Antiprotozoal activity of major constituents from the bioactive fraction of Verbesina encelioides

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Antiprotozoal activity of major constituents from the bioactive fraction of *Verbesina encelioides*

Shahira M Ezzat[1], Maha M Salama[*1], Engy A Mahrous[1], Louis Maes[2], Cheol-Ho Pan[3] and Essam AbdelSattar[1]

[1]*Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo11562, Egypt*

[2]*Laboratory for Microbiology, Parasitology and Hygiene (LMPH), Antwerp University, B-2610 Antwerp, Belgium*

[3]*Functional Food Center, Korea Institute of Science and Technology, Gangneung 210-340, Republic of Korea*

Abstract

The bioactive petroleum ether fraction of *V. encelioides*, previously studied by the authors, was chosen for the isolation of antiprotozoal metabolites. Pseudotaraxasterol-3β-acetate (**1**), benzyl 2,6-dimethoxy benzoate (**2**), 16β-hydroxy-pseudotaraxasterol-3β-palmitate (**3**)and pseudotaraxasterol (**4**), in addition to *β*-sitosterol glucoside (**5**) and *β*-sitosterol galactoside (**6)** were isolated and identified based on 1D and 2D spectral analysis. This is the first report describing **(3)** & **(6)** in genus *Verbesina*. The isolated compounds were tested *in vitro* against *Plasmodium falciparum*, *Trypanosoma brucei*, *T. cruzi* and *Leishmania infantum.* Cytotoxicity was evaluated on MRC-5 cells. Compound **1** showed moderate to weak activity against *L. infantum T. brucei* and *P. falciparum* and was inactive against *T. cruzi*. Compound **3** showed moderate activity against *L. infantum*; compound **4** revealed weak activity against *T. cruzi*, while **5** and **6** were inactive against all tested protozoa. All compounds were non-cytotoxic. The isolated constituents showed less antiprotozoal activity than the crude fraction.

Keywords: Antimalarial; Antitrypanosomal; Antileishmanial; Cytotoxicity.

[*] **Corresponding author**: [*]Maha M. Salama, Department of Pharmacognosy, College of Pharmacy,Cairo University, Kasr El-Ainy St., Cairo11562, Egypt. Tel.: +201o66823076; fax: +225320005. E-mail: maha.salama@pharma.cu.edu.eg

Introduction

Infections caused by protozoa are a major worldwide health problem causing significant morbidity and mortality in Africa, Asia and Latin-America. African sleeping sickness, leishmaniasis, Chagas' disease and malaria are all regarded as neglected tropical diseases (NTDs) as they afflict the world's poor and do not receive attention as other diseases. NTDs tend to thrive in developing regions where water quality, sanitation and access to health care are substandard. The WHO estimates that about 1/6 of the world's population suffer from at least one NTD. It is estimated that 7 to 8 million people have Chagas disease (Chagas disease, WHO Fact sheet, 2014) and that there were about 198 million cases of malaria in 2013 and an estimated 584,000 deaths (Malaria, WHO Fact sheet, 2014). NTDs take a tremendous toll on global health. Conventional medicines for NTDs are relatively expensive, often unaffordable and not devoid of side effects, which reinforces the need of finding more efficient and less toxic drugs. Treatment of Chagas disease remains a challenge since benznidazole possesses side effects and is only active in the acute phase of the disease (Fairlamb 1999). In the context of efforts to improve the therapy of Chagas disease, higher plants are thought to be a potential source of new drugs (Sepúlveda-Bozas 1996).

Verbesina encelioides (Cav.) Benth. & Hook. fil ex Gray (golden crown beard, cowpen daisy, butter daisy) is a flowering plant in the family Asteraceae and regarded as ornamental garden plant (Kaul 1987). Sesquiterpenoides, triterpenoides and flavonoids are the major constituents of *V. encelioides* and were reported for having antimicrobial, antiviral, antitumor and hypoglycemic activities (Jain *et al.* 2008a). Cinnamic esters of borneol were found in *Verbesina rupestris* (Urb.) S.F. Blake and in *Verbesina turbacensis* Kunth. (Venditti et al.2015).The plant is used in folk medicine as analgesic, emetic, febrifuge, insecticide and anti-inflammatory remedy (Jain et al. 2008b). Recently, the fresh extract was incorporated in biogenic nanoparticles to enhance its antimicrobial activity (Kushwaha 2012). In our ongoing screening program for antiprotozoal natural products, the bioactive petroleum ether fraction of the aerial parts of *V. encelioides* (Abdel-Sattar *et al.*, 2010) was subjected to purification and isolation of its major metabolites. The *in vitro* activity of the isolated constituents was evaluated against *Plasmodium falciparum*,

