Trypanocidal Constituents in Plants 4.¹⁾ Withanolides from the Aerial Parts of *Physalis angulata*

Shinya NAGAFUJI,^a Hikaru OKABE,^a Hiroshige AKAHANE,^b and Fumiko ABE^{*,a}

^a Faculty of Pharmaceutical Sciences, Fukuoka University; ^b Fukuoka University School of Medicine; 8–19–1 Nanakuma, Jonan-ku, Fukuoka 814–0180, Japan. Received October 7, 2003; accepted November 4, 2003

The constituents of the aerial parts of *Physalis angulata* (Solanaceae) were investigated based on the plant's trypanocidal activity against epimastigotes of *Trypanosoma cruzi*, the etiologic agent for Chagas' disease. Four new withanolides were isolated, along with six known ones, from the active fraction. Their structures were determined by spectroscopic analysis. Trypanocidal activity against trypomastigotes, an infectious form of *T. cruzi*, was also estimated, as well as cytotoxic activity against human uterine carcinoma (HeLa) cells *in vitro*. Evaluation of trypanocidal activity using the colorimetric reagent Cell Counting Kit-8 was also examined.

Key words *Trypanosoma cruzi*; *Physalis angulata*; withanolide; trypanocidal activity; Chagas' disease; Solanaceae

In the previous paper in this series,²⁾ we described the identification of trypanocidal xanthenes in *Garcinia subelliptica* against epimastigotes and trypomastigotes of the protozoan *Trypanosoma cruzi*, which is the etiologic agent of Chagas' disease (American trypanosomiasis). In the course of our search for trypanocidal constituents in plants, we have found that the MeOH extract of the aerial parts of *Physalis angulata* L. (Solanaceae) showed considerable activity.

P. angulata is indigenous to tropical America and was naturalized in Japan in the Edo era. It is an annual small herb and has been traditionally utilized for antipyretic purposes in Japan.³⁾ The isolation of ergostane-type steroidal compounds, physagulins A–G,^{4–6)} withangulatin A⁷⁾ and physalins,⁸⁾ has been reported from this plant.

T. cruzi is transformed into three stages in its life cycle: epimastigote in the insect gut; trypomastigote, an infectious form in the mammalian blood stream; and amastigote, a proliferative form in mammalian cells. During the isolation procedure, trypanocidal activity was monitored against epimastigotes, because it is easy to cultivate them and they are not infectious. Ten withanolides (**1**–**10**) were isolated from the active fraction. To check the therapeutic possibility of these withanolides, trypanocidal activity against trypomastigotes, an infectious form, was examined. Furthermore, evaluation of the IC₅₀ value against epimastigotes was performed using Cell Counting Kit-8 in order to compare with cytotoxicity at the IC₅₀ value. In this paper we describe the structural elucidation of the four new withanolides (**2**, **5**, **9**, **10**) and the trypanocidal activity of the isolates. The possibility of chemotherapeutic utility is also discussed.

MATERIALS AND METHODS

Plant Materials *P. angulata* was cultivated in the medicinal plant garden of Fukuoka University and harvested in September 2002. The aerial parts were air-dried and powdered.

Instruments and Reagents The instruments used in this study were a JEOL JNM-A500 for NMR spectra (500 MHz for ¹H-NMR, 125 MHz for ¹³C-NMR), a JEOL JMS-HX 110 for MS spectra, a JASCO DIP 360 polarimeter for optical rotations, and a Labsystems Multiskan JX Microplate Reader for measuring the absorbance of a formazan product. The

following materials were used for chromatography: Diaion HP-20 (a styrene polymer, Mitsubishi Chemical Industries, Ltd.), Silica gel 60N (Cica), Sephadex LH-20 (Pharmacia Fine Chemicals), and YMC gel (ODS, YMC Co. Ltd.). New-born calf serum (NCS) was purchased from Nacalai Tesque. Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium were purchased from Sigma. Gentian violet, ketoconazole, and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Wako. A Cell Counting Kit-8 was purchased from Dojindo. Other reagents are the same as in the previous papers.^{2,9)}

