



NOTE

Trypanocidal and leishmanicidal activity of six limonoids

Dietmar Steverding¹ · Lazare S. Sidjui^{2,3} · Édén Ramalho Ferreira^{4,5} · Bathelemy Ngameni⁶ · Gabriel N. Folefoc³ · Valérie Mahiou-Leddet⁷ · Evelyne Ollivier⁷ · G. Richard Stephenson⁸ · Thomas E. Storr⁸ · Kevin M. Tyler⁴Received: 3 February 2020 / Accepted: 31 March 2020
© The Author(s) 2020

Abstract

Six limonoids [kotschyienone A and B (**1**, **2**), 7-deacetylgedunin (**3**), 7-deacetyl-7-oxogedunin (**4**), andirobin (**5**) and methyl angolensate (**6**)] were investigated for their trypanocidal and leishmanicidal activities using bloodstream forms of *Trypanosoma brucei* and promastigotes of *Leishmania major*. Whereas all compounds showed anti-trypanosomal activity, only compounds **1–4** displayed anti-leishmanial activity. The 50% growth inhibition (GI₅₀) values for the trypanocidal and leishmanicidal activity of the compounds ranged between 2.5 and 14.9 μM. Kotschyienone A (**1**) was found to be the most active compound with a minimal inhibition concentration (MIC) value of 10 μM and GI₅₀ values between 2.5 and 2.9 μM. Only compounds **1** and **3** showed moderate cytotoxicity against HL-60 cells with MIC and GI₅₀ values of 100 μM and 31.5–46.2 μM, respectively. Compound **1** was also found to show activity against intracellular amastigotes of *L. major* with a GI₅₀ value of 1.5 μM. The results suggest that limonoids have potential as drug candidates for the development of new treatments against trypanosomiasis and leishmaniasis.

Keywords Limonoids · African trypanosomiasis · *Trypanosoma brucei* · Leishmaniasis · *Leishmania major*

Introduction

Trypanosomiasis and leishmaniasis are devastating diseases for both humans and their domestic animals. Trypanosome parasites cause sleeping sickness in humans and nagana disease in cattle in Africa and Chagas disease in humans in Latin America [1, 2]. The different *Leishmania* parasites cause a variety of clinical conditions (localised skin lesions,

mucosal ulcers, and internal organ damage) in humans worldwide [3]. These parasites are kinetoplastid protozoans and are transmitted to their mammalian host by insect vectors. Treatment of these parasitoses relies on chemotherapy but only a few drugs are available. However, most of the drugs are not well tolerated or show toxic side effects, are not very effective, and are being increasingly subject to drug resistance. Therefore, effective and better-tolerated chemotherapies are urgently needed for the treatment of trypanosomiasis and leishmaniasis.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s11418-020-01408-7>) contains supplementary material, which is available to authorized users.

✉ Dietmar Steverding
dsteverding@hotmail.com

✉ Bathelemy Ngameni
bath_ngameni@yahoo.fr

¹ Bob Champion Research and Education Building, Norwich Medical School, University of East Anglia, Norwich NR4 7UQ, UK

² Institute of Medical Research and Medicinal Plant Studies, P.O. Box 13033, Yaoundé, Cameroon

³ Bioorganic and Medicinal Chemistry Laboratory, Department of Organic Chemistry, Faculty of Sciences, University of Yaoundé I, Yaoundé, Cameroon

⁴ BioMedical Research Centre, Norwich Medical School, University of East Anglia, Norwich NR4 7TJ, UK

⁵ Departamento de Microbiologia, Imunologia e Parasitologia, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil

⁶ Department of Pharmacognosy and Pharmaceutical Chemistry, Faculty of Medicine and Biomedical Science, University of Yaoundé I, Yaoundé, Cameroon

⁷ Aix-Marseille University, Avignon University, CNRS, IRD, IMBE, FAC PHARM, Marseille, France

