47.2%，25.4%，14.1%で，これまで考えられてきたよりも予後不良である[7]．

皮膚が病変の首座である場合は，外用薬，光化学療法や局所放射線照射など皮膚指向性治療が行われる．これらの治療は皮膚局所の症状緩和の手段としては有効であるが，生存期間の改善に貢献するエビデンスはない．

III 海外でのATL治療

海外ではインターフェロンα/ジドブジン(IFN/AZT)併用療法が，急性型，慢性型，くすぶり型に対する標準治療とされている[8]．特筆すべきことは，単純に比較すると海外でIFN/AZT併用療法を受けた慢性型，くすぶり型患者の予後は，本邦で無治療経過観察をされた患者より明らかに良好な可能性があることである[9]．そこでJCOG-LSGではindolent ATLを対象に本療法の有用性を科学的に検証する第III相試験を先進医療として実施することを計画しており，2013年夏には開始見込みである．

おわりに

Aggressive ATLとindolent ATLではそれぞれ異なった治療戦略がとられ，前者では多剤併用化学療法と可能な症例では同種造血幹細胞移植，後者ではaggressive ATLになるまで無治療経過観察を行うことが本邦の標準治療である．Aggressive ATLは近年同種造血幹細胞移植や新規治療薬の導入などによりその治療に大きな進歩がみられている．今後はindolent ATLに対する早期治療介入の有用性の検討に関心がもたれる．

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NOTE

Screening of promising chemotherapeutic candidates from plants against human adult T-cell leukemia/lymphoma (II): apoptosis of antiproliferactive principle (24,25-dihydrowithanolide D) against ATL cell lines and structure–activity relationships with withanolides isolated from solanaceous plants

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Abstract Adult T-cell leukemia/lymphoma (ATL) is an incurable peripheral T-cell malignancy caused by human T-cell lymphotropic virus type I. In our preceding paper, 214 extracts from 162 plants were screened to elucidate the antiproliferative principles against ATL cell lines. Several withanolides were isolated and the structure–activity relationships (SAR) examined. To extend the search for SAR, 31 further withanolides, previously isolated from solanaceous plants, were tested against ATL cell lines. The presence of a 4β-hydroxy group as well as a 5β,6β-epoxy group appeared to be essential for the activity. In contrast, the presence of a sugar moiety at either the 3- or the 27-position led to a reduction in the activity. Furthermore, 24,25-dihydrowithanolide D (**13**) was identified as the most potent inhibitor, showing selective toxicity against ATL cell lines by inducing apoptotic cell death.

Keywords Adult T-cell leukemia/lymphoma · Solanaceae · Withanolides · Structure–activity relationships

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Introduction

Adult T-cell leukemia/lymphoma (ATL) is a peripheral T-cell malignancy caused by human T-cell lymphotropic virus type I (HTLV-1). Clinical manifestations of ATL range from smoldering to chronic, lymphoma and acute subtypes. Combinations of conventional chemotherapeutic agents used against other types of malignant lymphoma have been administered to patients with acute- and lymphoma-type ATL, but the therapeutic outcomes remain very poor [1]. In the preceding paper [2], 214 extracts from 162 plants belonging to 65 families were screened to elucidate the antiproliferative effect against two HTLV-1-infected T-cell lines (MT-1 and MT-2). Extracts from aerial parts of *Physalis pruinosa* (Solanaceae) have shown potent antiproliferative effect. We isolated five withanolides from the extracts by activity-guided fractionation and examined the structure–activity relationships (SAR). SAR indicated that the presence of a 5β,6β-epoxy group was important for the activity and identified 4β-hydroxywithanolide E as the most potent principle, showing selective toxicity to HTLV-1-infected T-cell lines. These results prompted us to investigate the antiproliferative activities of various other withanolides previously isolated from the extracts of solanaceous plants. Herein we report the extended search for SAR and identify 24,25-dihydrowithanolide D as the most potent principle for the induction of apoptosis.