Trypanosoma brucei, T. cruzi and *Leishmania infantum*. Cytotoxicity was evaluated against human embryonic lung fibroblasts (MRC-5)

Results and discussion

The petroleum ether extract of *V. encelioides* previously studied by the authors indicated antiprotozoal potential (Abdel-Sattar *et al.,* 2010). This bioactive fraction was purified for the isolation and identification of its major antiprotozoal constituents. Six compounds were isolated (Fig. 1S); pseudotaraxasterol-3β-acetate (**1**), benzyl 2,6-dimethoxy benzoate (**2**), 16β-hydroxy-pseudotaraxasterol-3β-palmitate (**3**) and pseudotaraxasterol (**4**), *β*-sitosterol glucoside (**5**) and *β*-sitosterol galactoside (**6**). Identification of the isolated compounds was based on physicochemical properties, 1D and 2D spectral analysis and comparison to published data (Silva 2011; Lu 1993; Mahato 1994) (Tables 1S &2S). The presence of 8 methyl groups (7 were singlet and one was doublet) along with the HMBC correlation suggested pseudotaraxasterol skeleton in all three compounds. This was confirmed by further analysis of 2D-COSY, HMBC and HSQC spectra. The downfield shift of the H-3 proton at 4.44 ppm in both **1** and **3** indicated possible esterification at the 3-hydroxyl group. The presence of an acetate group at the 3-position of **1** was evident from the HMBC correlation between the proton at 3-position at δ_H 4.44 ppm to the carbonyl group at δ_C171 ppm which in turn showed strong correlation to a methyl group at δ_H 2.03 ppm. Accordingly, **1** was identified as pseudotaraxasterol-3-acetate (Mahato 1994, Silva 2011). Similarly, NMR spectra of compound **3** indicated esterification at the hydroxyl group in the 3-position. Following HMBC correlation and integration of ^{1}H-NMR signals, position-3 was found to be esterified with palmitic acid. HSQC and HMBC spectra indicated the presence of another hydroxyl group at the C-16 position due to the presence of a methine group at δ_H 3.42 and δ_C 76.9. The splitting pattern and coupling constant of the proton signal for H-16 (*dd*, *J*=5, 12 Hz) was identical to that reported for 16β-hydroxy-pseudotaraxasterol-3β-palmitate previously isolated (Yahara 1990). Compound **3** was identified as 16β-hydroxy-pseudotaraxasterol-3β-palmitate. Meanwhile, spectral data analysis of **4** led to its identification as pseudotaraxasterol (Silva 2011). Compound **2** was distinguished from other compounds by the down field shift of its ^{1}H and ^{13}C signals which appeared in the aromatic region. It also showed an $[M+H]^+$ ion at *m/z* 273.104 using high resolution Q-TOF MS which was indicative of the molecular formula $C_{16}H_{16}O_4$, a fragment

at *m/z* 165.05 $[C_9H_9O_3]^+$ indicated the presence of 2,6 dimethoxy benzonium ion which was also confirmed from HMBC correlations. Thus, compound **2** was identified as benzyl 2, 6-dimethoxy benzoate (Lu 1993). Compounds **1**, **2, 4** and **5** were previously reported in *V. encelioides,* (Jain et al. 2008b); **3** & **6** were not reported in the plant before.