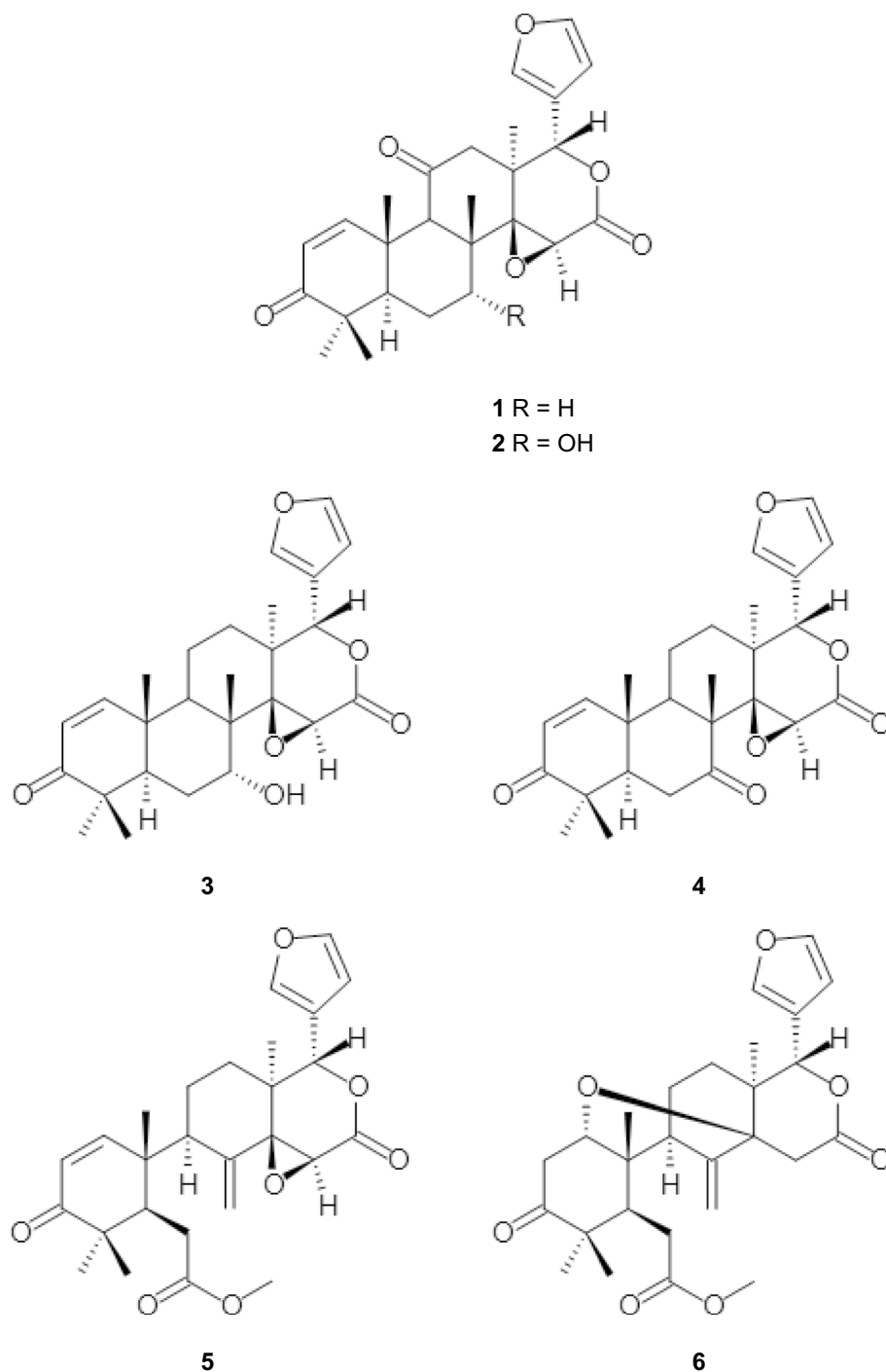
⁸ School of Chemistry, University of East Anglia, Norwich NR4 7TJ, UK

