Two New Antiprotozoal Diterpenes From the Roots of *Acacia nilotica*

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Introduction

The powdered roots of the medicinal plant *Acacia nilotica* were extracted with hexane and ethyl acetate. Extracts were subjected to column chromatography for the isolation of potentially bioactive compounds and screened against kinetoplastid pathogens.

Materials and Methods

General Experimental Procedures. Column chromatography was carried out using silica gel 60 (0.040–0.063 mm) (230–400 mesh ASTM). Thin-layer chromatography (TLC) was performed on silica gel F250 (Merck, Germany). Nuclear magnetic resonance (NMR) experiments were carried out on a Bruker AVIII (500 MHz) spectrophotometer using CDCl3 as the solvent and TMS as the internal standard. Mass spectral data were acquired on a JEOL MStation JMS-700 mass spectrometer.

Plant Material
Roots of Acacia nilotica were collected . The plant was authenticated at the Department of Forestry and Wildlife of the University of Makurdi .

Isolation of Compounds
Dried roots of the plant were ground to powder (250 g) and extracted with hexane and ethyl acetate. The extracts were combined (based on similarity on TLC) and subjected to column chromatography using silica gel in a glass column. The column was packed wet in a hexane: ethyl acetate (95:5) mixture and eluted with ethyl acetate in hexane gradient starting with 5% ethyl acetate in hexane and increasing the amount of ethyl acetate by 5% until 100% ethyl acetate collecting 10-ml vials to obtain 186 fractions. The fractions were examined by TLC, and similar ones were combined and allowed to dry in a fume hood to obtain a mixture of compounds 1 and 3 (fractions 76–79), mixture of compounds 2 and 5 (fractions 18–19), compound 4 (fractions 64–67), a mixture of compounds 6a and 6b (fractions 59–60), and compound 7 (fractions 25–28) as white crystalline solids. The compounds were analyzed by NMR (1D and 2D) spectroscopy and mass spectrometry.

Determination of Antiprotozoal and Cytotoxic Activity
Parasites, Mammalian Cells, and Culture Conditions
Two strains of *Trypanosoma brucei brucei* bloodstream form (BSF) were used in this study: 1) wild-type (WT) *T. b. brucei* strain Lister 427 and 2) a multidrug resistant strain, B48, which was derived from a TbAT1-KO strain. The two *T. b. brucei* strains and drug-sensitive (WT) strains of *T. evansi* and *T. equiperdum* were used throughout as bloodstream trypomastigotes and cultured according to standard procedures. Bloodstream forms of T. congolense savannah type strain IL3000 and T. congolense strain 6C3 [diminazene resistant (Alenezi et al., 2020)] were cultured also. Leishmania mexicana promastigotes (MNYC/BZ/62/M379 strain) were grown in hemoflagellate modified minimal essential medium (HOMEM) (Gibco®, Life technologies, Ghent, Belgium) (pH 7.4) supplemented with 10% heat-inactivated FBS at 27°C. Human embryonic kidney (HEK) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma D-5671) supplemented with 10% heat-inactivated FBS, 10 ml/L penicillin/ streptomycin (Gibco 15140-122), and 10 ml/L of 200 mM glutamine (Gibco 25030-024). The cells were maintained at 37° C in 5% CO2 atmosphere.

Test Compounds/Fractions
All compounds and mixtures were dissolved in DMSO at 10 mg/ ml, and the stock solutions were stored at −20°C.

In vitro Drug Sensitivity Assay Using Resazurin (alamarBlue) in Bloodstream Forms of T. b. brucei, T. equiperdum, T. evansi, and T. Congolense
The susceptibilities of bloodstream formtrypanosomes to the compounds and mixtures were determined using resazurin (alamarBlue)-based assay, as described previously (Nvau et al., 2020). Fluorescence was measured in 96-well plates with a FLUOstar Optima (BMG Labtech, Durham, NC, United States) at wavelengths of 544 nm for excitation and 590 nm for emission. EC50 values were calculated by nonlinear regression using an equation for a sigmoidal dose–response curve with variable slope (GraphPad 7.0, GraphPad Software Inc., San Diego, CA, United States).

Drug Sensitivity Using alamarBlue in L. mexicana Promastigotes
Drug sensitivity assay in L. mexicana was carried out using a similar method as described above. However, a seeding density of 2 × 105 cells/well was used for this species. The plate containing the cells and drug dilutions was incubated for a period of 72 h at 27° C, followed by the addition of 20 µL 125 µg/ml resazurin and a further 48 h of incubation. Pentamidine was used as a control drug. Fluorescence was measured as above.

