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ORIGINAL RESEARCH

Phytochemical study of *Piliostigma thonningii*, a medicinal plant grown in Nigeria

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Abstract

Piliostigma thonningii (Schumach.) Milne-Redhead. (Leguminosae) is used for various medicinal purposes in African countries. Phytochemical investigation of *P. thonningii* yielded two compounds newly isolated from natural sources, 2β-methoxyclovan-9α-ol (**1**), and methyl-*ent*-3β-hydroxylabd-8(17)-en-15-oate (**2**), along with 14 known compounds (**3**–**16**). Compounds **1** and **4** (alepterolic acid) showed potential selectivity towards *Trypanosoma brucei brucei* with IC_{50} 7.89 and 3.42 µM, respectively. Compound **2** showed activity towards *T. brucei* and *Leishmania donovani* Amastigote with IC_{50} 3.84 and 7.82 µM, respectively. The structure activity relationship (SAR) of the isolated metabolites suggested that hydroxylation at C-2 enhances the antiprotozoal activity towards *T. brucei* in sesquiterpenes **1** and **3**. Similarly hydroxylation at C-3 in labdane diterpenes elevates the antiprotozoal activity towards *T. brucei*.

Keywords: *Piliostigma thonningii* · Sesquiterpene · diterpene · *Trypanosoma brucei* · *Leishmania donovani*

Introduction

Utilization of plants for different medicinal purposes has been known for thousands of years (Samuelsson 2004). Plants initially used in crude forms such as teas, powders, tinctures, poultices, and other herbal formulations (Samuelsson 2004). In the early 19th century, the use of plants as medicines has involved the isolation of active compounds, beginning with the isolation of morphine from opium (Kinghorn 2001; Samuelsson 2004). Several known active compounds were isolated from African medicinal plants such as Betulinic acid, Combretastatin A4 phosphate, and Harpagoside (Salim et al. 2008). The West African plant Piliostigma thonningii, (Milne-Redhead) belongs to the subfamily Caesalpinioideae in the legume family, Leguminosae/Fabaceae. In African countries P. thonningii is used for various medicinal purposes (Silva et al. 1997). The decoction of the leaves and bark is used for the treatment of ulcers, wounds, heart pain, arthritis, malaria, pyrexia, leprosy, sore throat, diarrhea, toothache, gingivitis, cough, and bronchitis (Ibewuike et al. 1996; Ighodaro and Omole 2012). Its roots and twigs are used in the treatment of dysentery, fever, wound infections, cough, and skin diseases (Asuzu and Onu 1994). The crude extract of *P. thonningii* was reported to possess antilipidemic (Ighodaro and Omole 2012), antibacterial (Akinpelu and Obuotor 2000), antihelminthic (Asuzu and Onu 1994), and anti-inflammatory (Ibewuike et al. 1997) activities.

Previous phytochemical studies on *P. thoningii* revealed the presence of diverse chemical classes of compounds that possibly accommodate for the various activities of this medicinal plant. Among the identified chemical classes are flavonoids, tannins, kaurane diterpenes, alkaloids, carbohydrates, saponins, terpenes, and volatile oils (Baratta et al.

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1999; Egharevba and Folashade 2010; Ibewuike et al. 1997; Ighodaro et al. 2012; Martin et al. 1997). A representative crucial metabolite isolated from *P. thonningii* is D-3-*O*-methylchiroinositol, which possesses anthelmintic activity (Asuzu et al. 1999), analgesic, antipyretic, antidiabetic, antioxidant, and antilipidemic activities (Asuzu and Nwaehujor 2013; Nwaehujor et al. 2015); another potential example is C-methyl flavanols, which was identified from the same species and showed antibacterial and anti-inflammatory activities (Ibewuike et al. 1997). In continuation to our studies on African medicinal plants (Afolayan et al. 2018; Mohamed et al. 2016a, 2017, 2016b; Mostafa et al. 2016), and based on our in-house battery of screening, we have perused leishmanial and trypnasomal studies on the chemical constituents of *P. thonningii*.