Many approved drugs are based on natural compounds and their derivatives [4]. In addition, a considerable number of natural products have been shown to display potent anti-trypanosomal and anti-leishmanial activity [5]. Plants that are used in traditional medicine are promising starting materials for the discovery of natural compounds with trypanocidal and leishmanicidal activity. One such plant is *Pseudocedrela kotschy* (Schweinf.) Harms (Meliaceae),

which is used for the treatment of trypanosomiasis in the Kaduna state of Nigeria [6]. Furthermore, dichloromethane extracts of the roots of *P. kotschy* have been shown to display anti-leishmanial activity against intracellular amastigotes of *L. major* [7].

In this study, we investigated the trypanocidal and leishmanicidal activity of six limonoids (Fig. 1, compounds 1–6) isolated from the roots of *P. kotschy*.

Fig. 1 Structures of compounds 1–6. Kotschyenone A (1), kotschyenone B (2), 7-deacetylgedunin (3), 7-deacetyl-7-oxogedunin (4), andirobin (5), methyl angolensate (6)



Experimental

Compounds

The limonoids **1–6** were isolated from the roots of *P. kotschyi* as previously described [8]. The compounds have been characterized by NMR (kotschyienone A and B (**1**, **2** [8], 7-deacetylgedunin (**3**) [9, 10], 7-deacetyl-7-oxogedunin (**4**) [9, 11], andirobin (**5**) [12] and methyl angolensate (**6**) [11]) and their purity was judged to be about 95% by TLC. The ¹H-NMR and ¹³C-NMR spectra of **1–6** are shown in Supplementary Figures S1–6.

Cell culture

Bloodstream forms of *Trypanosoma brucei* (clone 427-221a [13]) and human myeloid leukaemia HL-60 cells [14] were maintained in Baltz medium [15] supplemented with 16.7% heat-inactivated bovine serum in a humidified atmosphere containing 5% CO₂ at 37 °C. Promastigotes of *Leishmania major* (strain MHOM/IL/81/Friedlin [16]) were cultured in Schneider's insect medium supplemented with 10% heat-inactivated foetal bovine serum in a humidified atmosphere containing 5% CO₂ at 27 °C.

In vitro toxicity assay

Toxicity assays were carried out as previously described with some modifications [17, 18]. In brief, cells were seeded in 96-well plates in a final volume of 200 µL of appropriate medium containing various concentrations of test compounds (tenfold dilution from 100 µM to 1 nM) and 1% DMSO. Wells just containing medium and 1% DMSO served as controls. The initial cell densities were 1 × 10⁴/mL for *T. brucei* bloodstream forms, 2.5 × 10⁵/mL for *L. major* promastigotes, and 5 × 10⁴/mL for HL-60 cells. After 24 h incubation, 20 µL of a 0.5 mM resazurin solution prepared in PBS was added and the cells were incubated for a further 48 h. Thereafter, the absorbance of wells was read on a BioTek ELx808 microplate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm. The 50% growth inhibition (GI₅₀) value, i.e., the concentration of a compound necessary to reduce the growth rate of cells by 50% compared to the control, was determined by linear interpolation [19]. The minimum inhibitory concentration (MIC) values, i.e. the concentration of a compound at which all trypanosomes and human cells were killed, was determined microscopically.