With regard to putative antiprotozoal activity potential, traditional herbal medicines have long been used in disease control and they continue to play a key role in daily health care, especially in endemic countries where access to modern health facilities is limited (Zofou 2013). While plant-derived biomolecules may be promising as novel antiprotozoals, the ascribed properties are often attributable to few selected constituents that must be properly identified (Sülsen 2006). The authors previously indicated the antiprotozoal potential of the petroleum ether fraction of *V. encelioides* (IC_{50}: 8.2, 10.1, 9.7 and 4.6 µg/mL against *T. cruzi* , *T. brucei* , *L. infantum* and *P. falciparum* respectively (Abdel-Sattar *et al.,* 2010). In the present study, the isolated compounds from the petroleum ether fraction were tested (Table 3S) against the same protozoa in addition to cytotoxicity evaluation on MRC-5 cells. Compound **2** was minimally active against *L. infantum* (IC_{50} 32.2 µg/mL), *T. brucei* (IC_{50} 38.1 µg/mL) and *P. falciparum* (IC_{50} 48.2 µg/mL) and was inactive against *T. cruzi* (IC_{50} >64 µg/mL) Compound **3** showed moderate activity against *L. infantum* (IC_{50} 38.1 µg/mL) while compound **4** revealed mild activity only against *T. cruzi* (IC_{50} 42.57 µg/mL). The activity of compound **2** has been previously tested against *P. falciparum* (Köhler 2002) but this is the first report for its activity against other tested protozoa. Conversely, compound **1** did not show any activity which is in agreement with a previous report for pseudotraxasterol acetate (Abreu 2011). However, substitution of C-3 with a palmitate group and C-16 with a hydroxyl group (compound **3**) slightly affected the activity. This was also shown in compound **4** which has free hydroxyl groups at C-3 & C-16 as regard to compound **1**. In addition, compounds **5** and **6** were inactive against all tested protozoa, this is in accordance with previous reports for antiprtozoal activity of sterols (Waechter et al. 1999). All compounds were non-cytotoxic on MRC-5 cells (CC_{50} >64 µg/mL). The cytotoxic results of the tested compounds competed that of the petroleum ether fraction which showed clear cytotoxicity (CC_{50} 6 µg/mL) (Abdel-Sattar 2010). Our findings indicated that the individual isolated compounds were much less active than the total petroleum ether extract, this might be attributed to the synergistic effect of the compounds

in the crude extract rather than acting individually. It is noteworthy to mention that this is the first report for isolation of *β*-sitosterol galactoside from *V. enceloides*.

Conclusion

The present work describes the isolation of six compounds from the bioactive non-polar fraction of the aerial parts of *V. enceloides* and their *in vitro* antiprotozoal activity. The isolated compounds showed weak to moderate activity against the tested protozoa

Acknowledgement

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SUPPLEMENTARY MATERIAL

Antiprotozoal activity of major constituents from the bioactive fraction of *Verbesina encelioides*

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[1]*Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo11562, Egypt*
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Keywords: Antimalarial; Antitrypanosomal; Antileishmanial; Cytotoxicity

Experimental

Materials and Methods

Plant material: Aerial parts of *V. encelioides* were collected from the western region of Saudi Arabia between March and April 2006. The plant was identified by members of Plant Taxonomy Department, College of Science, King Abdulaziz University, Saudi Arabia. Voucher specimen

(VE-2006-39) was deposited at the Herbarium of the Department of Natural Products, College of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia. Plant material was air-dried in the shade, then grinded into powder.

Preparation and fractionation of the plant extract: Dried powdered plant materials (500g) were refluxed with methanol (2x 2L). The solvent was distilled off under reduced pressure to give 40 g methanolic extract. The methanolic extract was suspended in distilled water and subjected to liquid–liquid fractionation against organic solvents of increasing polarity. The petroleum ether fraction yielded (15g). Five grams were stored for the biological study.

Isolation and purification of the compounds: Five grams petroleum ether extract were applied onto VLC column (10 cm L X 3 cm W) of Silica gel H (70 g). Gradient elution was performed starting with *n*-hexane 100% and increasing the polarity by addition of 5% $CHCl_3$ until 100%. Then 5% increments of EtOAc, up to 100% EtOAc. Fractions, each of 100 mL, were collected and monitored by TLC plates using different solvent systems. Similar fractions were pooled together to yield four collective fractions (Fr. I - Fr. IV). For isolation and purification of individual compounds, these fractions were further subjected to different chromatographic techniques as follows: Fr. I (5-10%) $CHCl_3$ /*n*-hexane was purified on silica gel 60 column, isocratic elution was performed by *n*-hexane-$CHCl_3$ (9.5-0.5 V/V) to yield 50.3 mg of white powder of compound **1**. Fr. II (15-35%) $CHCl_3$ /*n*-hexane was further purified on silica gel column using *n*-hexane-EtOAc (9.5-0.5 V/V)) to yield 20 mg of white needle crystals of compound **2**. Fr. III (65-75%) $CHCl_3$ /*n*-hexane was purified repeatedly on different silica columns using *n*-hexane-EtOAc to yield white crystalline powder of 13.6 mg of compound **3** and 44.9 mg of compound **4**. Purification of Fr. IV (65-85%) EtOAc /$CHCl_3$ was achieved on silica gel column using $CHCl_3$-MeOH (9.5-0.5 V/V)) to yield 12 mg of compound **5** (white powder) and 15 mg compound **6** (white needles).