Cultivation of *T. cruzi* The strain of *T. cruzi* used in this study and the cultivation methods of epimastigotes and trypomastigotes were the same as those described in the previous papers.^{2,9)}

Assay of Trypanocidal Activity The activity was expressed as the MC₁₀₀ value (μg/ml or μM), which was defined as the minimum concentration at which all the epimastigotes or trypomastigotes become immobilized after 24 h incubation.¹⁰⁾ MC₁₀₀ values of compounds **1**–**10** against epimastigotes and trypomastigotes were estimated by the same method as mentioned in the previous papers,^{2,9)} and are summarized in Table 3. Gentian violet¹¹⁾ and ketoconazole¹²⁾ were also tested for comparison. The results are shown in Table 3.

Assay of Trypanocidal Activity against Epimastigotes Using Cell Counting Kit-8 Cultivation medium of epimastigotes was changed from liver infusion tryptose (LIT) medium to DMEM after centrifugation. Test samples were first dissolved in dimethyl sulfoxide (DMSO) and then diluted with DMEM. Fifty microliters of cell suspension (ca. 6 × 10⁷ epimastigotes/ml) was placed over 50 μl of the sample solution prepared by the serial two-fold dilution method in 96-well microplates in quadruplicate, followed by incubation at 37 °C for 24 h under 5% CO₂. The cells without the sample were used as a control. To eliminate the influence of the medium, 100 μl of medium without the sample and epimastigotes was used as a reference. After cultivation, 10 μl of Cell Counting Kit-8 was added to each well. After incubation for 4 h under the above-mentioned conditions, the absorbance at 450 nm (ref. 630 nm) was measured on a microplate reader. The percent cell growth inhibition (IC) was calculated by the formula: [1 – (T – R)/(C – R)] × 100 (T: OD₄₅₀ of epimastigotes with the test sample, C: OD₄₅₀ of epi-

* To whom correspondence should be addressed. e-mail: abefumi@fukuoka-u.ac.jp

mastigotes without the test sample, R : OD₄₅₀ of medium). The IC₅₀ value was defined as the concentration of sample necessary to inhibit the growth to 50% of the control. The IC₅₀ values (μM) of the isolated compounds are the means of four determinations and are shown in Table 3.

Assay of Cytotoxicity The cytotoxicity of isolated withanolides against human uterine carcinoma (HeLa) cells was examined using a modified MTT assay.¹³⁾ Ninety microliters of HeLa cell suspension (*ca.* 2×10^5 cells/ml) in RPMI 1640 medium was seeded into 96-well microplates and incubated for 24 h at 37 °C under 5% CO₂. Test samples were dissolved in DMSO and diluted with RPMI 1640 medium. Ten microliters of test sample solutions were added to adjust final concentrations to 50, 25, 12, 6 and 3 $\mu\text{g/ml}$. To the control well, only RPMI medium was added. Following continuous incubation of cells for 24 h, MTT (10 μl , 5 mg/ml) was added to each well and incubated at 37 °C for another 2 h. The medium was removed and then 100 μl DMSO was added to solubilize the MTT-formazan product. The absorbance at 570 nm (ref. 650 nm) was measured on a microplate reader. The percent cell growth inhibition (IC) was calculated by the formula: $(1 - A/B) \times 100$ (A : OD₅₇₀ of the HeLa cells with the test sample, B : OD₅₇₀ of the HeLa cells without the test sample). The IC₅₀ value was defined as the concentration of sample necessary to inhibit the growth to 50% of the control. IC₅₀ values against HeLa cells are shown in Table 3.

Extraction and Isolation of the Active Constituents The dried and powdered aerial parts of *P. angulata* (500 g) were extracted with MeOH (1000 ml) under reflux for 1 h and filtered. The residue was further extracted for 30 min. Another 450 g of the sample was extracted in the same manner and all filtrates were concentrated and dried *in vacuo* to give a dark brown residue (125 g). All epimastigotes were terminated at the concentration of 1 mg/ml of the MeOH extract after 48 h incubation.