Material and methods

General experimental

A Bruker model AMX 500 NMR and 400 NMR spectrometer operating on a standard pulse system collected ^{1}H and ^{13}C NMR spectra. The instrument ran at 500 and 400 MHz for ^{1}H and 125–100 MHz for ^{13}C. $CDCl_3$, CD_3OD, DMSO-d_6, and C_5D_5N were used as solvents, and TMS was used as an internal standard. HR-MS was performed on Agilent 1100 HPLC coupled to a JOEL AccuTOF (JMS-T100LC) (Peabody, MA). FT-IR spectrum 100 was used to record neat IR spectra for the isolated compounds. ESI-MS was analyzed in Orbitrap (Mass error on the instrument <2 ppm). TLC was performed on precoated silica gel GF_{254} plates and Column Chromatography was performed on silica gel (200–300 mesh) (Sorbent Technologies, Atlanta, GA, USA).

Plant material

P. thoningii leaves were collected during the rainy season (June 2016) from the medicinal plant garden at the Sheda Science and Technology Complex (SHESTCO), Abuja, Nigeria. The leaves were identified and authenticated at the Forestry Research Institute of Nigeria (FRIN), Ibadan, Oyo state, Nigeria, by Mr. A. Adeyemo, where a voucher specimen was deposited with the assigned number FHI 110688.

Extraction and isolation

P. thoningii leaves were dried and grounded and the ground leaves (1.5 kg) were extracted using MeOH (7 L). The extract was filtered and concentrated at 40 °C yielding 235.6 g of crude methanolic extract. The crude methanolic extract (80 g) was triturated with water: MeOH (50:50, 500 mL) and partitioned successively using CH_2Cl_2 (500 mL), EtOAc (500 mL), and *n*-butanol (500 mL). Each fraction was evaporated to yield 8.7 g CH_2Cl_2 fraction (A), 8.5 g EtOAc fraction (B), and 32.4 g *n*-butanol fraction (C).

Fraction A (8 g) was loaded onto a silica gel column and eluted using *n*-hexanes-acetone gradient to yield 19 fractions (A1–A19). Fraction A1 (240 mg) was purified over silica gel column using *n*-hexanes— EtOAc gradient yielded 5.5 mg of *α*-tocopherol (vitamin E, **8**) and 2.6 mg of *β*-amyrin (**7**). Fraction A2 (900 mg) was loaded on silica gel column and eluted with *n*-hexanes—EtOAc gradient yielded stigmasterol (**15**, 150 mg) and 5.6 mg of 2*β*-methoxyclovan-9*α*-ol (**1**, about 90% purity based on its NMR spectral data). Fractions A3 and A4 were pooled together (150 mg) and purified over silica gel column using EtOAc —*n*-hexanes to yield 11.3 mg of methyl ent-3*β*-hydroxylabd-8(17)-en-15-oate (**2**). Fraction A12 was identified to be piliostigmin (**9**, 3.0 mg). Fraction A13 (150 mg) yielded two compounds while purifying it over silica gel column using *n*-hexanes—EtOAc gradient with increasing polarity, which were identified as alepterolic acid (**4**, 19.5 mg) and chlorae-2*β*, 9*α*-diol (**3**, 2.4 mg).

Fraction B (8 g) was further fractionated on normal phase VLC using a mixture of EtOAc, CH_2Cl_2, MeOH, and H_2O in three ratios (15:8:4:1; 10:6:4:1; 6:4:4:1) to give three fractions (B1–B3). The first fraction B1 (2 g) was loaded on a normal phase column and eluted with CH_2Cl_2:MeOH gradient with increasing polarity to yield six fractions (D1–D6). Fraction D1 was identified as anticopalic acid (**5**, 14.2 mg), Fraction D2 (500 mg) was subjected to column chromatography over silica gel using CH_2Cl_2 and MeOH gradient with increasing polarity yielded 3.5 mg of (3*R*,5*R*,6*R*)-trihydroxy-7*E*-megastigmen-9-one (**6**), 21.9 mg of (+)-epicatechin (**10**), and 2.4 mg of quercetin (**11**). Fraction D3 (200 mg) was loaded on silica gel column and eluted with CH_2Cl_2 and MeOH gradient with increasing polarity yielded 12.2 mg of *β*-sitosterol glucoside (**16**), 3.5 mg of kampferol-3-*O*-rhamnoside (afzelin, **13**), and 31.4 mg of quercetin-3-*O*-rhamnoside (quercitrin, **12**). Fraction B2 (2.5 g) was loaded on silica gel column and eluted with CH_2Cl_2 and MeOH gradient with increasing polarity to yield eight fractions (E1–E8). Fraction E2 was identified as 3-hexenyl-1-*O*-*β*-D-glucopyranoside (**14**, 3.5 mg).

