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Trypanocidal constituents of *Dracocephalum komarovi*

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Abstract—Trypanocidal constituents of *Dracocephalum komarovi* were investigated. Under guidance of the in vitro trypanocidal activity against epimastigotes of *Trypanosoma cruzi*, the causative agent of Chagas' disease, two new diterpenes, dracocequinones A (**1**) and B (**2**), and two known triterpene acids, ursonic acid and ursolic acid, were isolated as trypanocidal constituents, in addition to previously reported diterpenes, cyclocoulterone (**4**), komaroviquinone (**5**), dracocephalone A (**6**) and komarovispirone (**7**). Furthermore a new diterpene, komarovinone A (**3**), was isolated, together with four known terpenes. Among these compounds, komaroviquinone (**5**) showed the most potent activity with minimum lethal concentration of 0.4 μM. Structure elucidation of the new diterpenes **1–3** was described.

1. Introduction

Chagas' disease is a major public health problem endemic in Central and South American countries, with 18–20 million infected people, 25% of the human population at risk of infection, ca. 200,000 new cases, and 21,000 deaths per year.[1] Its causative agent is *Trypanosoma cruzi*, a parasitic protozoan transmitted to mammalian host by blood-sucking triatomine bugs. *T. cruzi* undergoes three main developmental stages during its life cycle, that is, the replicative epimastigote form in insect vectors and the trypomastigote and amastigote forms in mammalian hosts. Non-dividing and infective trypomastigotes circulate in the blood with their free flagellum before invading host cells, preferably muscle cells, where they lose their flagellum to differentiate into replicative amastigotes.[2] Infections by *T. cruzi* result in a life-threatening, acute and/or chronic disease with severe cardiac complications. This situation is worsened by the lack of effective vaccines, undesirable side effects of anti-chagasic drugs in use such as nifurtimox and benznidazole, and the emergence of parasite resistance to these drugs. Therefore, development of new chemotherapeutic agents is urgently needed.

The genus *Dracocephalum* is an annual or perennial herb of the Labiatae family, occurring widely in Southern Europe and temperate Asia. Some of its species are used as an astringent and a carminative,[3] and are reported to show antihyperlipidemic effect,[4] immunomodulatory effect[5] and antinociceptive effect.[6] *Dracocephalum komarovi* Lipsky is a perennial semishrub that grows at around 2300–3600 m above sea level in the West Tien Shan mountain system.[7] It is called 'buzbosh' in Uzbekistan and the local people use the aerial parts in a tea to cure various disorders such as inflammatory diseases and hypertony. During our screening of medicinal plants of Uzbekistan for trypanocidal activity, this plant showed trypanocidal activity, and we previously reported the isolation of four new diterpenes, cyclocoulterone (**4**), komaroviquinone (**5**), dracocephalone A (**6**)[8] and komarovispirone (**7**)[9] from the hexane and EtOAc extracts. In this paper, we report a full account of the elucidation of trypanocidal constituents of *D. komarovi*, including the isolation and structure elucidation of three new diterpenes.

2. Results and discussion

Dried whole plants of *D. komarovi* were extracted as described previously[8] (Scheme 1). The hexane and EtOAc

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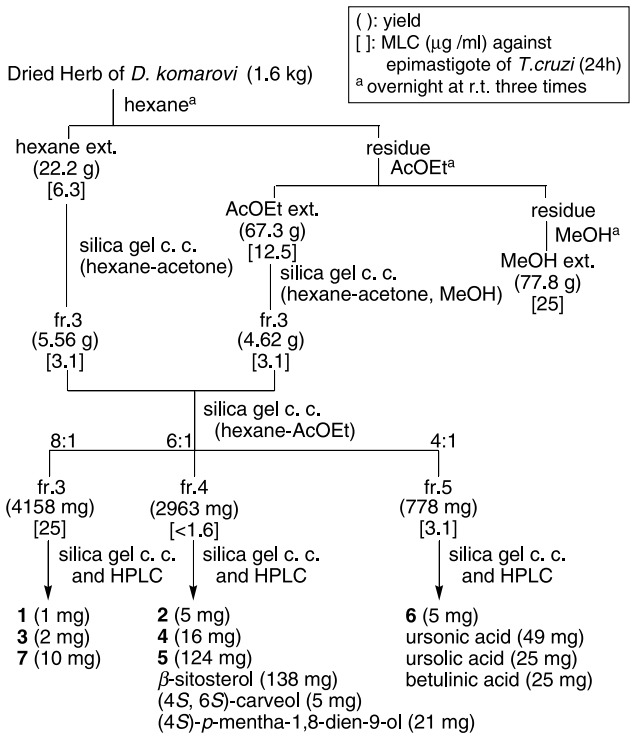