Chemicals and equipment: For the different tests, appropriate reference drugs were used as positive control: vinblastine for MRC-5, chloroquine for *P. falciparum*, miltefosine for *L. infantum*, benznidazole for *T. cruzi* and suramin for *T. brucei*. All reference drugs were either obtained from the fine chemical supplier Sigma Chemical Co. (St Louis, MO, USA) or from WHO-TDR. Testing for antiprotozoal activity was performed in 96-well plates (Greiner, Bio-One Ltd, UK), each plate containing 16 samples at 4-fold dilutions in a dose-titration range of 64 mg/mL to 0.25 mg/mL. Dilutions were carried out by a programmable precision robotic station (BIOMEK 2000, Beckman, USA). Each plate also contained medium-controls (blanks: 0% growth), infected

untreated controls (negative control: 100% growth) and reference controls (positive control). All tests were run in duplicate. Silica gel H (Merck, Darmstadt, Germany) for vacuum liquid chromatography (VLC), silica gel 60 (70–230 mesh ASTM, Fluka, Steinheim, Germany), silica gel (40-63µm, Fluka) were used for column chromatography. Thin-layer chromatography (TLC) was performed on silica gel GF254 pre-coated plates (E-Merck) using different solvent systems The chromatograms were visualized after spraying with *p*-anisaldehyde/sulphuric acid reagent. NMR experiments were performed on a Bruker DRX-600 spectrometer (Bruker Inc., Billerica, MA) relative to TMS in $CDCl_3$ or CD_3OD. Mass spectra were measured on Acquity UPLC system (Waters, Milford, MA) using a MicrOTOF-Q hybrid quadrupole time-of-flight mass spectrometer (Bruker Daltonics, Billerica, MA).

Antimicrobial assays: The integrated panel of microbial screens and standard screening methodologies were adopted as previously described (Cos et al. 2006). Compounds were tested at dilutions ranging from 64 µg/mL to 0.25 µg/mL using automated robotics with 10-fold serial dilution strategy. Initially, two-fold serial dilutions were made in 100% DMSO to ascertain complete solubility during the dilution process. An immediate dilution step was performed in Milli-Q water before transferring the respective compound dilutions to the test plates (1/20 dilution: 10 µL compound solution + 190 µL cell medium and test system) so that the final in-test concentration of DMSO did not exceed 1%.

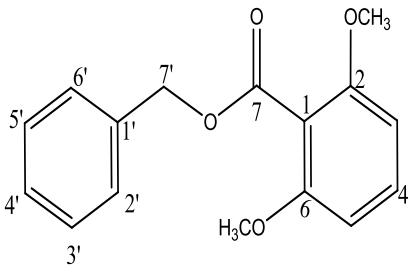
Antileishmanial assay: Mouse macrophages were stimulated by intraperitoneal injection of starch. Two days after injection, macrophages were collected and seeded in each well (3×10^4) of a 96-well plate. The plates were incubated at 37°C and 5% CO_2. After 2 days of outgrowth, *ex vivo* amastigotes were used to infect primary peritoneal mouse macrophages at a 10:1 infection ratio. The plates were further incubated for two hours before the compound dilutions were added. After 5 days of incubation, cells were dried, fixed with methanol and stained with 20% Giemsa to assess total intracellular amastigote burdens through microscopic reading. The results are expressed as percentage reduction of amastigote burden compared to untreated control cultures and IC_{50}-values were calculated.