2*β*-methoxyclovan-9*α*-ol (1)

Yellow oil; $[\alpha]_D^{25} = +52.8$ (*c* 0.009, MeOH); IR (neat): ν_{max} 3416, 2928, 1453 cm^{-1}; for ^{1}H and ^{13}C NMR data, see Table 1; HR-MS $[M+Na]^+$ *m/z* 275.1869 (calc. for $C_{16}H_{28}NaO_2$ 275.1987).

Table 1 ^{13}C and ^{1}H NMR data for compounds **1** and **2** in $CDCl_3$ (δ_C and δ_H in ppm; *J* in Hz)

Position	1[a]		2[a]	
	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR
1	44.3	–	37.2	1.77 m, 1.17 m
2	90.3	3.32 m	28.1	1.70 m, 1.60 m
3	44.2	1.70 m, 1.45 m	79.0	3.25 dd (4.5, 12.0)
4	37.1	–	39.3	–
5	50.7	1.40 m	54.7	1.08 dd (2.5, 12.5)
6	20.7	1.40 m	24.1	1.73 m, 1.34 m
7	33.2	2.33 m	38.3	2.38 m, 1.96 m
8	34.9	–	148.2	–
9	75.4	3.30 m	56.8	1.53 m
10	26.1	1.61 m	39.5	–
11	26.7	1.98 m, 1.66 m	21.1	1.40 m
12	36.7	1.61 m, 1.25 m	35.8	1.32 m, 1.11 m
13	25.5	0.85 s	31.0	1.91 m
14	31.4	1.02 s	42.0	2.27 dd (6, 15), 2.12 dd (8, 15)
15	28.5	0.96 s	173.9	–
16	–	–	19.8	0.94 d (6.5)
17	–	–	106.8	4.82 d (1.5), 4.48 d (1.5)
18	–	–	28.4	0.99 s
19	–	–	15.5	0.77 s
20	–	–	14.6	0.68 s
2-OCH_3	58.4	3.35 s	–	–
15-OCH_3	–	–	51.5	3.66 s

[a]^{1}H NMR carried out at 500 MHz, ^{13}C NMR carried out at 125 MHz
[b]The assignments were based on ^{1}H–^{1}H COSY, HSQC, and HMBC experiments.

Methyl ent-3*β*-hydroxylabd-8(17)-en-15-oate (2)

Yellow oil; IR (neat): ν_{max}3419, 2924, 1733 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 1; HR-MS $[M+Na]^+$ *m/z* 359.2524 (calc. for $C_{21}H_{36}NaO_3$ 359.2562).

Biological evaluation

In vitro antitrypanosomal assays

Blood stage forms of *Trypanosoma brucei brucei* was grown in IMDM medium supplemented with 10% fetal bovine serum. The culture was maintained at 37 °C in 5% CO_2 incubator. Two-day-old culture of *T. brucei* was diluted to 5000 parasites/ml. Diluted *T. brucei* parasite culture was dispensed in clear flat-bottom culture well plates and treated with test compounds. The antitrypanosomal screening assay was based on Alamar blue-based fluorometric growth analysis at a concentration range of 10–0.4 µg/ml. Active compounds were further screened at a concentration range of 10–0.0032 µg/ml. Difluoromethylornithine was used as positive drug controls. IC_{50} values were computed from the dose response growth inhibition curve by XLfit version 5.2.2 (Mohamed et al. 2016a; Tarawneh et al. 2018).

In vitro antileishmanial assays

Promastigote culture of *Leishmanial donovani* was grown in RPMI medium with 10% fetal bovine serum (FBS) with pH 7.4 at 26 °C. Axenic amastigote culture of *Leishmanial donovani* was grown in RPMI medium with 10% FBS with pH 5.5 at 37 °C in 5% CO_2 incubator. The antileishmanial activity of the compounds was tested in vitro against promastigotes, axenic amastigotes, and macrophage internalized amastigote form of Leishmania donovani parasite. Promastigotes and axenic amastigotes assays were based on Alamar blue fluorometric growth analysis. Differentiated THP1 cells were been used in the macrophage internalized amastigote assay. The macrophage internalized amastigote method was based on parasite rescued and transformation assay described earlier; pentamidine was used as positive standards (Jain et al. 2012; Tarawneh et al. 2018).