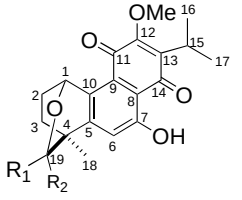
Scheme 1.

extracts were fractionated by silica gel column chromatography using hexane–acetone and MeOH as eluents. The fractions that were eluted with hexane–acetone (6/1) from the hexane extract, and hexane–acetone (8/1) from the EtOAc extract, showed strong in vitro trypanocidal activity against epimastigotes of *T. cruzi*. These fractions were further separated by silica gel column chromatography and HPLC to give seven new compounds **1** (1 mg), **2** (5 mg), **3** (2 mg), **4** (16 mg), **5** (124 mg), **6** (5 mg) and **7** (10 mg), together with ursonic acid,[10] ursolic acid,[11] betulinic acid,[12] β-sitosterol,[13] (4*S*,6*S*)-carverol[14] and (4*S*)-*p*-mentha-1,8-diene-9-ol.[15] The structures of compounds **4** (cyclocoulterone), **5** (komaroviquinone), **6** (dracocephalone A), **7** (komarovispirone) were reported previously.[8,9] The known compounds were identified by comparisons of the physical and spectroscopic data with those reported.

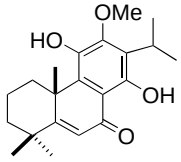
Compound **1** was obtained as an orange oil. The molecular formula $C_{20}H_{22}O_5$ was revealed by high-resolution electron-impact mass spectrum (HREIMS). The presence of a tetra-substituted *p*-benzoquinone moiety (δ_C 112.4, 124.5, 183.4, 159.2, 138.5, 191.3) with methoxy (δ_C 60.9) and isopropyl (δ_C 20.2, 20.4, 24.3) groups, which is similar to that found in komaroviquinone (**5**),[8] was concluded from its ^{13}C NMR and HMBC spectra (Table 1, Fig. 1). However, the chemical shifts of the ring juncture carbons (C-8, δ_C 112.4; C-9, δ_C 124.5) suggested the presence of further conjugation. In fact, HMBC correlations from the chelated hydroxy (δ_H 12.97) and olefin (δ_H 7.12) protons connected this enol system to the *p*-benzoquinone part to form a hydroxy naphthoquinone moiety (Fig. 1). Homo-gated decoupling (HOM) experiments revealed an ^1H–^1H coupling network between an oxymethine proton (δ_H 6.09; δ_C 64.7) and protons of two methylenes (δ_H 1.36, 1.64, 1.80, 2.41). This part structure was connected to the hydroxy naphthoquinone moiety through a quarterly carbon (δ_C 35.4), which also had a methyl (δ_C 18.6) and an oxymethylene (δ_C 71.6) groups. Finally, the two oxygen-bearing carbons (δ_C 64.7 and 71.6) were connected through an ether linkage, because there was only one oxygen atom left in the molecule. Irradiation of the H-6 proton (δ_H 7.12) resulted in a nuclear Overhauser effect (NOE) on H-18 (δ_H 1.34, α-methyl) (Figure 2). Thus, compound **1** was concluded to have the structure indicated, and was named dracocequinone A.

Compound **2** was obtained as an orange oil. This compound showed very similar NMR spectra to those of **1**. However, compound **2** showed no oxymethylene protons corresponding to H-19 in **1**, and instead of the oxygen-bearing carbon at δ_C 71.6 in **1**, compound **2** had an ester carbonyl at δ_C 174.2. This was compatible with its molecular formula $C_{20}H_{20}O_6$ revealed by HRMS. Thus, compound **2** was concluded to be a 19-keto derivative of **1**, and was named dracocequinone B.