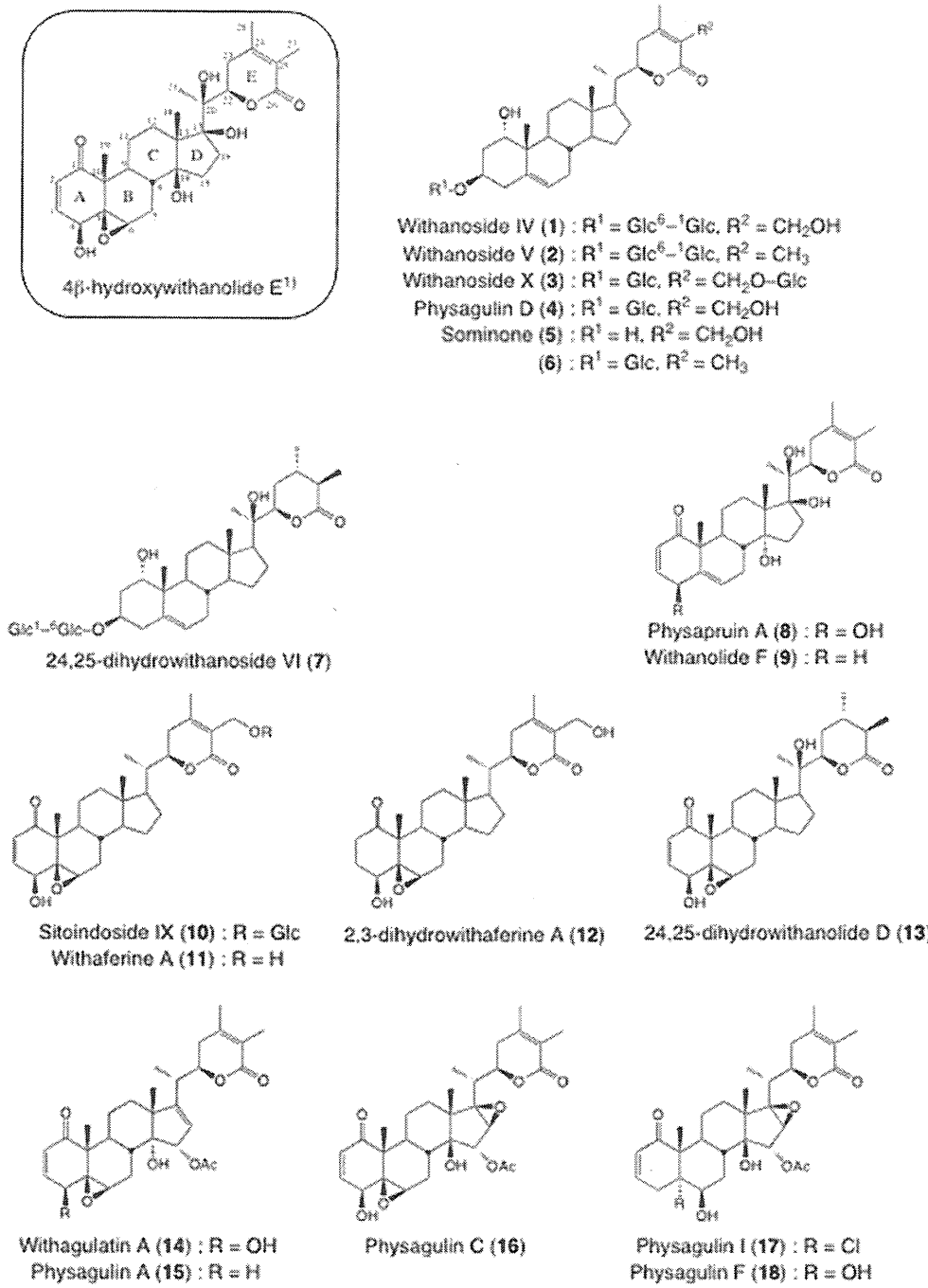


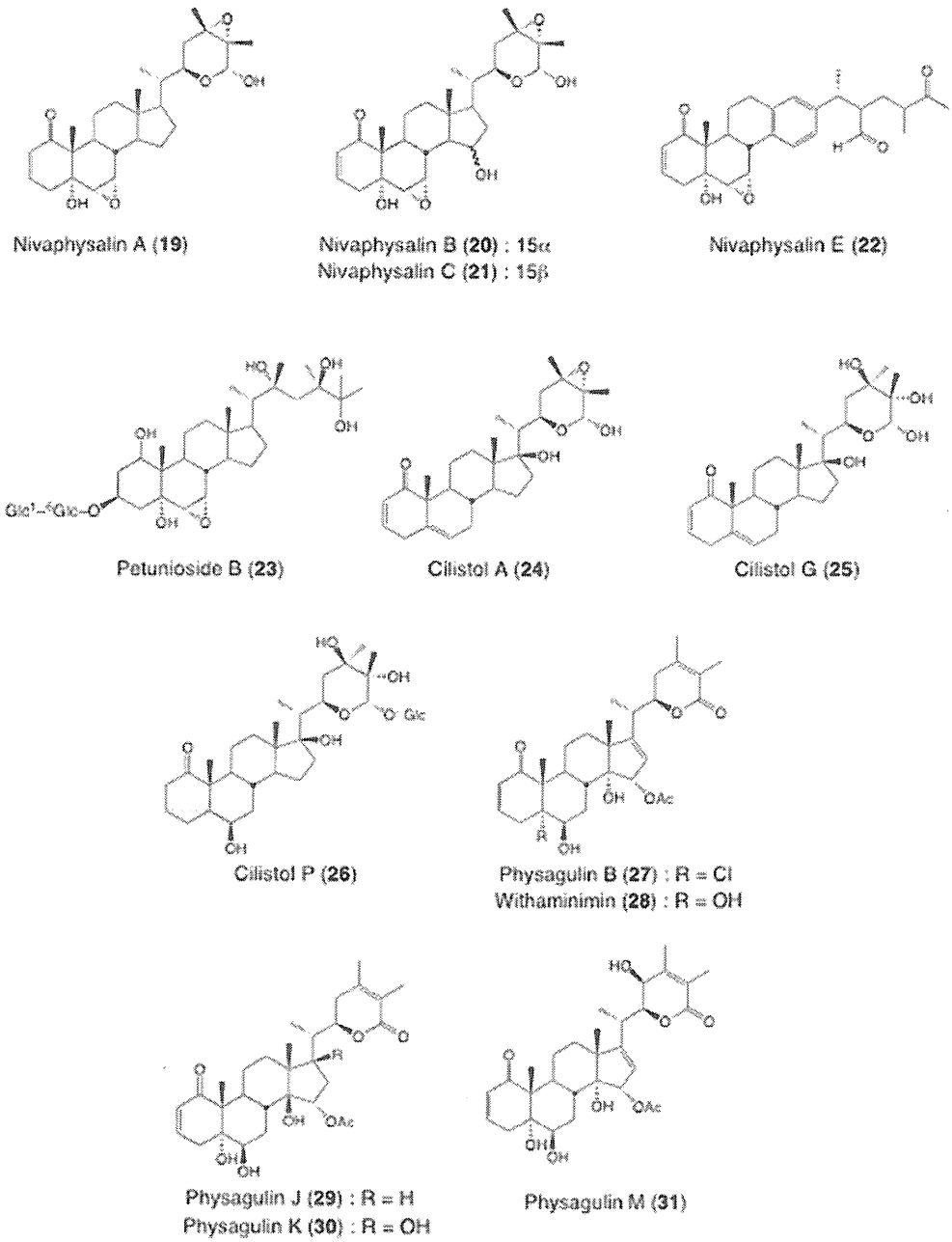
Fig. 1 Structures of the withanolides examined

Materials and methods

Materials

The withanolides tested in this study are shown in Fig. 1. Withanoside IV (**1**), withanoside V (**2**), withanoside X (**3**), physagulin D (**4**), sominone (**5**), compound **6**, 24,25-dihydrowithanoside VI (**7**), sitoindoside (**10**), withaferin A (**11**), 2,3-dihydrowithaferin A (**12**) and 24,25-dihydrowithanolide D (**13**) were previously isolated from *Withania somnifera* [3–9]. Withanolide F (**8**) and sitoindoside IX (**9**) were previously isolated from *Physalis pruinosa* [10] and *Withania coagulans* [11], respectively. Withagulatin (**14**), physagulin A (**15**), physagulin C (**16**), physagulin I (**17**), physagulin (**18**), physagulin B (**27**), withaminimin (**28**), physagulin J (**29**), physagulin K (**30**) and physagulin M (**31**) were previously isolated from *Physalis angulata* [5, 12–14]. Nivaphysalin A (**19**), nivaphysalin B (**20**), nivaphysalin C (**21**) and nivaphysalin E (**22**) were previously isolated from *Nicandra physaloides* [15]. Petunioside

Fig. 1 continued



B (**23**) was previously isolated from *Petunia hybrida* [16]. Cilistol A (**24**), cilistol G (**25**) and cilistol P (**26**) were previously isolated from *Solanum cilistum* [17, 18].

RPMI-1640 medium, fetal bovine serum, Dulbecco's phosphate-buffered saline (PBS) and kanamycin sulfate were purchased from Lifetech (Rockville, MD, USA). WST-8, APO2.7-PC5 and poly(adenosine diphosphate[ADP]-ribose) polymerase (PARP) antibody were purchased from Dojindo (Kumamoto, Japan), MBL (Nagoya, Japan), and Cell Signaling (Beverly, MA, USA), respectively.