Results and discussion

Compound **1** was obtained as yellow oil. The HR-MS data indicated a molecular formula $C_{16}H_{28}O_2$, based on the $[M+Na]^+$ ion signal at *m/z* 275.1869 (calc. 275.1987). The ^{1}H NMR data (Table 1), showed three singlets at δ_H 0.85, 0.96, and 1.02 attributed to three methyls CH_3-13, 15, and 14, respectively. Based on HSQC and HMBC correlations, the multiplets at δ_H 3.27–3.34 [2H] were assigned to CH-2 and 9, the methoxy group appears as singlet at δ_H 3.35 is assigned to [2-OMe]. The ^{13}C NMR data (Table 1) of **1** showed resonances of 16 carbon atoms, which were classified by DEPT 135 and HSQC experiments as three methyls, one methoxy, six methylenes, three methines, and three quaternary carbons. The HMBC spectrum of compound **1** showed the following key correlations: methoxy protons singlet at δ_H 3.35 showed ^{3}J correlation with δ_C 90.3 (C-2), indicated the methoxylation at C-2. The methyl singlet at δ_H 0.96 exhibited ^{2}J and ^{3}J correlation with carbons at δ_C 33.2 (C-7), 75.4 (C-9), and 36.7 (C-12) indicated the attachment of this methyl group at C-8. The orientations of the two stereo centers at C-2 and 9 were assigned to be *β* and *α* respectively, by comparison with the previously reported data (Collado et al. 1996, 1998). The overall NMR data were in full agreement with the data of 2*β*-methoxyclovan-9*α*-ol (Collado et al. 1996), which were obtained from the biotransformation of (−)- caryophyllene oxide. However, this is the first time to be isolated from natural source.