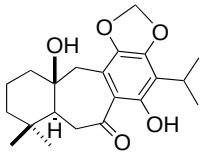
Compound **3** was obtained as a yellow amorphous solid. The ^{13}C NMR spectrum showed very similar chemical shifts for C-1 to C-7 carbons to those of salvinolone (**8**)[16] (Table 1). However, the molecular formula $C_{21}H_{28}O_4$ (HREIMS) together with the ^1H and ^{13}C NMR spectra indicated the presence of an additional methoxy group



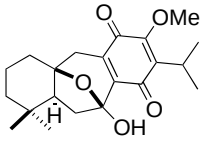
dracocequinone A (**1**): R_1=R_2=H
dracocequinone B (**2**): R_1,R_2= =O



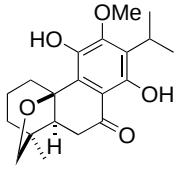
komarovinone A (**3**)



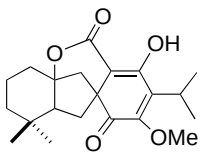
cyclocoulterone (**4**)



komaroviquinone (**5**)



dracocephalone A (**6**)



komarovispirone (**7**)

Table 1. NMR Data of **1**–**3**[a]

No.	**1**			**2**			**8**[b]	**3**		
	^{13}C	^{1}H	HMBC[c]	^{13}C	^{1}H	HMBC[c]	^{13}C	^{13}C	^{1}H	HMBC[c]
1	64.7	6.09, m		73.1	6.87, d, J=2.8 Hz		33.9	34.3	3.33, overlap	20
									1.45, td, J=12.5, 4.0 Hz	
2	25.9	2.41, m		25.9	2.49, dddd, J=14.0, 10.4, 5.5, 2.8 Hz	3	18.2	18.7	1.95, m	
		1.64, m			1.88, br t, J=14.0 Hz				1.61, dt, J=14.3, 4.6 Hz	
3	30.1	1.80, ddd, J=14.0, 10.7, 3.7 Hz	18, 19	29.2	2.04, ddd, J=13.1, 10.4, 3.1 Hz	18	39.0	40.4	1.73, dt, J=13.2, 4.6 Hz	18, 19
		1.36, m			1.60, br td, J=12.8, 5.5 Hz				1.43, overlap	
4	35.4		6, 18	45.1		3, 6, 18	37.6	38.2		6, 18, 19
5	153.6		18, 19	149.2		1, 3, 18	173.9	176.5		18, 19, 20
6	117.3	7.12, s	OH	118.2	7.16, s	OH	122.6	123.2	6.36, s	18
7	161.4		6, OH	162.0		6, OH	183.8	190.9		
8	112.4		6	113.0		6, OH	122.6	111.1		6
9	124.5			125.7			137.6	135.4		20, 11-OH
10	134.6		6	130.3		6	41.6	43.0		6, 20
11	183.4			182.9			142.5	138.1		11-OH
12	159.2		OMe	159.0		15, OMe	147.5	150.8		15, OMe, 11-OH
13	138.5		16, 17	139.2		15, 16, 17	134.2	125.8		15, 16, 14-OH
14	191.3		15	190.9		15	114.1	156.7		15, 14-OH
15	24.3	3.42, sept, J=7.0 Hz	16, 17	24.4	3.42, sept, J=7.4 Hz	16, 17	24.6	26.1	3.33, overlap	16
16	20.4[d]	1.29, d, J=7.0 Hz	15, 17	20.4[d]	1.29, d, J=7.4 Hz	17	22.8[d]	20.4[d]	1.43, d, J=7.3 Hz	15, 17
17	20.2[d]	1.27, d, J=7.0 Hz	16	20.2[d]	1.28, d, J=7.4 Hz	15, 16	22.6[d]	20.3[d]	1.41, d, J=7.3 Hz	15, 16
18	18.6	1.34, s		16.2	1.68, s		29.1	33.0	1.26, s	19
19	71.6	3.80, d, J=7.9 Hz	18	174.2		1, 3, 18	26.1	29.4	1.35, s	1, 18
		3.20, dd, J=7.9, 3.4 Hz								
20							32.9	24.8	1.65, s	
OMe	60.9	4.05, s		61.1	4.09, s			62.1	3.80, s	
OH		12.97, s			12.93, s				5.81, s (C-11); 13.52, s (C14)	

[a] Recorded in $CDCl_3$ at 500 MHz (^{1}H) and 125 MHz (^{13}C), respectively; data in δ ppm (J in Hz).
[b] Recorded in DMSO-d_6; *Phytochemistry*, **1989**, *28*, 177.
[c] Protons correlated with the carbon.
[d] The assignments may be interchanged within each column.