The MeOH extract was suspended in 60% MeOH and centrifuged. The precipitates were extracted with MeOH, and then with AcOEt. The 60% MeOH solution was passed through a column of Diaion HP-20, and the column was washed with 60% MeOH (fr. 1, 70.1 g). The MeOH solution was passed through the same column and the column was washed again with MeOH (fr. 2, 19.9 g). The AcOEt solution was treated in the same way (fr. 3, 20.9 g). MC₁₀₀ values ($\mu\text{g/ml}$) of frs. 1, 2 and 3 were >1000, 50 and 300, respectively. Fraction 2 was chromatographed successively on silica gel (hexane–AcOEt), Sephadex LH-20 (CHCl₃), and YMC gel (MeOH–H₂O) columns to afford physagulin C⁴⁾ (**1**, 35 mg), **2** (20 mg), withangulatin A⁷⁾ (**3**, 180 mg), physagulin A⁵⁾ (**4**, 140 mg), **5** (15 mg), physagulin F⁶⁾ (**6**, 290 mg), physagulin B⁵⁾ (**7**, 51 mg), withaminimin¹⁴⁾ (**8**, 140 mg), **9** (39 mg), and **10** (90 mg).

Compound **2**: A solid, $[\alpha]_{\text{D}}^{27} + 61.4^\circ$ ($c=0.87$, MeOH), high-resolution (HR)-FAB-MS (m/z): 549.2475 $[\text{M}+\text{Na}]^+$ (Calcd for C₃₀H₃₈NaO₈: 549.2464). ¹³C- and ¹H-NMR data: see Tables 1 and 2.

Compound **5**: A solid, $[\alpha]_{\text{D}}^{27} + 66.0^\circ$ ($c=0.85$, MeOH), HR-FAB-MS (m/z): 585.2238 $[\text{M}+\text{Na}]^+$ (Calcd for C₃₀H₃₉NaO₈Cl: 585.2232). ¹³C- and ¹H-NMR data: see Tables 1 and 2.

Compound **9**: A solid, $[\alpha]_{\text{D}}^{27} + 30.9^\circ$ ($c=1.15$, MeOH), HR-FAB-MS (m/z): 553.2772 $[\text{M}+\text{Na}]^+$ (Calcd for

Table 1. ¹³C-NMR Spectral Data for Compounds **1**, **2**, **5**, **6**, **9** and **10** (δ ppm from TMS in Pyridine-*d*₅)

No.	1	2	5	6	9	10
1	202.7	203.6	201.3	204.8	204.9	204.7
2	131.4	128.5	128.4	128.9	129.0	129.1
3	145.9	145.9	142.7	142.3	142.0	141.9
4	70.1	33.1	37.8	36.7	36.8	36.8
5	63.6	61.5	83.1	77.1	77.2	77.3
6	60.8	63.6	74.2	74.8	74.9	74.8
7	26.1	26.2	29.0	29.1	29.6	27.9
8	35.6	35.5	36.1	36.4	38.1	36.2
9	40.0	39.3	36.9	35.9	37.0	35.3
10	48.2	48.5	53.4	52.4	52.7	52.6
11	20.2	22.8	22.4	22.9	24.7	23.2
12	31.3	31.9	32.7	33.3	44.2	31.7
13	46.7	46.6	47.0	47.1	47.1	51.3
14	81.3	81.0	81.6	82.3	84.9	87.7
15	78.2	78.1	77.6	77.3	80.4	79.2
16	59.0	59.1	59.5	59.5	35.1	48.2
17	76.1	76.1	76.3	76.7	53.3	86.2
18	15.3	15.4	15.9	15.9	18.4	15.9
19	16.9	15.4	16.1	15.2	15.3	15.1
20	34.3	34.1	33.9	33.6	38.1	42.4
21	13.9	13.8	14.0	14.0	15.3	10.0
22	76.8	76.8	76.9	77.0	78.6	77.3
23	32.9	32.8	32.8	32.8	30.5	32.3
24	149.6	149.2	149.2	149.3	149.6	150.3
25	121.8	121.8	121.8	121.8	121.7	121.6
26	165.8	165.8	165.8	165.9	166.5	166.6
27	12.5	12.5	12.5	12.5	12.6	12.5
28	19.9	19.9	19.9	20.0	19.9	20.1
-OAc	20.7	20.7	21.1	21.1	21.6	21.4
	169.5	169.5	169.8	170.2	170.4	170.3