Antiplasmodial assay: Chloroquine-resistant *P. falciparum* 2/K 1-strain was cultured in human erythrocytes O^+ at 37°C under micro-aerophilic atmosphere (3% O_2, 4% CO_2, and 93%N_2) in RPMI-1640 supplemented with 10% human serum. Two hundred µL of infected RBC (1% parasitaemia, 2% haematocrit) was added in each well of a 96 well plate containing pre-diluted

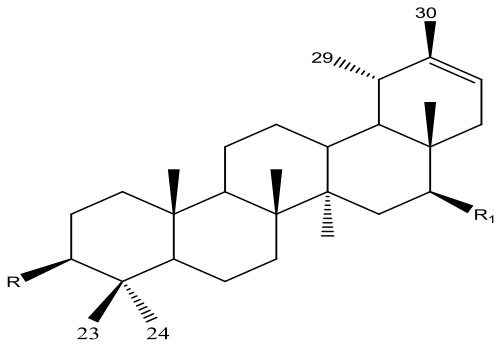
extract. The test plates were kept in the modular incubator chamber for 72 h at 37°C and subsequently put at -20°C to lyse the red cells upon thawing. Next, 100 µL of Malstat ^{TM} reagent were put in new micro titer plate to which 20µL of haemolysed parasite suspension was added. After 15 minutes incubation at room temperature, 20 µl of NBT/PES solution was added. The plate was incubated in the dark for another two hours at room temperature and spectrophotometrically read at 655nm. The IC_{50} was calculated from the drug concentration – response curves. According to WHO guidelines, antiplasmodial activity is very good with IC_{50} less than 1 µg/ml; good to moderate, if IC_{50} of 1–10 µg ⁄ ml; weak if 15–50 µg ⁄ ml and inactive if IC_{50} >50 µg⁄ml, always taking into account a SI higher than 10.

Antitrypanosomal Activity. T. brucei Squib-427 strain (suramin-sensitive) was cultured at 37∘C and 5% CO_2 in Hirumi-9 medium supplemented with 10% fetal calf serum (FCS). About 1.5 × 104 trypomastigotes were added to each well and parasite growth was assessed after 72 h at 37∘C by adding resazurin. For Chagas disease, the beta-galactosidase reporter strain *T. cruzi* Tulahuen CL2 (benznidazole-sensitive) was maintained on MRC-5 cells in minimal essential medium (MEM) supplemented with 20mML-glutamine, 16.5mM sodium hydrogen carbonate, and 5% FCS. In the assay, 4×10^3 MRC-5 cells and 4 × 104 parasites were added to each well. After incubation at 37∘C for 7 days, parasite growth was assessed by adding the β-galactosidase substrate chlorophenol red β-D-galactopyranoside. The color reaction was read at 540 nm after 4 h and absorbance values were expressed as a percentage of the blank controls.

Cytotoxicity assay: MRC-5 cells were cultivated in MEM medium, supplemented with L-glutamine (20 mM), 16.5 mM $NaHCO_3$ and 5% FCS at 37°C and 5% CO2. For the assay, 104 MRC-5 cells/well were seeded onto the test plates containing the pre-diluted samples and incubated at 37°C and 5% CO_2 for 72 h. Cells viability were determined fluorimetrically after addition of resazurin.



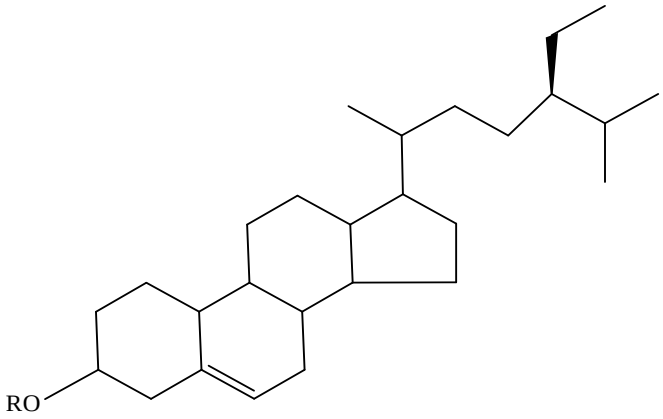
(2)



(1): R= acetate, R_1=H

(3): R=palmitate, R_1= OH

(4): R= OH, R_1=H



(5): R= glucose
(6): R= galactose

Fig.1S: Structures of the isolated compounds

Table 1. ^{1}H (400 MHz) and ^{13}CNMR (100 MHz) of compounds (1), (3) and (4)