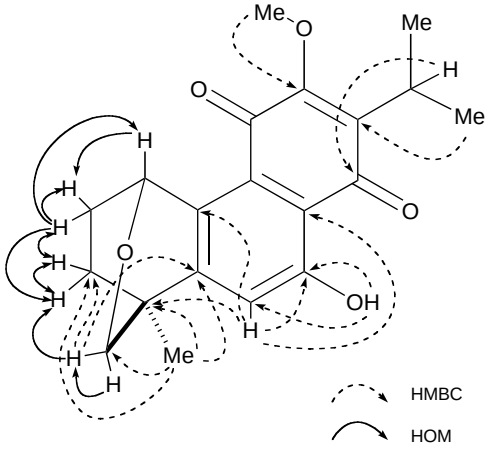


Figure 1. Key HMBC correlations and ^{1}H–^{1}H coupling network revealed by HOM experiments in **1**.

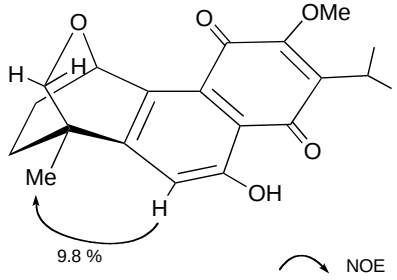


Figure 2. Observed NOEs in **1**.

(δ_H 3.80; δ_C 62.1). From the HMBC spectrum, the hydroxy groups were located at C-11 and C-14 and the methoxy group was concluded to be at C-12. In NOE difference experiments (Fig. 3), irradiation of the H-18 proton (δ_H 1.26, α-methyl) resulted in NOEs on H-6 (δ_H 6.36) and H-19 protons (δ_H 1.35, β-methyl), whereas irradiation of the H-20 proton (δ_H 1.65) enhanced the signal intensity

of H-19. These results indicated the stereochemistry at C-10 to be 10β. Thus, **3** was determined to have the indicated structure, and was named komarovinone A.

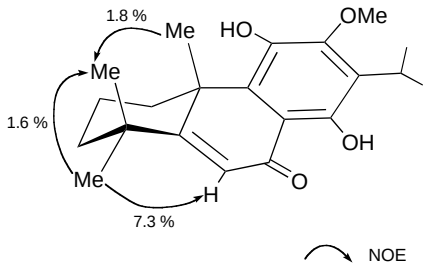


Figure 3. Observed NOEs in **3**.

Trypanocidal activities of the isolated compounds are summarized in Table 2. Dracocequinone A (**1**) and B (**2**) showed trypanocidal activity against epimastigotes of *T. cruzi* with a minimum lethal concentration (MLC) of 12.5 and 25 μM, respectively. The MLC of **1** and **2** are similar to that of **4** (20 μM) and **7** (23 μM), but higher than that of **5** (0.4 μM) under the same conditions. On the contrary, komarovinone A (**3**), which lacks the quinone moiety the same as **6** (200 μM), did not show trypanocidal activity even at 200 μM. Two triterpenes showed moderate trypanocidal activity: ursonic acid, MLC=50 μM; ursolic acid, MLC=100 μM. Betulinic acid, β-sitosterol and monoterpene alcohols; (4*S*,6*S*)-carveol and (4*S*)-*p*-mentha-1,8-dien-9-ol did not show trypanocidal activity even at 200 μM. These results indicated that **5** was the major trypanocidal component of *D. komarovi*. The MLC of gentian violet, which is used to disinfect trypanosomes from transfusion blood in Latin America, was 6.3 μM under the same assay conditions. Several types of natural quinones have been reported to show trypanocidal activity, and their activities have been partly ascribed to the production of a reactive oxygen species in the parasite.[17] In fact, we found that **5** underwent one electron reduction by *T. cruzi* old yellow enzyme to produce its semiquinone radical, which subsequently generates superoxide anion radicals.[18] Thus, the trypanocidal activity of **1**, **2** and **5** may be due to the generation of a reactive oxygen species. Previously, trypanocidal activity of several types of diterpenes and triterpenes has been reported. Da Costa et al. reported that kaurane diterpenes; (−)-*ent* kaur-16-en-19-oic acid, (−)-trachyloban-19-oic acid, (−)-kaur-16-en-19-ol and (−)-kauran-16-α-ol were effective against trypomastigotes of *T. cruzi* with IC_{50} of 1.66, 1.66, 0.69 and 1.72 mM, respectively.[19,20] Cassane diterpenes were also reported to show trypanocidal activity against trypomastigotes and amastigotes of *T. cruzi* with IC_{50} in the range of 11.5 to 104 μM and 16.6 to 95.5 μM, respectively.[21,22] Thus, trypanocidal activity of **1**, **2**, **4** and **7** were in the same range as those of cassane diterpenes. However, **5** showed more potent activity than the other diterpenes.