Intra-macrophages amastigote assay

The intra-macrophages amastigote assay was performed as previously described with some modification [20]. In brief,

1 mL of J774 cells (7 × 10⁴) suspended in RPMI supplemented with 10% foetal bovine serum were pipetted into wells of a 24 well plate containing sterile glass coverslips. Subsequently, cells were incubated overnight at 37 °C and 5% CO₂ in a humidified incubator. The next day, metacyclic *L. major* promastigotes (MOI 20:1) were added and the plates incubated for 24 h at 34 °C and 5% CO₂ in a humidified incubator. After 24 h incubation, cells were washed 4 times with PBS to remove parasites that had not invaded any macrophage. Then, cells were incubated with compound **1** at the following concentrations: 10, 2.5, 1.25, 0.625 and 0.1 µM. Amphotericin B (0.1 µM) was used as a positive control and no drug treatment as a negative control. After 72 h of drug incubation, cells were gently washed 4 times with PBS and fixed with 4% paraformaldehyde for 30 min. Thereafter, cells were washed once with water and stained with Giemsa for 40 min. Cells on coverslips were then destained using the acetone-xylene protocol as follows: first, pure acetone; second, pure acetone; third, acetone/xylene 9:1; fourth, acetone/xylene 7:3; fifth, acetone/xylene 3:7; sixth, pure xylene. Finally, coverslips were mounted on slides with Entellan new mounting medium. Infection index (parasites/cell) were determined by counting approximately 300 cells per coverslips using an Olympus BX50 microscope with a 100× N.A. 1.35 oil immersion objective.

Results and discussion

All six limonoids **1–6** showed a dose-dependent inhibitory effect on the growth of bloodstream forms of *T. brucei* with MIC values ranging from 10 to > 100 µM and GI₅₀ values ranging from 2.48 to 14.5 µM (Table 1). Similar activities were also observed with promastigotes of *L. major* for the four gedunin-type limonoids **1–4** (Table 1). In contrast to their trypanocidal activity, the two andirobin-type limonoids **5** and **6** displayed no leishmanicidal activity (MIC and GI₅₀ > 100 µM, Table 1). The most active compound against both parasites was **1** with a MIC value of 10 µM and GI₅₀ value of 2–3 µM (Table 1). On the other hand, only compounds **1** and **3** showed some cytotoxic activity against human HL-60 cells with MIC values of 100 µM and GI₅₀ values in the mid micromolar range (Table 1). The other four compounds (**2**, **4**, **5** and **6**) displayed no cytotoxicity against HL-60 cells (MIC and GI₅₀ > 100 µM, Table 1). Based on the antiparasitic and cytotoxic activities, only the gedunin-type limonoid **1** exhibited moderated selectivity indices with MIC and GI₅₀ ratios of ≥ 10 (Table 2).

Compared with suramin and amphotericin B, two drugs used in the treatment of sleeping sickness and cutaneous leishmaniasis, respectively, the six limonoids were 10–1000 times less active (Table 1). The higher antiparasitic activity

Table 1 In vitro trypanocidal, leishmanicidal and cytotoxic activity of the limonoids 1–6

Compound	<i>T. brucei</i>		<i>L. major</i>		HL-60 cells	
	MIC (μM)	GI ₅₀ (μM)	MIC (μM)	GI ₅₀ (μM)	MIC (μM)	GI ₅₀ (μM)
1	10	2.48 \pm 0.12	10	2.86 \pm 0.89	100	31.5 \pm 3.7
2	100	14.2 \pm 2.9	100	14.9 \pm 5.9	> 100	> 100
3	100	14.5 \pm 3.2	100	11.6 \pm 1.6	100	46.2 \pm 6.7
4	> 100	3.18 \pm 0.48	> 100	7.63 \pm 2.78	> 100	> 100
5	> 100	11.5 \pm 6.3	> 100	> 100	> 100	> 100
6	> 100	6.04 \pm 2.25	> 100	> 100	> 100	> 100
Suramin	1	0.042 \pm 0.004	nt	nt	> 100	> 100
Amphotericin B	nt	nt	0.1	0.036 \pm 0.001	> 100	50.7 \pm 9.3

nt not tested

Table 2 Selectivity indices of the limonoids 1–6

Compound	<i>T. brucei</i>		<i>L. major</i>	
	MIC ratio	GI ₅₀ ratio	MIC ratio	GI ₅₀ ratio
1	10	12.7	10	11.0
2	> 1	> 7.2	> 1	> 6.7
3	1	3.2	1	4.0
4	1	> 31.4	1	> 13.1
5	1	> 8.7	1	1
6	1	> 16.6	1	1
Suramin	> 100	> 2381	nd	nd
Amphotericin B	nd	nd	> 1000	1408