Cells

Two HTLV-1-infected T-cell lines, MT-1 and MT-2, were kindly provided by Dr I. Miyoshi, Kochi University, Nangoku, Japan. MT-1 cells were established from peripheral blood (PB) tumor cells of ATL patients, whereas MT-2 cells were established from cord blood T cells by co-cultivation of normal human cord lymphocytes and PB tumor cells of an ATL patient [19, 20]. Culture conditions were as previously described [1]. Fresh ATL cells and normal mononuclear cells (PB-MNCs) obtained from the

Table 1 The antiproliferative activity of compounds **1–31** (EC_{50}) and cytotoxity of **13** towards normal cells

Compounds	EC_{50} (μM)		
	MT-1	MT-2	Normal
1	>100	>100	ND
2	>100	>100	ND
3	>100	>100	ND
4	>100	>100	ND
5	37.8 ± 7.0	35.3 ± 4.3	ND
6	>100	>100	ND
7	>100	>100	ND
8	0.050 ± 0.016	0.28 ± 0.14	ND
9	1.40 ± 0.39	1.58 ± 0.30	ND
10	0.828 ± 0.067	6.05 ± 0.55	ND
11	0.156 ± 0.044	1.33 ± 0.11	ND
12	0.022 ± 0.006	0.508 ± 0.112	ND
13	0.008 ± 0.001	0.008 ± 0.000	0.860 ± 0.262
14	2.28 ± 0.65	3.42 ± 0.19	ND
15	2.29 ± 0.22	2.23 ± 0.10	ND
16	1.60 ± 0.10	1.60 ± 0.21	ND
17	2.55 ± 0.36	2.35 ± 0.23	ND
18	3.73 ± 0.21	3.00 ± 0.10	ND
19	>100	>100	ND
20	>100	>100	ND
21	58.9 ± 16.2	58.3 ± 1.70	ND
22	11.0 ± 5.47	16.2 ± 2.82	ND
23	>59.7	>59.7	ND
24	26.1 ± 0.12	17.5 ± 2.32	ND
25	>42.2	>42.2	ND
26	>76.3	>76.3	ND
27	7.06 ± 0.42	6.70 ± 0.45	ND
28	>37.9	>37.9	ND
29	50.8 ± 6.87	41.1 ± 8.90	ND
30	17.6 ± 1.91	17.6 ± 7.25	ND
31	>100	>100	ND
4β-Hydroxywithanolide E [1]	0.219 ± 0.019	0.146 ± 0.025	1.534 ± 0.012
Dox	0.017 ± 0.000	0.023 ± 0.001	ND

Dox doxorubicin (positive control), *ND* not done

PB of healthy individuals after informed consent were separated by Ficoll-Hypaque density sedimentation.

Evaluation of cell growth

Measurements of cytotoxicity towards cell lines and normal PB-MNCs were performed as previously described [1].

Induction of apoptosis

Induction of apoptosis was determined by flow cytometric APO2.7-PC5 assay by detecting mitochondrial membrane protein 7A6 on apoptotic cells using an EPICS XL flow cytometer (Beckman Coulter, Hialeah, FL, USA). MT-1, MT-2 and fresh ATL cells were cultured for 72 h in media alone or at different concentrations (0.5, 1 and 2 μM) of compound **13**. Cells were incubated with APO2.7-PC5 reagent for 15 min at room temperature in the dark and analyzed using a flow cytometer.

Western blot analysis

MT-1, MT-2 and fresh ATL cells were treated with or without compound **13** for 24 h and then lysed in sodium dodecyl sulfate (SDS) sample buffer containing 2 mM Na_3VO_4, 5 mM NaF, 1 mM phenylmethyl sulfonyl fluoride, 5 mg/mL leupeptin and 5 mg/mL aprotinin. Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5 % non-fat dried milk for 1 h at room temperature and then incubated overnight at 4 °C with an antibody for PARP. Following incubation, the membranes were treated with a secondary antibody and visualized with chemiluminescence.

Results and discussion

Proliferative activity of withanolides and structure–activity relationships

The antiproliferative activities of compounds **1–31** against MT-1 and MT-2 cells, as indicated by their EC_{50} values, are listed in Table 1.

With the exception of **10**, the glycosides (**1–4**, **6**, **7**, **23** and **26**) did not inhibit the growth of either of the tumor cell lines. Compound **10**, having a 5β,6β-epoxy group, showed strong activity in accordance with the previous report [1]. However, the activity of the corresponding desglycosidic compound (**11**) was approximately three times greater than that of **10**. Similarly, compound **5** showed moderate antiproliferative activity, although the corresponding 3-*O*-glycosidic compounds (**1**, **3** and **4**) did not show any activity. These results indicated that the presence of a sugar moiety at the 3- or the 27-position of withanolides leads to a reduction in the antiproliferative activity shown in MT-1 and MT-2 cells (Fig. 2).

The importance of the 5β,6β-epoxy group was further supported by the potent antiproliferative activities shown by compounds **11** and **12**, which both contained the same group. The activities of **11**, with a double bond between C-2 and C-3, were three-fold weaker than those of **12**.

In contrast to compounds containing the 5β,6β-epoxy group, the activities of the compounds containing 6α,7α- and/or 24α,25α-epoxy groups, such as compounds **19–24**, were significantly lower.

In addition to the 5β,6β-epoxy group, the presence of a 4β-hydroxy group also led to an enhancement in

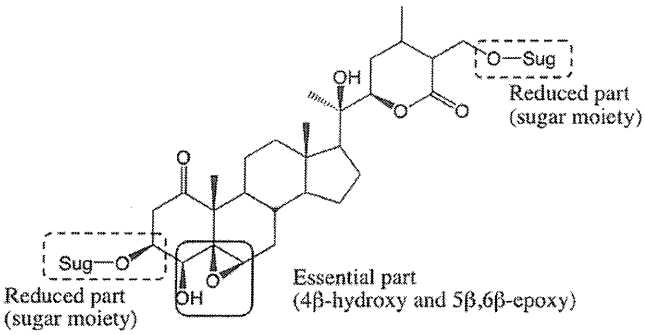


Fig. 2 Summary of structure–activity relationships

antiproliferative activities, as exemplified by the 10-fold greater activity of **8** in comparison to **9**. In contrast, the presence of a 4β-hydroxy group in **14** made no difference to the antiproliferative activities relative to the corresponding 4β-deshydroxy compound (**15**).

Compound **21** with a 15β-hydroxy group showed potent activities, whereas **20** with a 15α-hydroxy group did not show any activities, indicating that the configuration of the C-15-hydroxy group influences the antiproliferative activities.

The cytotoxic effect of the most active principle (compound **13**) toward normal PB-MNCs was observed at a concentration 10–20 times higher than that which induced growth inhibition in MT-1 and MT-2 cells (Table 1). Therefore, compound **13** has more selective toxicity to MT-1 and MT-2 cells than 4β-hydroxywithanolide E (Table 1).