Signal assignments are based on heteronuclear multiple-quantum connectivity (HMQC) spectra and heteronuclear multiple bond connectivity (HMBC) spectra.

C₃₀H₄₂NaO₈: 553.2777). ¹³C- and ¹H-NMR data: see Tables 1 and 2.

Compound **10**: Colorless needles, mp 190–193 °C, $[\alpha]_{\text{D}}^{27} + 92.8^\circ$ ($c=2.00$, MeOH), HR-FAB-MS (m/z): 569.2734 $[\text{M}+\text{Na}]^+$ (Calcd for C₃₀H₄₂NaO₉: 569.2726). ¹³C- and ¹H-NMR data: see Tables 1 and 2.

RESULTS AND DISCUSSION

Trypanocidal activity-guided fractionation of the MeOH extract of the aerial parts of *P. angulata* resulted in the isolation of 10 withanolides (Chart 1). Six (**1**, **3**, **4**, **6**–**8**) of ten withanolides have already been isolated from the same plant.^{4–7)} They were identified by comparison of the spectral data with those reported. Compounds **2**, **5**, **9** and **10** are new isolates.

Structures of 2, 5, 9 and 10 Four new compounds were proved to be C₃₀ ones judging from HR-FAB-MS analysis. Each NMR spectrum showed signals due to two tertiary methyl groups, a secondary methyl group, an acetoxyl group and a 1-one-2-ene system on ring A in addition to signals due to a typical α,β -unsaturated δ -lactone in the side-chain. These structural features indicated that these compounds were also withanolides like the other known ones. The molecular formula of **2** was determined to be C₃₀H₃₈O₈ by HR-FAB-MS, one oxygen atom less than **1**. In the NMR spectra, signals in rings C–E were in good agreement with those of **1**, suggesting the same substitution pattern in those rings.

Table 2. ^1H -NMR Spectral Data for Compounds **1**, **2**, **5**, **6**, **9** and **10** (δ ppm from TMS in Pyridine- d_5)