MIC ratio MIC_{HL-60}/MIC_{parasite}, GI₅₀ ratio GI_{50,HL-60}/GI_{50,parasite}, MIC ratios and GI₅₀ ratios were calculated from MIC and GI₅₀ values shown in Table 1

nd not determined

of the drugs is also reflected in their greater selectivity indices of at least > 100 (Table 2).

As compound **1** was found to be the most potent leishmanicidal limonoid, we investigated its ability to kill *L. major* amastigotes within macrophages. The compound was able to reduce the parasite burden of infected macrophages with an ED₅₀ (50% effective dose) of 1.51 μM (Fig. 2). Thus, compound **1** was almost twice as effective in affecting intracellular amastigotes than extracellular promastigotes (GI₅₀(promastigotes) = 2.86 μM vs ED₅₀(amastigotes) = 1.51 μM). At 10 μM , compound **1** killed completely intracellular amastigotes and only vacuoles containing fragments of parasites could be seen (Supplementary Figure S7). However, compared with amphotericin B, compound **1** was less potent in killing amastigotes within macrophages. The licensed drug was able to completely destroy intracellular amastigotes at a concentration of 0.1 μM (Fig. 2).

The trypanocidal and leishmanicidal activity of compounds 1–6 are in good agreement with previously reported antiprotozoal activities. For instance, the GI₅₀ values for their anti-plasmodial activity were found to be within the

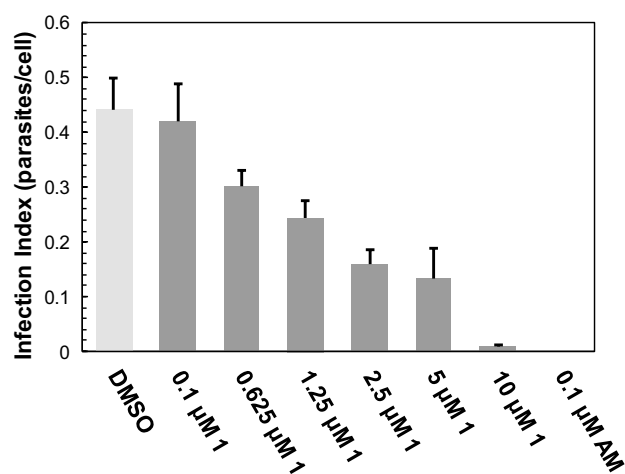


Fig. 2 Effect of compound **1** on intracellular *L. major* amastigotes. After infection of J774 macrophages with metacyclic promastigotes of *L. major*, cells were treated with compound **1** or amphotericin B (AM) or with DMSO alone (DMSO) 24 h post infection. After 72 h incubation, coverslips with cells were washed with PBS, fixed with paraformaldehyde and stained with Giemsa. Subsequently, cells were destained and coverslips mounted, and the infection index determined. Mean values \pm SD of three independent experiments run in triplicate are shown

range of 1.7–19.3 μM [8]. Compounds **3** and **4** have been previously reported to inhibit the growth of bloodstream forms of *T. brucei rhodesiense* with GI₅₀ values of 3.4 μM [21]. Axenic grown amastigotes of *L. donovani* were affected by **3** and **4** with GI₅₀ values of around 1 μM [21]. These activities are somewhat lower than those we found for **3** and **4** with promastigotes of *L. major*. The observed differences may be due to that axenic grown amastigotes are more sensitive as they are removed from their natural environment of a host cell.