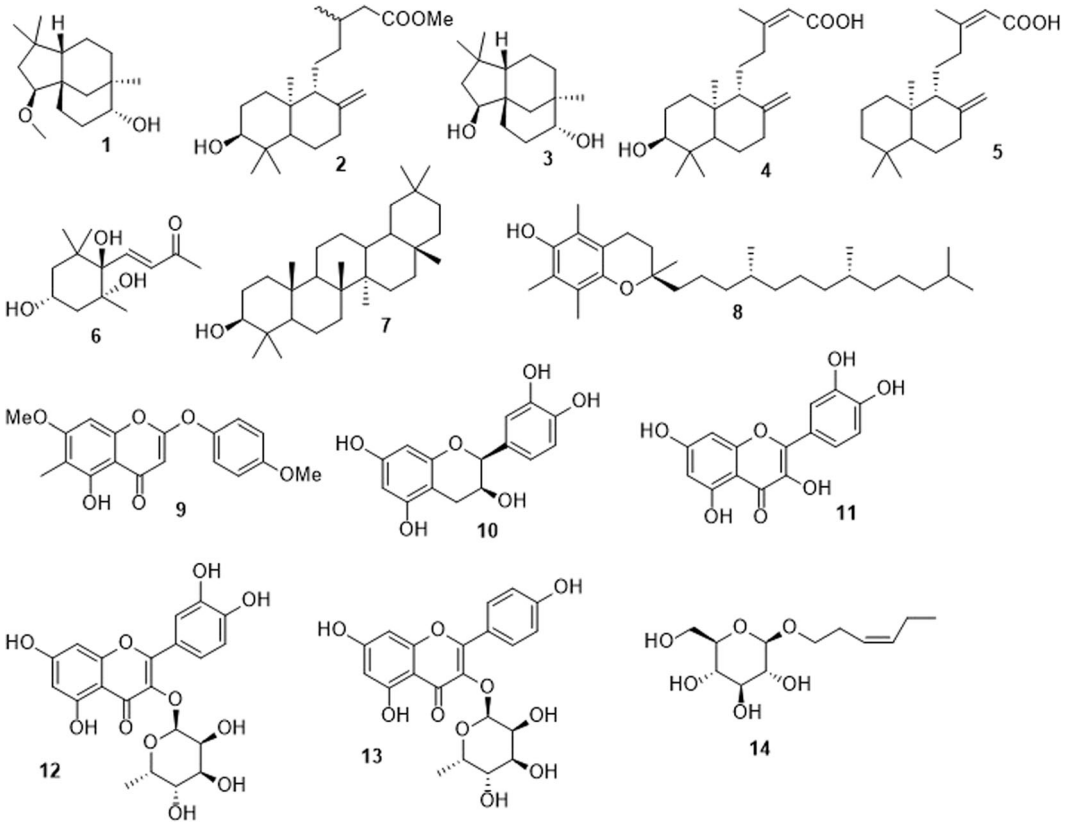

Fig. 1 Isolated compounds from *P. thonningii*

Compound **2** was also isolated as yellow oil. Its HR-MS data showed a molecular formula $C_{21}H_{36}O_3$, based on the $[M+Na]^+$ ion signal at *m/z* 359.2524 (calc. 359.2562). The ^{1}H NMR data (Table 1), showed three singlets at δ_H 0.67, 0.76, and 0.98 for three methyls CH_3-20, 19, and 18, respectively. The doublet at δ_H 0.93[$J = 6.6$ Hz] to be assigned to the methyl CH_3-16. The singlet at δ_H 3.65 was attributed to the methoxy group at C-15. A doublet of doublet of the appeared at δ_H 3.25 [$J = 4.4$, 11.8 Hz], was attributed to oxymethine CH-3. Two singlets observed at δ_H 4.82 and 4.48 were assigned to exomethylene CH_2-17.

The ^{13}C NMR data of **2** (Table 1) exhibited the resonances of 21 carbons, which were classified as four methyls, one methoxy, eight methylenes, four methines, and four quaternary carbons via DEPT 135 and HSQC experiments. The exocyclic methylene protons doublets at δ_H 4.82 and 4.48 showed ^3J HMBC correlations with δ_C 38.3 (C-7), and 56.8 (C-9) indicated the presence of double bond between C-8 and C-17. The methoxy protons at δ_H 3.66 showed ^3J correlation to δ_C 173.9 (C-15), indicated the presence of methyl ester at C-15. The methyl singlet at δ_H 0.99 exhibited ^2J and ^3J HMBC correlations with carbons at δ_C 79.0 (C-3), 39.3 (C-4), 54.7 (C-5), and 15.5 (C-19), the methyl singlet at δ_H 0.77 exhibited ^2J and ^3J HMBC correlations with carbons at δ_C 79.0 (C-3), 39.3 (C-4), 54.7 (C-5), and 28.4 (C-18) indicated that these two geminal methyl groups are directly attached to C-4. The doublet at δ_H 0.94 showed ^2J and ^3J HMBC correlations to carbons at δ_C 31.0 (C-13), 35.8 (C-12), and 42.0 (C-14) confirmed the presence of the methyl group at C-13. The structure proposed for the major component is consistent with previously synthesized compound methyl-*ent*-3β-hydroxylabd-8(17)-en-15-oate (**2**, Fig. 1). This compound has been synthesized as part of confirming the carboxyl functional group in *ent*-3β-hydroxylabd-8(17)-en-15-oic acid by reaction with diazomethane (Branco et al. 2004). However, this is the first time that this is being reported from a natural source.

The known isolated compounds **3**–**16** (Fig. 1) were identified by comparing their spectral data to those in the literature and were identified as clovane-2β,9α-diol (**3**) (Collado et al. 1998), alepterolic acid (**4**) (Braun and Breitenbach 1977), anticopalic acid (**5**) (Villegas Gómez et al. 2009), (3*S*,5*R*,6*S*)-trihydroxy-7*E*-megastigmen-9-one (**6**) (Park et al. 2011), β-amyrin (**7**) (Okoye et al. 2014), Vitamin E (**8**) (Matsuo and Urano 1976), piliostigmin (**9**) (Ibewuike et al. 1996), (+)-epicatechin (**10**) (Foo et al. 1996), quercetin (**11**), quercitrin (**12**) (Aderogba et al. 2013), Afzelin (**13**) (Aderogba et al. 2013), 3-hexenyl-1-O-