Compound **13** induces apoptosis in MT-1, MT-2 and fresh ATL cells

To determine the mechanism for the cytotoxicity of **13** against MT-1 and MT-2 cells, induction of apoptosis was examined by flow cytometric APO2.7 assay. MT-1, MT-2 and fresh ATL cells were cultured for 72 h in media alone or at different concentrations (0.5, 1 and 2 μM) of **13**. APO2.7 positive proteins were barely detectable in control cells (Fig. 3). In contrast, **13** induced dose-dependent apoptosis in MT-1, MT-2 and fresh ATL cells.

Next, the PARP cleavage was examined. After 24 h of incubation, **13** induced dose-dependent apoptosis in MT-1, MT-2 and fresh ATL cells, as evidenced by Western blot analysis (Fig. 4).

In conclusion, 31 withanolide analogs were tested and the SAR further elucidated (Fig. 2). The presence of a 4β-hydroxy group as well as a 5β,6β-epoxy group appeared to be essential for the activity. In contrast, the presence of a sugar moiety at either the 3- or the 27-position led to a reduction in the activity. The most active principle (compound **13**) showed selective toxicity and also induced

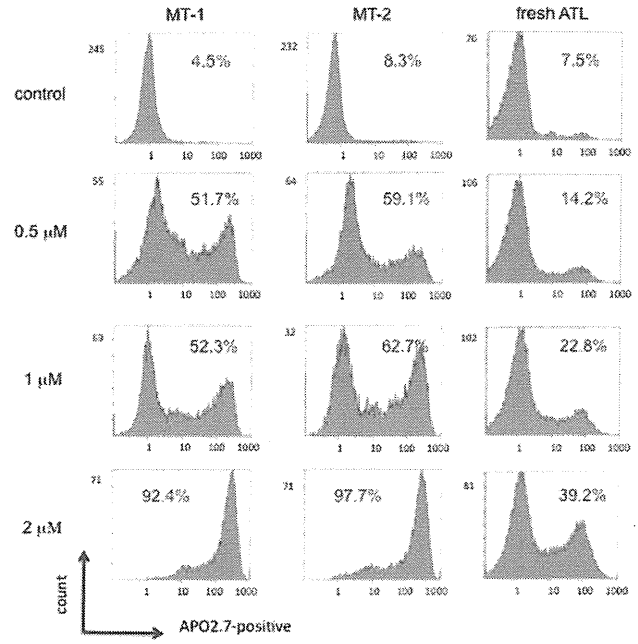


Fig. 3 Induction of apoptosis by compound **13** determined by cytometric APO2.7-PC5 assay

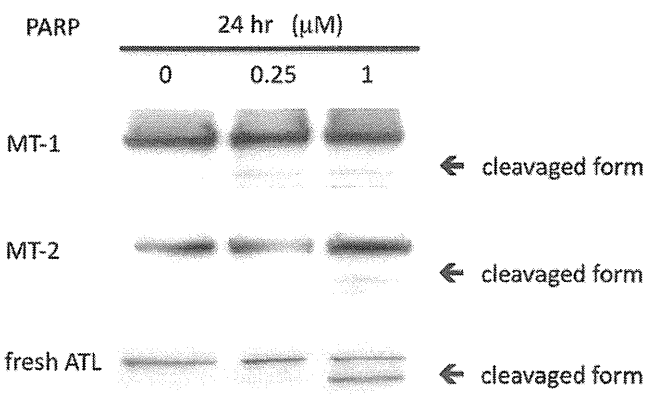


Fig. 4 Induction of apoptosis by compound **13** evidenced by PARP cleavage

apoptosis against MT-1, MT-2 and fresh ATL cells. Withanolides have therefore been demonstrated to be promising candidates for the treatment of ATL.

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NATURAL RESOURCE LETTER

Screening of promising chemotherapeutic candidates from plants against human adult T-cell leukemia/lymphoma (III)

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Abstract Adult T-cell leukemia/lymphoma (ATL) is a malignancy of mature peripheral T lymphocytes caused by human T-cell lymphotropic virus type I (HTLV-I). In our previous paper, 214 extracts from 162 plants were screened to elucidate the anti-proliferative principles against HTLV-I-infected T-cell lines. In this study, 245 extracts from 182 plants belonging to 61 families were further tested against two HTLV-I-infected T-cell lines (MT-1 and MT-2). Potent anti-proliferative effects were exhibited against MT-1 and MT-2 cells by 52 and 60 of the 245 extracts tested, respectively. Of these, two extracts showed strong inhibitory activity (EC_{50} values 0.1–1 μg/mL; +++) against both cells, 7 extracts showed moderate inhibitory activity (EC_{50} values 1–10 μg/mL; ++), and 43 extracts showed weak inhibitory activity (EC_{50} values 10–100 μg/mL; +), whereas the remaining extracts did not show any activity (EC_{50} values >100 μg/mL; –) against MT-1 cells. On the other hand, 10 extracts showed moderate inhibitory activit and, 48 extracts showed weak inhibitory activity, whereas the remaining extracts did not show any activity against MT-2 cells. Extracts from the aerial parts of *Annona reticulata* and *A. squamosa* showed the most potent inhibitory activity and three aporphine alkaloids were isolated from their extracts as the active principles by activity-guided fractionation.

Keywords Screening · Adult T-cell leukemia/lymphoma · *Annona* spp. · Aporphine alkaloid

Introduction

Adult T-cell leukemia/lymphoma (ATL) is a malignancy of mature peripheral T lymphocytes caused by human-cell lymphotropic virus type I (HTLV-I). Conventional chemotherapeutic regimens used against other malignant lymphoma have been administered to ATL patients, but the therapeutic outcomes of acute and lymphoma-type ATL remain very poor [1]. In our previous paper [2], extracts from aerial parts of *Physalis pruinosa* (Solanaceae) demonstrated a potent anti-proliferative effect against two HTLV-I-infected T-cell lines (MT-1 and MT-2) in the 214 extracts from 162 plants, and withanolides were identified as potent principles against ATL cell lines. Moreover, 36 withanolides were tested against ATL cell lines and their structure–activity relationships examined in the preceding paper [3]. These results showed that the natural product represented a rich source of chemotherapeutic candidates against ATL.

Following the previous screening program [2], 245 extracts from 182 plants belonging to 61 families were screened and two of the extracts showed promising anti-proliferative effects against MT-1 and MT-2 cells. Herein, we report the screening and isolation of active principles from the aerial parts of *Annona squamosa* and *A. reticulata* by activity-guided fractionation.

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Materials and methods

Plant materials

Plant materials used in this study were taken from the same place as indicated in the previous paper [2]. Voucher specimens were deposited in the Laboratory of Pharmacognosy at Fukuoka University.

Extraction and isolation

The 245 samples obtained from a variety of plant parts from 182 plants were powdered and extracted according to the procedure described in the previous paper [2]. The anti-proliferative effects of the extracts against cultured MT-1 and MT-2 cells were then evaluated (Table 1). One of the most effective plant materials, the leaves of *Annona reticulata* (577 g), was extracted with MeOH under reflux. The extract (55.7 g) was initially partitioned between EtOAc and a 3 % aqueous tartaric acid solution. The aqueous phase was collected, neutralized with Na_2CO_3, and extracted with $CHCl_3$. The $CHCl_3$ fraction (fr.) showed higher potency than either the aqueous or EtOAc fractions.