No.	1	2	5	6	9	10
2	6.46 (d, 10)	6.14 (dd, 10, 2)	6.12 (dd, 10, 2)	6.12 (dd, 10, 2)	6.12 (dd, 10, 2)	6.13 (dd, 10, 2)
3	7.27 (dd, 10, 6)	6.82 (ddd, 10, 6, 2)	6.70 (ddd, 10, 5, 2)	6.67 (ddd, 10, 5, 2)	6.70 (ddd, 10, 5, 2)	6.65 (ddd, 10, 5, 2)
4	4.02 (d, 6)	2.98 (dt, 19, 2)	3.84 (dt, 20, 2)	3.72 (dt, 20, 2)	3.84 (dt, 20, 2)	3.72 (dt, 20, 2)
		1.92 (dd, 19, 6)	2.62 (dd, 20, 5)	2.36 (dd, 20, 5)	2.37 (dd, 20, 5)	2.36 (dd, 20, 5)
6	3.27 (br s)	3.16 (d, 3)	4.28 (t, 3)	4.12 (t, 3)	4.16 (t, 2)	4.14 (brt, 2)
8	2.03 (td, 12, 4)	2.1	2.43 (td, 13, 3)	2.49 (td, 13, 3)	2.70 (td, 13, 3)	2.72 (td, 13, 4)
9	1.82 (td, 12, 4)	2.21 (td, 12, 3)	2.97 (td, 13, 3)	3.16 (td, 13, 3)	3.19 (td, 13, 3)	3.51 (td, 13, 4)
15	5.31 (s)	5.30 (s)	5.47 (s)	5.61 (s)	5.74 (d, 4)	5.89 (dd, 9, 4)
16	3.76 (s)	3.73 (s)	3.77 (s)	3.71 (s)	2.40—2.50 (m)	2.92 (dd, 15, 9)
						2.12 (dd, 15, 4)
18	1.30 (s)	1.28 (s)	1.35 (s)	1.39 (s)	1.44 (s)	1.47 (s)
19	1.79 (s)	1.33 (s)	1.58 (s)	1.60 (s)	1.66 (s)	1.67 (s)
20	2.51 (qd, 7, 6)	2.52 (qd, 7, 6)	2.56 (qd, 7, 6)	2.62 (qd, 6, 5)	2.36 (m)	2.42 (qd, 6, 2)
21	0.98 (d, 7)	0.97 (d, 7)	1.01 (d, 7)	1.02 (d, 6)	1.13 (d, 6)	1.26 (d, 6)
22	4.46 (ddd, 13, 6, 4)	4.47 (ddd, 13, 6, 4)	4.48 (m)	4.50 (ddd, 13, 5, 4)	4.51 (dt, 13, 2)	5.06 (dt, 13, 2)
27	1.90 (br s)	1.90 (br s)	1.90 (br s)	1.91 (br s)	1.92 (br s)	1.96 (br s)
28	1.74 (br s)	1.74 (br s)	1.74 (br s)	1.75 (br s)	1.64 (br s)	1.74 (br s)
-OAc	2.10 (s)	2.08 (s)	2.20 (s)	2.23 (s)	2.19 (s)	2.24 (s)

The olefinic proton signal at δ 6.82 (ddd, $J=10, 6, 2$ Hz) coupled with the other olefinic proton signal at δ 6.14 (dd, $J=10, 2$ Hz) as well as with the methylene proton signals at δ 2.98 (dt, $J=19, 2$ Hz) and 1.92 (dd, $J=19, 6$ Hz). In the heteronuclear multiple-bond connectivity (HMBC) spectrum, methylene proton signals showed connectivity with signals of the olefinic carbons (C-2, 3) and the epoxide ring carbons (C-5, 6). Therefore, **2** was determined to be 4-dehydroxyphysagulin C, viz. (20*R*,22*R*)-15 α -acetoxy-5 β (6),16 β (17)-diepoxy-14 β -hydroxy-1-oxo-witha-2,24-dienolide. Compound **2** is called physagulin H.¹⁵⁾

Compound **5** has the molecular formula $\text{C}_{30}\text{H}_{39}\text{O}_8\text{Cl}$. ^1H - and ^{13}C -NMR spectra of **5** were similar to those of **6**, except for lower-field shifts of C-5 (+6.0 ppm), H-4 (+0.12 ppm, +0.26 ppm) and H-6 (+0.16 ppm), and higher-field shifts of C-1 (−3.5 ppm) and H-9 (−0.19 ppm). These data suggested that the 5 α -hydroxyl group in **6** was replaced with chlorine in **5**. Since the signals due to rings A and B of **5** were in good agreement with those of **7**, orientations of a chlorine atom and a hydroxyl-group were determined to be α and β , respectively. Thus, the structure of **5** was elucidated to be (20*R*,22*R*)-15 α -acetoxy-5 α -chloro-16 β ,17 β -epoxy-6 β ,14 β -dihydroxy-1-oxo-witha-2,24-dienolide, and it is called physagulin I.