Position	(1)		(3)			(4)	
	H	C	H	C		0.94, 1.7	38.7
1	1.01, 1.69	38.3	1.02,1.71	38.4		1.61	27.1
2	1.63	23.8	1.62	23.7		3.2,dd,5, 11	79.1
3	4.44, dd, 5.5, 10.5	81.0	4.44, dd, 5.5,11	80.7		-	38.9
4	-	38.5	-	37.9		0.69	55.1
5	0.79	55.5	0.8	55.4		1.38, 1.52	18.1
6	1.38, 1.50	18.10	1.4, 1.52	18.2		1.39	34.2
7	1.38	34	1.4	34		-	41.1
8	-	41.7	-	41.1		1.28	50.3
9	1.32	50.5	1.3	49.9		-	37.1
10	-	37.6	-	37.3		1.24, 1.53	21.5
11	1.24, 1.53	21.5	1.23, 1.54	21.5		0.98, 1.76	26.7
12	0.96, 1.67	26.5	1.22, 1.62	27.1		1.6	39.2
13	1.58	39.1	1.6	38.7		-	42.4
14	-	42.2	-	42.3		1.02, 2.0	26.9
15	1.0, 1.75	26.9	1.32, 1.64	36.5		1.22, 1.30	36.6
16	1.21, 1.29	36.7	3.42, dd, 5,12	76.9		-	34.2
17	-	34.4	-	39.9		1.04	48.6
18	0.96	48.7	1.04	47.6		1.57	36.2
19	1.56	39.3	1.65	35.7		-	139.9
20	-	139.8	-	139.8		5.25, d 6.9	119
21	5.24 d,6.7	118.9	5.29 d, 7	118.3		1.54,1.70	42.1
22	1.54,1.73	42.1	1.68, 2.1	37.5		0.97, s	27.9
23	0.85, s	28.2	0.84, s	28.1		0.76, s	15.3
24	0.84, s	16.6	0.84, s	16.5		0.85, s	16.2
25	0.86, s	16.3	0.87, s	16.4		1.04, s	15.9
26	1.02, s	15.9	1.05, s	15.9		0.94, s	14.5
27	0.92, s	14.6	0.99, s	16.3		0.73, s	17.7
28	0.73, s	17.7	0.72, s	11.3		0.98, s	22.4
29	0.98, d	22.5	1.00, d,6.3	22.4		1.63, s	21.6
30	1.63, s	21.6	1.64 , s	21.5		0.94, 1.7	38.7
31	-	171		173.6			
32	2.05	21.3	2.29, t,7	34.8	2.29		
33			1.26	29.5	1.26		
34			1.33	30.17	1.33		
35			1.28	31.2	1.28		
36			0.87, t,7	14.17	0.87		

Table 2. ^{1}H (400 MHz) and ^{13}CNMR (100 MHz) of compound (2)

Position	^{1}H (ppm)	^{13}C (ppm)	HMBC correlations
1	-	112.9, s	
2,6	-	157.5, s	
3,5	6.54 (d ,8.4Hz)	103.9, d	C1, C2, C3, C5,C6, C7
4	7.27 (d, 8.4 Hz)	131.1, d	C2, C6
7	-	166.5, s	
1′	-	136.1, s	
2′,6′	7.45 (d,7.5Hz)	128.5, d	C2′, C6′
3′,5′	7.38 (t,7.5Hz)	128.1, d	C1′, C3′, C5′
4′	7.29 (d, 7.5Hz)	127.9, d	C1′, C2′, C6′
7′	5.39 (s)	66.8, t	C7, C1′, C2′, C6′
-OCH3	3.79 (s)	55.9, q	C2, C6

Table (3): Antiprotozoal activity of the isolated compounds

Compounds	MRC-5	*T. cruzi*	*L. infantum*	*T. brucei*	*Pf-K1*
Compound (1)	>64.00	> 64.00	> 64.00	>64.00	>64.00
Compound (2)	>64.00	> 64.00	32.22	38.05	48.08
Compound (3)	>64.00	> 64.00	38.05	>64.00	>64.00
Compound (4)	>64.00	42.57	> 64.00	>64.00	>64.00
Compound (5)	>64.00	> 64.00	> 64.00	>64.00	>64.00
Compound (6)	>64.00	> 64.00	> 64.00	>64.00	>64.00

MRC-5 = diploid human embryonic lung fibroblast; *T. brucei* = *Trypanosoma brucei*; *T. cruzi* = *Trypanosoma cruzi*; *L. infantum* = Leishmania infantum; Pf- K1 = *Plasmodium falciparum* (K1 strain)

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