The $CHCl_3$ fr. was subjected to an MCI gel CHP-20 column (Mitsubishi Chemical, Japan) using 70 % MeOH and 80 % MeOH. The 80 % MeOH elute was subjected to a Sephadex LH-20 column (GE Healthcare, NJ, USA) using 70 % MeOH, to give compound **1** (1.4 mg). Otherwise, the 80 % MeOH elute was HPLC purified using 40 % MeCN, to give compound **2** (3.0 mg). The overall yields of **1** and **2** were 4.6 mg and 6.3 mg, respectively, from not only this fr. but also the remaining fr.

The leaves of one of the most effective plant materials, *Annona squamosa* (159 g), were extracted with MeOH under reflux. The extract (30 g) was then partitioned according to the procedure outlined above. The $CHCl_3$ fr. was subjected to silica gel column chromatography using $CHCl_3$:MeOH:H_2O (90:10:1) and a Sephadex LH-20 column using 50 % MeOH, to give compound **3** (2.5 mg). The overall yield of **3** was 12.9 mg from not only this fr. but also the remaining fr.

Identification of compounds

Compounds **1**–**3** were identified as liriodenine [4], lysicamine [4], and lanugiosine [4], by comparison of their physical data with data reported in the literature.

Compound **1**

$[\alpha]_D^{23}$ −203.8° (*c* 0.10, $CDCl_3$); FAB–MS *m/z* 276 $[M + H]^+$; HR-FAB–MS *m/z* 276.0661 $[M + H]^+$ (calcd. for $C_{17}H_{10}O_3N$, 276.0661); ^{1}H-NMR ($CDCl_3$) δ 8.91 (1H, d, *J* = 5.2 Hz, H-5), 8.66 (1H, d, *J* = 7.9 Hz, H-11), 8.58 (1H, d, *J* = 7.9 Hz, H-8), 7.79 (1H, d, *J* = 5.2 Hz, H-4), 7.76 (1H, t, *J* = 7.0 Hz, H-10), 7.58 (1H, t, *J* = 7.9 Hz, H-10), 7.21 (1H, s, H-3), 6.38 (2H, s, $-CH_2-$); ^{13}C-NMR δ 147.9, 108.4, 123.3, 155.5, 103.3, 145.3, 124.3, 144.7, 136.0, 178.0, 131.3, 128.7, 128.9, 134.0, 127.4, 132.9 (C1–C11′), 102.5 ($-CH_2-$).

Compound **2**

$[\alpha]_D^{23}$ −8.6° (*c* 0.10, $CDCl_3$); FAB–MS *m/z* 314 $[M + Na]^+$; HR-FAB–MS *m/z* 314.07937 $[M + Na]^+$ (calcd. for $C_{18}H_{13}O_3NNa$, 314.0793); ^{1}H-NMR ($CDCl_3$) δ 9.19 (1H, d, *J* = 7.9 Hz, H-11), 8.93 (1H, d, *J* = 5.2 Hz, H-5), 8.61 (1H, d, *J* = 7.2 Hz, H-8), 7.78 (1H, d, *J* = 5.2 Hz, H-4), 7.77 (1H, t, *J* = 7.2 Hz, H-9), 7.58 (1H, t, *J* = 7.9 Hz, H-10), 7.22 (1H, s, H-3), 4.11 (3H, s, $-OCH_3$), 4.03 (3H, s, $-OCH_3$); ^{13}C-NMR δ 151.7, 121.0, 123.5, 157.5, 106.5, 144.5, 123.2, 145.1, 134.5, 181.2, 132.3, 128.9, 129.0, 134.0, 128.5, 134.3 (C1–C11′), 60.8 ($-OCH_3$), 56.2 ($-OCH_3$).

Compound **3**

$[\alpha]_D^{23}$ −230.4° (*c* 0.22, $CDCl_3$); FAB–MS *m/z* 306 $[M + H]^+$; HR-FAB–MS *m/z* 306.0770 $[M + H]^+$ (calcd. for $C_{18}H_{12}O_4N$, 306.0776); ^{1}H-NMR ($CDCl_3$) δ 8.90 (1H, d, *J* = 5.2 Hz, H-5), 8.59 (1H, d, *J* = 8.9 Hz, H-11), 8.04 (1H, d, *J* = 3.0 Hz, H-8), 7.78 (1H, d, *J* = 5.2 Hz, H-4), 7.30 (1H, dd, *J* = 3.0, 8.9 Hz, H-10), 7.15 (1H, s, H-3), 6.35 (2H, s, $-CH_2-$), 4.00 (3H, s, $-OCH_3-$); ^{13}C-NMR δ 147.0, 108.3, 122.8, 151.8, 102.5, 145.4, 124.3, 144.9, 135.8, 182.3, 126.2, 110.3, 159.8, 122.6, 129.1, 133.2 (C1–C11′), 102.3 ($-CH_2-$), 55.8 ($-OCH_3$).

Cells

Two HTLV-I-infected T-cell lines, MT-1 and MT-2, were kindly provided by Dr I. Miyoshi of Kochi University in Nangoku, Japan. Culture conditions were as previously described [1]. The cells were cultured in RPMI-1640 medium with L-glutamine and $NaHCO_3$, containing 15 % fetal bovine serum and kanamycin. Cells were cultured at 37 °C in humidified 5 % CO_2/95 % air.

Measurement of anti-proliferative effects against MT-1 and MT-2 cell

Cellular growth was determined using the MTT assay. The cells were maintained in RPMI-1640 medium containing fetal bovine serum (15 %). A 50-μL aliquot of the cell suspension and 50 μL of the test sample solution or suspension were plated in flat-bottomed microtiter wells

Table 1 The anti-proliferative activities of plant extracts against MT-1 and MT-2 cells