The molecular formula of **9** was afforded as $\text{C}_{30}\text{H}_{42}\text{O}_8$ in HR-FAB-MS analysis. The presence of a 2-ene-1-one sys-

Chart 1. Chemical Structures of Compounds **1**—**10**

tem, 5 α ,6 β -glycol moiety, 15 α -acetoxy-14 β -hydroxy system, and the normal withanolide side chain was suggested from the comparison of NMR spectra with those of **6**. In the aliphatic region in ^{13}C -NMR spectra of **9**, one more methine signal (δ 53.3) was observed in addition to C-8, 9 and 20. The corresponding proton signal (δ 1.97, m) coupled with an H-20 signal (δ 2.36, m), which suggests that this proton was located at C-17. The orientation of the side chain was considered to be β , since nuclear Overhauser effect (NOE) was observed between H-18/H-21 in the differential nuclear Overhauser effect (DIF-NOE) experiment. The lower-field shift of the C-12 signal (+10.9 ppm) was observed by the disappearance of a shielding effect of the C17–C20 bond in comparison with that of **6**, which is suggestive of the β -orientation of the side chain and α -orientation of C₁₇-H. Compound **9** was determined to be (20*S*,22*R*)-15 α -acetoxy-5 α ,6 β ,14 β -trihydroxy-1-oxo-witha-2,24-dienolide, and it is called physagulin J.

Compound **10** has the molecular formula C₃₀H₄₂O₉, one oxygen atom more than **9**. Comparison of the ^{13}C -NMR spectrum of **10** with that of **9** revealed the presence of one more tertiary hydroxyl group in addition to 5 α - and 14 β -hydroxyl groups (δ 77.3 and 87.7 ppm, respectively). The corresponding carbon signal (δ 86.2 ppm) was correlated to H-20 and H-15 in the HMBC spectrum, indicating the hydroxyl group to be at C-17. The orientation of the hydroxyl group was deduced to be β based on an NOE between H-18 and 17-hydroxyl proton (δ 5.68, s), and the higher-field shift of C-12 signal (δ 31.7 ppm), as similarly observed in **1**, **2**, **5** and **6** (δ 31.7–33.3 ppm). Thus, the structure of **10** was elucidated to be (20*R*,22*R*)-15 α -acetoxy-5 α ,6 β ,14 β ,17 β -tetrahydroxy-1-oxo-witha-2,24-dienolide. Compound **10** is called physagulin K.

Trypanocidal Activity Compounds **1–10** were tested for their trypanocidal activity against epimastigotes and trypomastigotes of *T. cruzi* *in vitro*. In these withanolides, trypanocidal activity against trypomastigotes is much higher than that against epimastigotes, suggesting their potentiality as chemotherapeutic agents for the treatment of Chagas' disease. The following are the effects of substitution patterns on the activity against trypomastigotes, although the number of withanolides examined might not be sufficient to clarify the structure–activity relationships.

- 1) Compounds **1–3** and **4**, which have a 5,6- β -epoxide group, showed almost the same strong activity as that of gentian violet. The β -epoxide group at C5–C6 might be a key structural factor to show the trypanocidal activity.
- 2) Compounds **6**, and **8–10** have a 5 α ,6 β -dihydroxy group in their structure. The activity of **8**, **9**, and **10** is rather small, whereas that of **6** is much greater. The β -epoxide group at C16–C17 might also contribute to the activity.
- 3) Replacement of a 5 α -hydroxyl group with a 5 α -chloro group enhances activity (2 times between **5** and **6**, 19 times between **7** and **8**).
- 4) Judging from the activity of **1** and **2**, and **3** and **4**, the influence of the presence of the 4 β -hydroxyl group would be negligible.
- 5) Compounds **6**, **9**, and **10** differ in ring D, and the ring cleavage of an epoxide to the hydroxyl group greatly

Table 3. Trypanocidal and Cytotoxic Activity (μM) of Compounds **1–10**, Gentian Violet and Ketoconazole *in Vitro*

	MC ₁₀₀ (E) ^{a)}	MC ₁₀₀ (T) ^{b)}	IC ₅₀ (E) ^{c)}	IC ₅₀ (H) ^{d)}
1 (Physagulin C)	46	2	14	12
2	38	3	17	11
3 (Withangulatin A)	48	2	19	11
4 (Physagulin A)	49	5	20	28
5	44	4	28	21
6 (Physagulin F)	>184	9	184	92
7 (Physagulin B)	91	5	16	14
8 (Withaminimin)	>189	94	>189	>95
9	>188	22	>188	>94
10	>183	183	>183	>92
Gentian violet	24	2		
Ketoconazole	94	377		

a) MC₁₀₀ values against epimastigotes of *T. cruzi*. b) MC₁₀₀ values against trypomastigotes of *T. cruzi*. c) IC₅₀ values against epimastigotes of *T. cruzi*. d) IC₅₀ values against HeLa cells.

reduces the activity.