Structure–activity relationship analysis revealed that the presence of a hydroxyl group at the C-7 position reduces the activity of the gedunin-type limonoids. For example, compound **2** with a hydroxyl group at C-7 displayed 5–6

times lower trypanocidal and leishmanicidal activity than compound **1** with a hydrogen atom at the position (Fig. 1 and Table 1). Likewise, compound **4** is 1.5–4.6 times less active than compound **3** which has a keto functional group instead of a hydroxyl group at the C-7 position (Fig. 1 and Table 1). These findings indicate that a slight increase in hydrophilicity reduces the trypanocidal and leishmanicidal activity of the gedunin-type limonoids.

This study has shown that phytochemical investigation of plants utilised in traditional medicine can yield the identification of compounds apparently responsible for the activity ascribed to their indigenous use. Here it was revealed that six limonoids isolated from the roots of *P. kotschyi*, a plant used for treating trypanosomiasis in domestic animals in Nigeria [6], display anti-trypanosomal activity. Kotschyienone A (**1**) was found to be not only the most active compound against *T. brucei* but also against *L. major*. Whereas compound **1** did not fully match the GI_{50} activity criteria for drug candidates for African trypanosomiasis [$< 0.2 \mu\text{g}/\text{mL}$ vs $1.09 \mu\text{g}/\text{mL}$ ($2.48 \mu\text{M}$)], it fulfilled the GI_{50} activity criteria for drug candidates for leishmaniasis [$< 1 \mu\text{g}/\text{mL}$ vs $0.66 \mu\text{g}/\text{mL}$ ($1.51 \mu\text{M}$)] [22]. The moderate selectivity indices of compound **1** did not correspond with selectivity index activity criteria for both parasite species (*Trypanosoma* > 100 ; *Leishmania* > 20 [22]). However, it should be pointed out that in this study a cancer cell line was used for determining selectivity and that, compared with non-malignant cells, the cytotoxicity of compound **1** may, therefore, be overestimated. For example, the cytotoxicity of **1** for the immortalised human embryonic kidney cell line HEK293T was previously determined to be $> 200 \mu\text{g}/\text{mL}$ [8]. Based on this cytotoxicity, compound **1** would match the selectivity index activity criteria for drug candidates for both bloodstream forms of *T. brucei* and intracellular amastigotes of *L. major*. In conclusion, the gedunin-type limonoids seem to be a promising class of compounds for further anti-trypanosomal and anti-leishmanial drug development.

Acknowledgements The authors are grateful to the Institute of Medical Research and Medicinal Plant Studies (IMPM) of Cameroon for their laboratory facilities; and to the University of Yaoundé I; the Aix-Marseille University; and the University of East Anglia for their research facilities and the award of a UEA Vice Chancellor Global Challenges Research Fellowship.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will