Family	Scientific name	Parts	MT-1	MT-2
Acanthaceae	*Dicliptera japonica*	Aerial parts	−	−
	Justicia procumbens	Whole parts	++	+
Aizoaceae	*Tetragonia expansa*	Whole parts	−	−
Amaranthaceae	*Celosia argentea*	Seeds	−	−
	Gomphrena globosa	Whole parts	−	−
Annonaceae	*Annona cherimola*	Leaves	+	+
		Barks	++	++
	Annona muricata	Stems	+	−
		Leaves	−	−
	Annona reticulata	Leaves	+++	+++
		Barks	++	++
	Annona squamosa	Leaves	+++	+++
		Twigs	+	−
Apocynaceae	*Apocynum venetum*	Whole parts	−	−
	Cerbera manghas	Barks	+	+
		Leaves	+	++
	Trachelospermum jasminoides	Aerial parts	+	+
	Trachelospermum liukiuense	Aerial parts	−	+
Aquifoliaceae	*Ilex cornuta*	Leaves	+	+
		Fruits	−	−
	Ilex latifolia	Leaves	−	−
	Ilex rotunda	Fruits	+	+
Araceae	*Pinellia ternata*	Tubers	−	−
Araliaceae	*Acanthopanax senticosus*	Roots	+	+
Aristolochiaceae	*Asiasarum sieboldii*	Roots	−	−
	Heterotropa nipponica	Roots	+	+
		Aerial parts	−	−
Balsaminaceae	*Impatiens textori*	Aerial parts	+	+
Berberidaceae	*Epimedium sagittatum*	Aerial parts	−	−
	Mahonia japonica	Stems	+	++
		Roots	+	+
		Leaves	−	+
	Nandina domestica	Leaves	−	−
		Barks	+	−
Bombacaceae	*Chorisia speciosa*	Immature Fruits	−	−
	Pachira macrocarpa	Leaves	−	+
Boraginaceae	*Lithospermum officinale*	Roots	−	−
Caprifoliaceae	*Lonicera japonica*	Flowers	−	−
	Sambucus chinensis	Leaves	−	−
		Stems	−	−
Caricaceae	*Carica papaya*	Roots	−	−
		Barks	−	−
Cecropiaceae	*Cecropia obtusifolia*	Leaves	−	−
Celastraceae	*Celastrus orbiculatus*	Aerial parts	−	−
Cephalotaxaceae	*Cephalotaxus harringtonia*	Stems	−	−
		Leaves	++	++
Compositae	*Achillea millefolium*	Leaves	−	−
		Stems	−	−

Table 1 continued

Family	Scientific name	Parts	MT-1	MT-2
	Adenocaulon bicolor var. *adhaerescens*	Aerial parts	−	+
	Adenocaulon himalaicum	Roots	−	−
	Adenostemma lavenia	Aerial parts	−	+
	Arctium lappa	Seeds	−	−
		Roots	−	−
	Artemisia absinthium	Roots	+	+
		Aerial parts	+	+
		Leaves	−	−
		Stems	−	−
	Artemisia campestris	Aerial parts	−	−
	Artemisia capillaris	Aerial parts	−	+
		Roots	−	−
	Artemisia ludoviciana var. *mexicana*	Aerial parts	−	−
	Aster spathulifolius	Leaves	+	+
		Stems	+	+
	Atractylodes japonica	Tubers	−	−
	Bidens frondosa	Roots	+	+
		Aerial parts	−	−
	Cacalia tebakoensis	Aerial parts	−	−
	Carthamus tinctorius	Flowers	−	−
	Chrysanthemum vulgare	Aerial parts	+	+
	Cichorium intybus	Aerial parts	−	−
		Roots	−	−
	Cnicus benedictus	Leaves	+	−
	Cosmos bipinnatus	Seeds	−	−
	Crassocephalum crepidioides	Aerial parts	−	−
		Roots	−	−
	Crepidiastrum lanceolatum	Roots	−	−
		Aerial parts	−	−
	Eclipta prostrata	Whole parts	−	−
	Eupatorium stoechadosmum	Roots	−	−
		Leaves	−	−
		Stems	−	−
	Euryops pectinatus	Leaves	−	−
		Stems	−	−
	Helianthus annuus	Aerial parts	+	+
	Ligularia japonica	Roots	−	−
		Leaves	+	−
	Rhynchospermum verticillatum	Aerial parts	−	−
	Santolina chamaecyparissus	Stems	−	−
		Leaves	−	−
	Saussurea lappa	Roots	+	++
	Senecio vulgaris	Whole parts	−	−
	Siegesbeckia glabrescens	Leaves	−	−
		Roots	−	−
	Sonchus asper	Aerial parts	−	−
	Tagetes patula	Roots	−	−
		Aerial parts	−	+

Table 1 continued

Family	Scientific name	Parts	MT-1	MT-2
	Tussilago farfara	Roots	−	−
	Wedelia prostrata	Whole parts	+	+
	Xanthium strumarium	Fruits	−	−
Cornaceae	*Cornus officinalis*	Fruits	−	−
Crassulaceae	*Bryophyllum pinnatum*	Roots	+	++
		Aerial parts	−	−
	Hylotelephium erythrostictum	Roots	−	−
	Orostachys japonicus	Whole parts	−	−
	Sedum aizoon var. *floribundum*	Roots	−	−
	Sedum tomentosum	Whole parts	−	−
Cruciferae	*Isatis indigotica*	Fruits	+	+
		Leaves	−	−
		Roots	−	−
	Lepidium virginicum	Whole parts	−	−
	Thlaspi arvense	Seeds	−	−
Cycadaceae	*Cycas revoluta*	Kernel	−	−
Daphniphyllaceae	*Daphniphyllum macropodum*	Leaves	−	−
		Barks	−	−
Eucommiaceae	*Eucommia ulmoides*	Barks	+	+
Euphorbiaceae	*Phyllanthus acidus*	Aerial parts	−	−
Guttiferae	*Garcinia subelliptica*	Leaves	−	−
		Woods	−	−
	Garcinia xanthochymus	Seeds	+	+
		Fruits	−	−
Iridaceae	*Crocosmiax crocosmiiflora*	Roots	−	−
Labiatae	*Ajuga decumbens*	Whole parts	−	−
	Ajuga reptans	Leaves	−	−
		Roots	−	−
	Glechoma longituba	Whole parts	−	−
	Lamium amplexicaule	Whole parts	−	−
	Leonurus sibiricus	Seeds	−	−
		Aerial parts	+	+
	Scutellaria barbata	Whole parts	+	+
Leguminosae	*Acacia melanoxylon*	Barks	−	−
		Leaves	−	+
	Apios americana	Flowers	−	−
	Astragalus membranaceus	Roots	−	−
	Canavalia gladiata	Roots	−	+
		Seeds	+	+
	Cassia obtusifolia	Seeds	−	−
	Erythrina variegata var. *orientalis*	Barks	−	−
	Euchresta japonica	Roots	−	+
	Eysenhardtia polystachya	Woods	−	−
	Gliricidia sepium	Leaves	−	−
	Glycyrrhiza pallidiflora	Roots	−	−
	Glycyrrhiza uralensis	Roots	−	−
	Haematoxylum brasiletto	Woods	++	++
	Medicago polymorpha	Whole parts	−	−