Table 2. Trypanocidal activity of isolated compounds from *D. komarovi*

Compound	MLC (μM)[a]
Dracocequinone A (**1**)	12.5
Dracocequinone B (**2**)	25
Komarovinone A (**3**)	>200
Cyclocoulterone (**4**)[8]	20
Komaroviquinone (**5**)[8]	0.4
Dracocephalone A (**6**)[8]	200
Komarovispirone (**7**)[9]	23
Ursonic acid	50
Ursolic acid	100
Betulinic acid	>200
β-Sitosterol	>200
(4*S*,6*S*)-Carveol	>200
(4*S*)-*p*-Mentha-1,8-dien-9-ol	>200
Gentian violet	6.3

[a] Minimum lethal concentration against epimastigotes of *T. cruzi*.

We will test the activity of the newly isolated diterpenes against trypomastigotes and the intracellular amastigotes of *T. cruzi*.

In this work, we isolated trypanocidal constituents from *D. komarovi* obtained in Uzbekistan, and trypanocidal constituents were also isolated from *D. kotschyi*[23] and *D. subcapitatum*[24] collected in Iran. Thus, we examined trypanocidal activity of some other *Dracocephalum* species (Table 3). The ethyl acetate extract of *D. integrifolium* collected in Uzbekistan showed moderate activity, whereas *D. nutans* collected in Kazakhstan and *D. argunense* grown in Japan showed weak trypanocidal activity. Elucidation of the trypanocidal constituents of these species will be a future interest.

Table 3. Minimum lethal concentration (MLC) of *Dracocephalum* extracts against epimastigotes of *T. cruzi*

Origin	MLC (μg/ml)		
	AcOEt	Acetone	MeOH
D. komarovi	—	<25	<25
D. integrifolium Bunge[a]	25	—	>100
D. nutans L.[b]	100	—	>100
D. ruyschiana L.[b]	>100	—	>100
D. argunense Fisch[c]	100	—	>100
D. argunense Fisch[d]	>100	—	>100

[a] Collected in Uzbekistan.
[b] Collected in Kazakhstan.
[c] Grown in Nagano prefecture, Japan.
[d] Grown in Hokkaido prefecture, Japan.

3. Experimental

3.1. General experimental procedures

Optical rotations were determined on a JASCO DIP-370 polarimeter. ^1H and ^{13}C NMR spectra were measured on a JEOL JNM-LA500 spectrometer with tetramethylsilane as an internal standard, and chemical shifts are given as δ values. Mass spectra were measured on a JEOL JMS-HX/HX110A spectrometer. UV and IR spectra were recorded on Hitachi U-3210 and Shimadzu FTIR-8700 spectrometers, respectively.

3.2. Extraction and isolation

Dried whole plants of *D. komarovi* were purchased at a local market in Kumyshkan, Uzbekistan, and identified by one of the authors (O.K.K.). A voucher specimen (ESM-4235) was deposited at the Experimental Station of Medicinal Plants, Faculty of Pharmaceutical Sciences, Kyoto University.