need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

1. Steverding D (2008) The history of African trypanosomiasis. *Parasit Vectors* 1:3
2. Steverding D (2014) The history of chagas disease. *Parasit Vectors* 7:317
3. Steverding D (2017) The history of leishmaniasis. *Parasit Vectors* 10:82
4. Cragg GM, Newman DJ (2013) Natural products: a continuing source of novel drug leads. *Biochim Biophys Acta* 1830:3670–3695
5. Cheuka PM, Mayoka G, Mutai P, Chibale K (2016) The role of natural products in drug discovery and development against neglected tropical diseases. *Molecules* 22:58
6. Atawodi SE, Ameh DA, Ibrahim S, Andrew JN, Nzelibe HC, Onyike EO, Anigo KM, Abu EA, James DB, Njoku GC, Sallau AB (2002) Indigenous knowledge system for treatment of trypanosomiasis in Kaduna state of Nigeria. *J Ethnopharmacol* 79:279–282
7. Ahua KM, Ioset J-R, Ioset KN, Diallo D, Mauël J, Hosettmann K (2007) Antileishmanial activities associated with plants used in the Malian traditional medicine. *J Ethnopharmacol* 110:99–104
8. Sidjui LS, Nganso YOD, Toghueo RMK, Wakeu BNK, Dameue JT, Mkounga P, Adhikari A, Lateef M, Folefoc GN, Ali MS (2018) Kotschyienones A and B, new antiplasmodial and cytotoxicity of limonoids from the roots of *Pseudocedrela kotschyi* (Schweinf.) Harms. *Z Naturforsch C J Biosci* 73:153–160
9. Ambrozini ARP, Leite AC, Bueno FC, Vieira PC, Fernandes JB, Bueno OC, das G. Fernandes da Silva MF, Pagnocca FC, Hebling MJA, Bacci Jr M (2006) Limonoids from andiroba oil and *Cedrela fissilis* and their insecticidal activity. *J Brz Chem Soc* 17:542–547
10. Ravangpai W, Theerawattananond T, Pengpreecha S, Muangsin N, Pudhom K (2010) 7-Deacetyl-gedunin. *Acta Crystallogr Sect E Struct Rep Online* 67:o158–o159
11. Taylor DAH (1974) ^{13}C nuclear magnetic resonance spectra of some limonoids, Part I. The structure of procerin, an extractive from *Carapa procea*. *J Chem Soc Perkin Trans* 1:437–441
12. Cheng Y-B, Chien Y-T, Lee J-C, Tseng C-K, Wang H-C, Lo I-W, Wu Y-H, Wang S-Y, Wu Y-C, Chang F-R (2014) Limonoids from the seeds of *Swietenia macrophylla* with inhibitory activity against dengue virus 2. *J Nat Prod* 77:2367–2374
13. Hirumi H, Hirumi K, Doyle JJ, Cross GAM (1980) *In vitro* cloning of animal-infective bloodstream forms of *Trypanosoma brucei*. *Parasitology* 80:371–382
14. Collins SJ, Gallo RC, Gallagher RE (1977) Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. *Nature* 270:347–349
15. Baltz T, Baltz D, Giroud C, Crockett L (1985) Cultivation in a semi-defined medium of animal infective forms of *Trypanosoma brucei*, *T. equiperdum*, *T. evansi*, *T. rhodesiense* and *T. gambiense*. *EMBO J* 4:1273–1277
16. Ivens AC, Blackwell JM (1996) Unravelling the *Leishmania* genome. *Curr Opin Genet Dev* 6:794–710
17. Merschjohann K, Sporer F, Steverding D, Wink M (2001) *In vitro* effect of alkaloids on bloodstream forms of *Trypanosoma brucei* and *T. congolense*. *Planta Med* 67:623–627
18. Mikus J, Steverding D (2000) A simple colorimetric method to screen drug cytotoxicity against *Leishmania* using the dye Alamar Blue®. *Parasitol Int* 48:265–269
19. Huber W, Koella JC (1993) A comparison of three methods of estimating EC_{50} in studies of drug resistance of malaria parasites. *Acta Trop* 55:257–261
20. Ferreira ER, Horjales E, Bonfim-Melo A, Cortez C, da Silva CV, De Groote M, Sobreira TJP, Cruz MC, Lima FM, Cordero EM,

- Yoshida N, da Silveira JF, Mortara RA, Bahia D (2016) Unique behaviour of *Trypanosoma cruzi* mevalonate kinase: a conserved glycosomal enzyme involved in host cell invasion and signalling. *Sci Rep* 6:26410
21. Hay A-E, Ioset J-P, Ahua KM, Diallo D, Brun R, Hostettmann K (2007) Limonoid orthoacetates and antiprotozoal compounds from the roots of *Pseudoedreia kotschyi*. *J Nat Prod* 70:9–13
 22. Nwaka S, Hudson A (2006) Innovative lead discovery strategies for tropical diseases. *Nat Rev Drug Discov* 5:941–955

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.