Table 1 continued

Family	Scientific name	Parts	MT-1	MT-2
	Melilotus officinalis	Whole parts	−	−
	Psoralea corylifolia	Seeds	−	+
	Rhynchosia volubilis	Seeds	+	−
	Sophora japonica	Fruits	+	+
	Trifolium dubium	Aerial parts	−	−
Liliaceae	*Allium sativum* var. *pekinense*	Bulbs	−	−
	Aloe ferox	Leaves	−	−
	Anemarrhena asphodeloides	Roots	+	+
	Fritillaria verticillata var. *thunbergii*	Bulbs	−	−
	Ophiopogon japonicus	Roots	−	−
Malvaceae	*Althaea cannabina*	Leaves	−	−
	Malvaviscus arboreus	Leaves	−	−
Menispermaceae	*Tinospora tuberculata*	Stems	−	−
Moraceae	*Morus alba*	Barks	−	−
Myristicaceae	*Myristica fragrans*	Whole parts	−	−
Myrsinaceae	*Ardisia crenata*	Roots	++	++
		Leaves	−	−
		Stems	−	−
	Ardisia japonica	Roots	−	−
		Leaves	−	−
Myrtaceae	*Psidium cattleianum*	Fruits	−	−
Nyctaginaceae	*Mirabilis jalapa*	Leaves	−	+
Oleaceae	*Ligustrum lucidum*	Leaves	−	−
		Fruits	−	−
	Ligustrum ovalifolium	Leaves	−	−
Orobanchaceae	*Cistanche deserticola*	Whole parts	−	−
Oxalidaceae	*Averrhoa carambola*	Leaves	−	−
		Barks	−	−
Paeoniaceae	*Paeonia lactiflora*	Roots	−	−
Phytolaccaceae	*Phytolacca americana*	Roots	−	−
	Rivina humilis	Aerial parts	−	−
Pittosporaceae	*Pittosporum tobira*	Barks	−	−
		Fruits	−	−
Polygonaceae	*Polygonum tinctorium*	Whole parts	+	+
Primulaceae	*Lysimachia japonica*	Whole parts	−	−
Pteridaceae	*Drynaria fortunei*	Whole parts	−	−
	Pteris multifida	Aerial parts	−	−
Rhamnaceae	*Zizyphus jujuba* var. *jujuba*	Fruits	−	−
Rosaceae	*Agrimonia pilosa*	Whole parts	−	−
	Crataegus cuneata	Fruits	−	−
	Duchesnea chrysantha	Whole parts	−	−
	Eriobotrya japonica	Leaves	−	−
		Seeds	−	−
	Potentilla fragarioides var. *major*	Aerial parts	−	−
	Prunus armeniaca	Kernel	−	−
	Rosa multiflora	Fruits	−	−
	Rubus hirsutus	Aerial parts	−	−
	Sanguisorba officinalis var. *carnea*	Roots	−	−

Table 1 continued

Family	Scientific name	Parts	MT-1	MT-2
Rubiaceae	*Damnacanthus macrophyllus* var. *macrophyllus*	Leaves	–	–
		Stems	–	–
		Roots	–	–
	Galium pogonanthum	Aerial parts	–	+
	Hamelia patens	Aerial parts	–	–
	Hedyotis diffusa	Whole parts	–	–
	Paederia scandens	Fruits	–	–
		Leaves	–	–
		Stems	–	–
	Rubia argyi	Roots	+	+
	Uncaria rhynchophylla	Hook	–	–
Sapindaceae	*Cardiospermum halicacabum*	Seeds	–	–
	Euphoria longana	Leaves	–	–
		Twigs	–	–
	Litchi chinensis	Leaves	–	–
		Twigs	–	–
	Sapindus mukurossi	Pericarps	++	+
		Seeds	–	–
Sapotaceae	*Pouteria sapota*	Seeds	–	–
Schisandraceae	*Schisandra chinensis*	Fruits	–	–
Scrophulariaceae	*Penstemon gloxinoides*	Leaves	–	–
		Stems	–	–
	Picrorhiza scrophulariiflora	Rhizomes	–	–
	Rehmannia glutinosa var. *purpurea*	Roots	–	–
	Scrophularia buergeriana	Roots	–	–
Solanaceae	*Nicandra physaloides*	Stems	–	–
		Fruits	–	–
	Physalis angulata	Roots	–	–
Sterculiaceae	*Sterculia nobilis*	Barks	–	–
		Woods	–	–
Tiliaceae	*Corchoropsis tomentosa*	Fruits	+	+
Umbelliferae	*Angelica acutiloba*	Roots	–	–
	Angelica decursiva	Aerial parts	–	–
	Coriandrum sativum	Leaves	–	–
	Osmorhiza aristata	Roots	+	+
	Peucedanum japonicum	Leaves	–	–
		Woods	–	–
	Peucedanum praeruptorum	Roots	+	+
	Urtica dioica	Aerial parts	–	–
	Urtica thunbergiana	Aerial parts	–	–
Verbenaceae	*Clerodendron trichotomum*	Flowers	–	–
	Clerodendrum bungei	Stems	–	–
		Flowers	–	–
		Leaves	–	–
	Verbena officinalis	Roots	–	–
	Vitex trifolia	Leaves	–	–
		Twigs	–	–
Vitaceae	*Cayratia japonica*	Aerial parts	–	–

Table 1 continued

Family	Scientific name	Parts	MT-1	MT-2
Zingiberaceae	*Alpinia japonica*	Seeds	–	+
		Fruits	–	–
	Curcuma zedoaria	Rhizomes	+	+
	Hedychium coronarium	Rhizomes	+	+
	Zingiber officinale	Rhizomes	+	++

EC_{50} values against ATL cells (MT-1 and MT-2) in vitro. Data presented as the mean of three independent experiments
+++, 0.1–1 μg/mL; ++, 1–10 μg/mL; +, 10–100 μg/mL; –, >100 μg/mL

(extracts: final concentrations 100, 10, 1, 0.1 μg/mL and control. Fraction: final concentrations 100, 50, 10, 5, 1, 0.5, 0.1, 0.05 μg/mL and control. Compounds: final concentrations 12.50, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.10 μg/mL and control) and incubated for 72 h at 37 °C in a humidified atmosphere of 5 % CO_2 in air. After cultivation, 10 μL of 3-(4,5)-dimetyl-2-thiazoyl-2,5-diphenyl-2H-tetrazolium bromide (MTT reagent) solution was added to the microtiter wells. After incubation for 4 h at 37 °C, 100 μL isopropanol was added to solubilize the MTT-formazan product. The absorbance at 450 nm was measured with a microplate reader.

Measurement of cytotoxicity toward normal cells

The cells were maintained in RPMI-1640 medium containing fetal bovine serum (20 %). A 50-μL aliquot of the cell suspension and 50 μL of the test sample solution were plated in flat-bottomed microtiter wells (final concentrations 100, 10, 1, 0.1 μg/mL and control) and incubated for 72 h at 37 °C in a humidified atmosphere of 5 % CO_2 in air. After cultivation, 10 μL of MTT reagent solution was added to the microtiter wells. After incubation for 4 h at 37 °C, 100 μL isopropanol was added to solubilize the MTT-formazan product. The absorbance at 450 nm was measured with a microplate reader.

