V., Koorbanally, N., Zimmermann, S., Hata, Y., 2013. Antiprotozoal screening of 60 South African plants, and the identification of the antitrypanosomal eudesmanolides schkurin I and II. Planta Med. 79, 1380.

Ng'ang'a, M.M., Chhabra, S., Langat-Thoruwa, C., Hussain, H., Krohn, K., 2008. Chemical constituents from the leaves of *Drypetes gerrardii*. Biochem. Syst. Ecol. 36, 320–322.

Ng'ang'a, M.M., Hussain, H., Chhabra, S., Langat-Thoruwa, C., Irungu, B.N., Al-Harrasi, A., Riaz, M., Krohn, K., 2012. Antiplasmodial activity of compounds from *Drypetes gerrardii*. Chem. Nat. Compd. 48, 339–340.

Ng'ang'a, M.M., Hussain, H., Chhabra, S., Langat-Thoruwa, C., Riaz, M., Krohn, K., 2011. Drypetdimer A: a new flavone dimer from *Drypetes gerrardii*. Nat. Prod. Commun. 6, 1115–1116.

Pajouhesh, H., Lenz, G.R., 2005. Medicinal chemical properties of successful central nervous system drugs. NeuroRx 2, 541–553.

Peters, W., 1987. Chemotherapy and Drug Resistance in Malaria. Academic Press, Inc., New York, pp. 145–273.

Renslo, A.R., 2013. Antimalarial drug discovery: from quinine to the dream of eradication. Chem. Lett. 4, 1126–1128.

Renslo, A.R., McKerrow, J.H., 2006. Drug discovery and development for neglected parasitic diseases. Nat. Chem. Biol. 2, 701–710.

Seephonkai, P., Pyne, S.G., Willis, A.C., Lie, W., 2013a. Bioactive compounds from the roots of *Strophioblachia fimbricalyx*. J. Nat. Prod. 76, 1358–1364.

Seephonkai, P., Pyne, S.G., Willis, A.C., Lie, W., 2013b. Fimbricalyx A, a novel phenanthrenone derivative having a rare 2H-benz[e]inden-2-one substructure. Tetrahedron Lett. 54, 2085–2088.

Seephonkai, P., Sangdee, A., Bunchalee, P., Pyne, S.G., 2009. Cytotoxic and antiplasmodial compounds from the roots of *Strophioblachia fimbricalyx*. J. Nat. Prod. 72, 1892–1894.

Severi, J.A., Fertig, O., Plitzko, I., Vilegas, W., Hamburger, M., Potterat, O., 2010. Oleanane Saponins and Glycerogalactolipids from the Leaves of *Guapira graciliflora*. Helv. Chim. Acta 93, 1058–1066.

Stuart, K., Brun, R., Croft, S., Fairlamb, A., Gurtler, R.E., McKerrow, J., Reed, S., Tarleton, R., 2008. Kinetoplastids: related protozoan pathogens, different diseases. J. Clin. Invest. 118, 1301–1310.

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Tang, G.-H., Zhang, Y., Yuan, C.-M., Li, Y., Gu, Y.-C., Di, Y.-T., Wang, Y.-H., Zuo, G.-Y., Li, S.-F., Li, S.-L., He, H.-P., Hao, X.-J., 2012. Trigohowilols A-G, degraded diterpenoids from the stems of *Trigonostemon howii*. J. Nat. Prod. 75, 1962–1966.

Uzair, B., Ahmed, N., Ahmad, V.U., Mohammad, F.V., Edwards, D.H., 2008. The isolation, purification and biological activity of a novel antibacterial compound produced by *Pseudomonas stutzeri*. FEMS Microbiol. Lett. 279, 243–250.

Vedani, A., Dobler, M., Smiesko, M., 2012. VirtualToxLab – a platform for estimating the toxic potential of drugs, chemicals and natural products. Toxicol. Appl. Pharmacol. 261, 142–153.

WHO, 2010. Working to Overcome the Global Impact of Neglected Tropical Diseases: First WHO Report on Neglected Tropical Diseases. World Health Organization, Geneva, pp. 91–95.

WHO, 2013a. World Malaria Report 2013. World Health Organization, Geneva, pp. v–xiv.

WHO, 2013b. Trypanosomiasis, Human African (sleeping sickness). Fact sheet N°259 World Health Organization, Geneva.

Zhu, Q., Tang, C.-P., Ke, C.-Q., Li, X.-Q., Liu, J., Gan, L.-S., Weiss, H.-C., Gesing, E.-R., Ye, Y., 2010. Constituents of *Trigonostemon chinensis*. J. Nat. Prod. 73, 40–44.

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