Assessment of Cytotoxicity of Test Compounds on Human Embryonic Kidney (HEK) 293T Cells
This was done according to standard procedures. The selectivity index (SI) was calculated for each compound/mixture as the ratio of the EC50 in HEK cells to the EC50 in a parasite species.

Determination of the Effect of HEAN 19b on L. mexicana Growth
L. mexicana cultures were set at 106 cells/ml in a 24-well plate with or without varying concentrations of HEAN-19b and pentamidine and a growth curve was plotted using cell density at each time recorded.

Conclusion

The compound displayed high activity, particularly against T. brucei, T. evansi, and L. mexicana (0.88–11.7 µM) but only a modest effect against human embryonic kidney cells and no cross-resistance with the commonly used melaminophenyl arsenical and diamidine classes of trypanocides. The effect of compound 4 against L. mexicana promastigotes was irreversible after a 5-h exposure, leading to the sterilization of the culture between 24 and 48 h.

Results

TABLE 1 | ^{1}H NMR data for compounds **1** (at 500 MHz) and **2** (at 400 MHz) in $CDCl_3$.

Position	Compound 1		Compound 2		Literature report (compd. 2)*
	^{1}H (δ in ppm; mult, J in Hz)	^{13}C (mult)	^{1}H (δ in ppm; mult, J in Hz)	^{13}C (mult)	^{13}C
1	0.97, 1.62	38.9 (CH_2)	0.98, 1.97	40.9 (CH_2)	0.94, 1.94	40.7
2	0.82 (m)	18.0 (CH)	1.44, 1.51	18.9 (CH_2)	1.40, 1.47	18.7
3	1.41, 1.30	35.4 (CH_2)	1.16, 1.42	41.8 (CH_2)	1.14, 1.43	41.6
4	–	37.7 (C)	–	34.5 (C)	–	34.3
5	1.22	48.4 (CH)	1.23 (br d)	47.8 (CH)	1.28	47.6
6	1.57 (m)	21.3 (CH_2)	1.83, 1.61	31.6 (CH_2)	1.65, 1.87	31.4
7	1.67 (m)	30.5 (CH_2)	5.21 (dd, 10.4,5.2)	95.1 (CH)	5.26	94.9
8	1.51	34.9 (CH)	4.47 (d, 8.9)	67.4 (CH)	4.52	67.2
9	1.38 (d, 3.7)	43.9 (CH)	1.23 (d, 4.0)	56.6 (CH)	1.21	56.4
10	–	36.7 (C)	–	39.2 (C)	–	39.0
11	2.23 (d,4.3), 2.08	26.1 (CH_2)	1.74, 1.21 (d, 4.0)	21.4 (CH_2)	1.76, 1.30	21.2
12	6.74 (t, 4.0)	141.0 (CH)	1.78 (m), 2.04 (d, 2.1)	32.0 (CH_2)	2.09, 1.83	31.8
13	–	142.0 (C)	–	135.9 (C)	–	135.8
14	2.70 (q, 6.2)	31.4 (CH)	–	129.9 (C)	–	129.6
15	–	198.0 (C)	2.31 (d, 7.1), 1.64 (d, 5.3)	30.4 (CH_2)	2.37, 1.30	30.2
16	4.44 (d, 17.6), 4.56 (d, 17.6)	64.4 (CH_2)	3.84 (td, 11.4, 3.8), 3.69 (dd, 11.0, 5.7)	56.4 (CH_2)	3.66, 3.82	56.2
17	0.86 (d, 6.2)	15.1 (CH_3)	5.72 (br s)	88.6 (CH)	5.70	88.4
18	3.12, 3.42	72.1 (CH_2)	0.89 (s)	33.2 (CH_3)	0.87	33.0
19	0.80	18.0 (CH_3)	0.87 (s)	22.5 (CH_3)	0.85	22.3
20	0.88	22.5 (CH_3)	0.90 (s)	15.7 (CH_3)	0.88	15.5

*Manríquez-Torres et al., (2013).

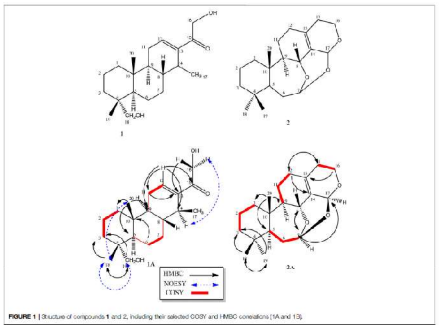

FIGURE 1 | Structure of compounds **1** and **2**, including their selected COSY and HMBC correlations (1A and 1B).