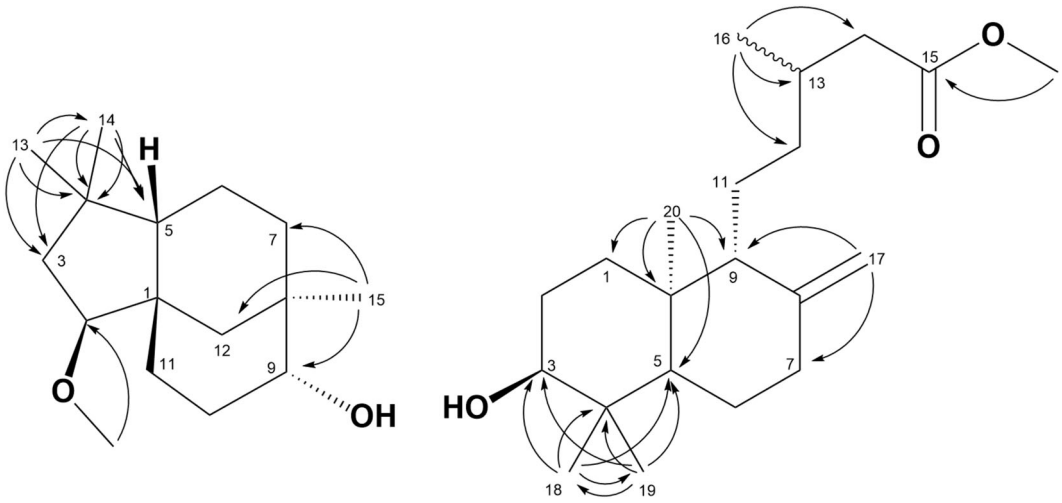

Fig. 2 Key HMBC correlations of compounds **1** and **2**

Table 2 Bioassay results of active compounds

Sample code	L. donovani Amastigote/ THP IC_{50}	T. brucei IC_{50}
Pentamidine	1.666	
Difluoromethylornithine		3.593
1	>10	7.89
2	7.82	3.84
4	>10	3.42

β-D-glucopyranoside (**14**) (Lee et al. 2005), stigmasterol (**15**), and *β*-sitosterol glucoside (**16**) (Fig. 2).

The total extract and isolated compounds were tested for their antiprotozoal activity (Table 2). Only the fractions containing major compounds **1**, **2**, and pure compound **4** showed activity against *Trypanosoma brucei* with IC_{50} values of 7.89, 3.84, and 3.42 µM, respectively (used standard for *T. brucei*, difluoromethylornithine IC_{50} 3.593 µM). In addition, the fraction contains major constituent as compound **2** showed activity towards *Leishmania donovani* with IC_{50} 7.82 µM (used standard for *L. donovani* Amastigote, Pentamidine IC_{50} 1.666 µM). The structure activity relationship (SAR) of sesquiterpenoids **1** and **3** suggested that the introduction of the hydroxyl group at C-2 enhanced the activity. Similarly, comparing the activities of **2**, **4**, and **5** towards *T. brucei* indicated the importance of the hydroxyl group at C-3 for the activity. There were few reports for the antiprotozoal activity of the labdane diterpenes (Fokialakis et al. 2006; Jassbi et al. 2016; Richomme et al.1991; Siheri et al 2014) and these compounds possess structural similarities to the active andrographolides (Sinha et al. 2000).

Conclusions

Phytochemical evaluation of *P. thonningii* yielded two new compounds **1**–**2**, and fourteen known compounds (**3**–**16**). Compounds **1** and **2** were isolated for the first time from the nature. Compounds **3**–**8**, **10**, **13**, and **14** were reported from this plant for the first time. Compounds **1** and **4** showed selectivity towards *T. brucei* with IC_{50} 7.89 and 3.42 µM, respectively. Compound **2** showed moderate activity towards *T. brucei* and *L. donovani* Amastigote with IC_{50} 3.84 and 7.82 µM, respectively. The structure activity relationship (SAR) suggested that hydroxylation at C-2 enhances the antileishmanial activity in sesquiterpenes **1** and **3**. Similarly the hydroxylation at C-3 in labdane diterpenes (**2**, **4**, and **5**) elevates the activity towards *T. brucei*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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