Evaluation of IC₅₀ Values against Epimastigotes Using Cell Counting Kit-8 In previous papers,^{1,2,9)} we assayed trypanocidal activity by observing the mobility of *T. cruzi* microscopically. The activity was shown by MC₁₀₀ value, which was defined as the minimum concentration at which all the epimastigotes or trypomastigotes become immobilized after 24 or 48 h incubation. In order to evaluate the IC₅₀ value, the colorimetric method of trypanocidal assay was examined using Cell Counting Kit-8. This assay is based on measuring the absorbance of the formazan product produced by the reduction of a tetrazolium-based compound by live cells. We checked the usefulness of this method first using epimastigotes because a large number of epimastigotes were obtainable. A cultivation medium of epimastigotes was changed to DMEM, since the LIT medium influenced the absorption at 450 nm. To optimize the experimental condition, the absorbance was measured by changing the number of epimastigotes under two conditions. Under condition 1, the plate was kept at 26 °C in the air, as in LIT medium, and under condition 2 the plate was kept at 37 °C under 5% CO₂ as in the case of trypomastigotes. A different number of epimastigotes (*ca.* 1.2 $\times$ 10⁸–0.5 $\times$ 10⁵/ml) suspended in DMEM (100 μl) was placed in 96-well microplates in quadruplicate, and these were incubated for 24 h under the two conditions. After incubation, 10 μl of Cell Counting Kit-8 was added to the wells. Following further incubation for another 4 h, the absorbance was measured on a microplate reader. Since almost the same absorbance was obtained from each condition, we incubated the epimastigotes under condition 2 in order to compare the activity against trypomastigotes. As shown in Fig. 1A, the absorbance of formazan changed depending on the number of epimastigotes seeded. The numbers of live epimastigotes from 8 $\times$ 10⁶ to 1.2 $\times$ 10⁸/ml linearly correspond to the absorbance from 0.6 to 2.6. Considering that the absorbance is reliable around 1.0–1.5, we placed the epimastigotes approximately 3 $\times$ 10⁷/ml in the control wells. Compounds **1–10** were tested as described above. The correlations between the concentration of the samples (μM) and percent inhibition are shown in Fig. 1 (B, C and D). IC₅₀ values were read from the graphs and are shown in Table 3. MC₁₀₀ values of **1–10** correspond to the concentrations at which the IC curves reach a plateau, suggesting that the

Fig. 1. Evaluation of Trypanocidal Activity against Epimastigotes Using the Cell Counting Kit-8 *in Vitro*

(A) Correlation between absorbance and number of epimastigotes. The value represents the mean $\pm$ S.D. ($n=4$). (B) Concentration-percent inhibition curves by 1, 2 and 8. (C) Concentration-percent inhibition curves by 3, 4 and 9. (D) Concentration-percent inhibition curves by 5, 6, 7 and 10.

MC₁₀₀(E) values reflect the concentration at which *T. cruzi* epimastigotes die.

The IC₅₀ values of trypanocidal activity against epimastigotes are almost compatible with cytotoxicity against HeLa cells. Selective toxicity [IC₅₀(HeLa)/IC₅₀(*T. cruzi*)] of withanolides is rather small (0.5–1.4) in the case of epimastigotes. However, stronger activity against trypomastigotes arouses interest in further *in vivo* investigation. Estimation of IC₅₀ values against trypomastigotes is now under progress. A bioassay against the intracellular amastigotes *in vitro* remains to be done.

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