Dried whole plants of *D. komarovi* (1.6 kg) were cut into small pieces and successively extracted with hexane and EtOAc at room temperature overnight to give hexane (22.2 g) and EtOAc (67.3 g) extracts. Each extract was subjected to silica gel column chromatography using hexane–acetone (10/1, 8/1, 6/1, 4/1, 0/1) and MeOH as eluents. The fractions from the hexane extract (eluted with 6:1, 5.6 g), and the EtOAc extract (eluted with 8:1, 4.6 g) were combined and fractionated by silica gel column chromatography (CC) with hexane–EtOAc to give six fractions: fr.1 (8:1, 0.23 g); fr.2 (8:1, 0.16 g); fr.3 (8:1, 4.16 g); fr.4 (6:1, 3.0 g); fr.5 (4:1, 0.78 g); fr.6 (0:1, 0.50 g). Repeated fractionation of fr. 3 by silica gel CC with benzene–EtOAc (10/0, 10/1), hexane–EtOAc (20/1), hexane–acetone (15/1), hexane–benzene (1/10) gave compounds **1** (1 mg), **3** (2 mg), **7** (komarovispirone, 10 mg). Repeated separation of fr. 4 by silica gel CC with $CHCl_3$–acetone (200/1, 100/1), benzene–EtOAc (30/1, 20/1), hexane–EtOAc (8/1), hexane–$CHCl_3$ (1/1) and HPLC (YMC Pack SIL-06, hexane–EtOAc=6:1, 5:1) afforded compounds **2** (5 mg), **4** (cyclocoulterone, 16 mg), **5** (komaroviquinone, 124 mg), (4*S*,6*S*)-carveol (5 mg),[14] (4*S*)-*p*-mentha-1,8-dien-9-ol (21 mg),[15] and β-sitosterol (138 mg).[13] Fractionation of fr. 5 by silica gel CC with $CHCl_3$–acetone (100/1), $CHCl_3$–EtOAc (100/1), HPLC (benzene–EtOAc=30:1, hexane–acetone=8:1) and silica gel CC with hexane–EtOAc (8/1) gave compound **6** (dracocephalone A, 5 mg), ursonic acid (49 mg),[10] ursolic acid (25 mg),[11] and betulinic acid (25 mg).[12]

3.2.1. Dracocequinone A (1). An orange oil; $[\alpha]_D^{25}$ +85.8 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 251 (4.07), 291 (3.94), 428 (3.60) nm; IR (KBr) ν_{max} 2932, 2858, 1666, 1632, 1601 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz): see Table 1; EIMS *m*/*z* 342 [M^+] (100), 328 (66), 314 (66), 298 (95), 285 (48); HREIMS *m*/*z* 342.1477 (calcd for $C_{20}H_{22}O_5$, 342.1461).

3.2.2. Dracocequinone B (2). An orange oil; $[\alpha]_D^{25}$ −42.9 (*c* 0.48, MeOH); UV (MeOH) λ_{max} (log ε) 255 (3.94), 292 (3.74), 421 (3.41) nm; IR (KBr) ν_{max} 2943, 1755, 1666, 1632, 1601 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz): see Table 1; EIMS *m*/*z* 356 [M^+] (25), 342 (6), 312 (100), 298 (36), 297 (49), 283 (21); HREIMS *m*/*z* 356.1254 (calcd for $C_{20}H_{20}O_6$, 356.1260).

3.2.3. Komarovinone A (3). Yellow amorphous solid, mp 193–195°C; $[\alpha]_D^{25}$ +29 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 238 (4.13), 258 (4.26), 298 (3.84), 393 (3.78) nm; IR (KBr) ν_{max} 3333, 2959, 2936, 1639, 1582, 1458, 1420, 1400 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz): see Table 1; EIMS *m*/*z* 344 [M^+] (95), 329 (100), 297 (15), 274 (43), 262 (45); HREIMS *m*/*z* 344.1993 (calcd for $C_{21}H_{28}O_4$, 344.1988).

3.3. Trypanocidal assay

Trypanocidal activity against epimastigotes of *T. cruzi* (Tulahuen strain) was determined as described previously.[25] Each assay was performed in duplicate.

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