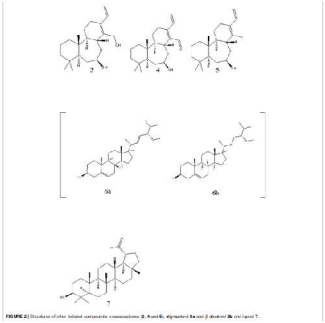

TABLE 2 | EC_{50} of two mixtures and compound **4** against *Trypanosoma* and *Leishmania* species ($n = 3$).

Compound/mixture	*T. brucei* s427	*T. congolense* IL3000 WT	*T. equiperdum*	*T. evansi*	*L. mexicana*
PAN-76 compounds 1 and 3 (µg/ml)	10.1 ± 1.0	44.1 ± 3.2	7.2 ± 0.6	4.3 ± 0.1	34.1 ± 11.2
HEAN-18 compounds 2 and 5 (µg/ml)	25.9 ± 2.6	194.8 ± 98.9	17.2 ± 2.6	ND	51.9 ± 3.8
HEAN 1 crude extract containing compound 4 (µg/ml)	5.7 ± 0.1	35.8 ± 4.3	7.3 ± 1.7	5.4 ± 0.1	10.1 ± 0.6
HEAN 19b compound 4 (µg/ml)	0.45 ± 0.02 (1.41 µM)	3.72 ± 0.54 (11.7 µM)	1.39 ± 0.27 (4.36 µM)	0.33 ± 0.05 (1.04 µM)	0.28 ± 0.05 (0.88 µM)
Diminazene (µM)	0.0878 ± 0.0355	0.228 ± 0.0446	0.0382 ± 0.0050	0.0438 ± 0.0005	ND
Suramin (µM)	0.0189 ± 0.0004	8.74 ± 1.634	0.021 ± 0.006	ND	ND
Pentamidine (µM)	ND	ND	ND	ND	0.786 ± 0.022

ND, not done.

TABLE 3 | Cross-resistance of two mixtures and compound 4 with existing drugs.

Compound/mixture	*T. brucei* B48		*T. congolense* 6C3	
	RF	*p* value	RF	*p* value
PAN-76 compounds 1 and 3	1.05	0.83	0.94	0.63
HEAN - 18 compounds 2 and 5	1.2	0.10	1.23	0.77
HEAN 1 crude extract	1.05	0.83	0.94	0.73
HEAN 19b compound 4	1.38	0.27	1.12	0.69
Diminazene	5.70	0.19	6.96	0.014
Suramin	0.62	0.50	0.37	0.74

RF, resistance factor, being the ratio of the EC50 values of the resistant and control strains. p value was obtained using unpaired Student's test between the EC50 values of the resistant line and control, obtained in parallel (n = 3).

TABLE 4 | Toxicity of mixtures and compound 4 to HEK cells.

Compound/mixture	EC_{50} for HEK cells ($n = 4$)	Selectivity index (SI)				
		SI *T. brucei* s427	SI *T. congolense* IL3000	SI *T. equiperdum*	SI *T. evansi*	SI *L. mexicana*
PAN-76 compound 1 and 3 (µg/ml)	56.8 ± 2.7	5.65	1.29	7.92	13.3	1.67
HEAN-1 crude (ug/ml)	75.5 ± 6.6	13.6	2.11	10.4	13.9	7.45
HEAN -19b compound 4 (µg/ml)	9.39 ± 1.37 (29.5 µM)	21.1	2.53	6.75	28.1	33.8
PAO (µM)	2.8 ± 0.08	–	–	–	–	–

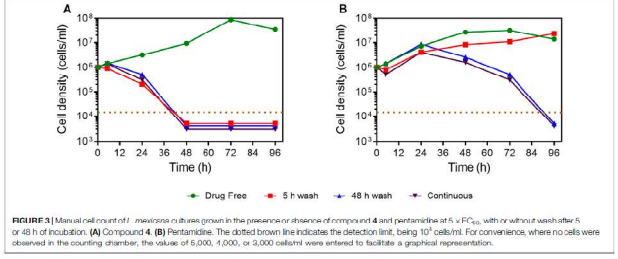

FIGURE 3 | Manual cell count of *L. mexicana* cultures grown in the presence or absence of compound **4** and pentamidine at 5 × EC_{50} with or without wash after 5 or 48 h of incubation. **(A)** Compound **4**. **(B)** Pentamidine. The dotted brown line indicates the detection limit, being 10^4 cells/ml. For convenience, where no cells were observed in the counting chamber, the values of 5,000, 1,000, or 2,000 cells/ml were entered to facilitate a graphical representation.

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