Measurement of apoptotic cells

MT-1 and MT-2 cells were cultured for 72 h with or without **1** (0, 2, 4, 8 μM), and propidium iodide (PI) staining was performed as described previously [5]. MT-1 and MT-2 cells cultured with **1** were harvested, fixed with 70 % ethanol, and pretreated with 250 μg/mL RNAse (Sigma). Cells were stained with PI 50 μg/mL (Sigma), and cell cycle profiles were determined by using M software on an EPICS XL flow cytometer.

Results and discussion

Table 1 shows the anti-proliferative activity of the 245 extracts against MT-1 and MT-2 cells. Positive effects

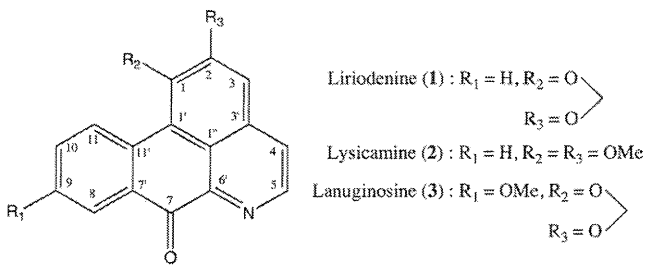

Fig. 1 Active principles from the leaves of *Annona reticulata* and *A. squamosa*

were exhibited against MT-1 and MT-2 cells by 52 and 60 of the 245 extracts tested, respectively.

Extracts from the leaves of the two annonaceous plants, *Annona reticulata* and *A. squamosa*, showed the strongest activity against MT-1 cells. Extracts of *Justicia procumbens* (Acanthaceae), *Annona cherimola* (Annonaceae), and *A. reticulata* (Annonaceae) barks, *Cephalotaxus harringtonia* (Cephalotaxaceae) leaves, *Haematoxylum brasiletto* (Leguminosae), *Ardisia crenata* (Myrsinaceae) roots, and *Sapindus mukurossi* (Sapindaceae) pericarps showed moderate activity. Interestingly, the two annonaceous extracts showed the strongest activity against MT-1 and MT-2 cells. With the exception of the extracts from *J. procumbens* and *S. mukurosi* pericarps, and in addition to the same extracts which showed moderate activity against MT-1 cells, the extracts of *Cerbera manghas* (Apocynaceae) leaves, *Mahonia japonica* (Berberidaceae) stems, *Saussurea lappa* (Compositae), *Bryophyllum pinnatum* (Crassulaceae) roots, and *Zingiber officinale* (Zingiberaceae) also showed moderate activity against MT-2 cells.

As described above, the two annonaceous plants, *Annona reticulata* and *A. squamosa*, showed the most potent activity against both MT-1 and MT-2 cells. On the other hand, the cytotoxicities (EC_{50}) toward normal peripheral blood mononuclear cells of *Annona reticulata* extract and *A. squamosa* extract were >100 μg/mL. Therefore, we attempted to isolate their active principles. Initially, the extract of *A. reticulata* leaves was partitioned between EtOAc and 3 % aqueous tartaric acid solution. The aqueous layer was collected, neutralized with Na_2CO_3,

Table 2 The anti-proliferative activities (EC_{50}) of the aporphine alkaloids, compounds **1–3**, against MT-1 and MT-2

Compounds	EC_{50} (μM)	
	MT-1	MT-2
1	3.09 ± 0.54	3.62 ± 0.25
2	31.61 ± 4.67	16.25 ± 1.29
3	1.34 ± 0.09	4.49 ± 0.24
Dox	0.017 ± 0.000	0.023 ± 0.001

Dox doxorubicin (positive control)

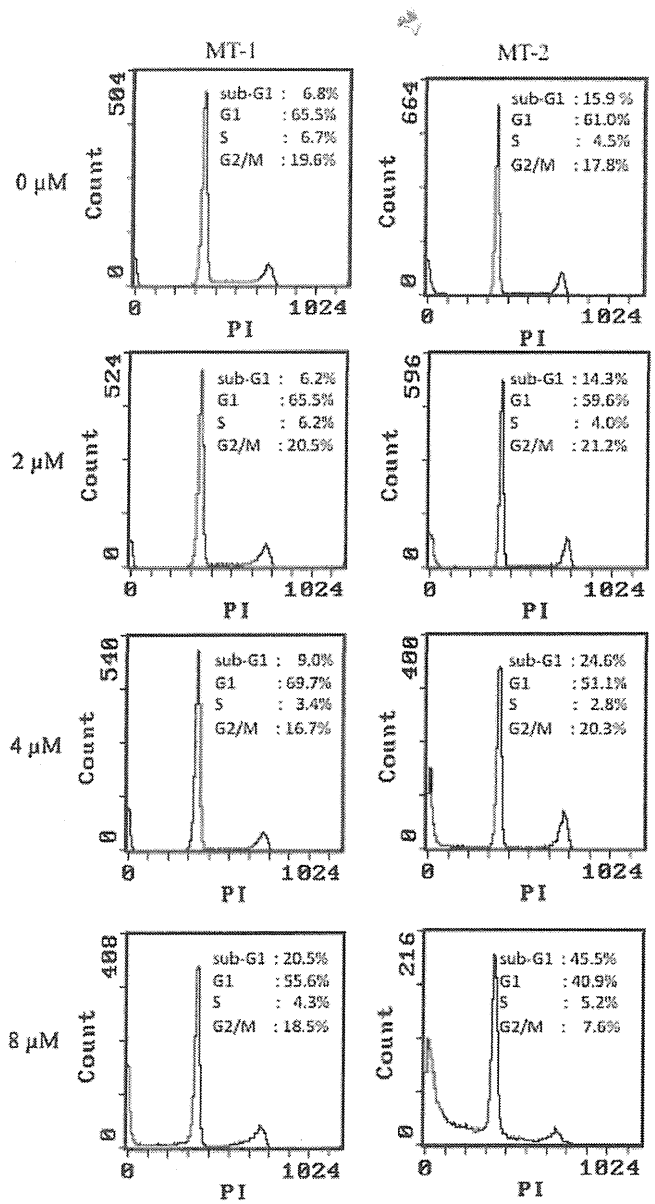

Fig. 2 Histograms obtained from propidium iodide staining

and extracted with $CHCl_3$. The anti-proliferative activities (EC_{50}) of each fr. were 0.4 μg/mL ($CHCl_3$ fr.), 4.3 μg/mL (EtOAc fr.), and >100 μg/mL (aqueous fr.), respectively. The $CHCl_3$ fr. was identified as the most potent fr. of all. The $CHCl_3$ extracts were then both subjected to several chromatographic purifications to obtain the active principles (**1** and **2**). The active principle (**3**) was also isolated in the same way from the extracts of *Annona squamosa* leaves. The anti-proliferative effects of **1–3** (Fig. 1) are listed in Table 2. All of the isolated principles inhibited the growth of both tumor cell lines. Accumulation of Sub-G1 cells were observed in MT-1 and MT-2 cells treated by **1**, suggesting induction of apoptosis (Fig. 2).

Aporphine alkaloids had cytotoxicity against MT-1 and MT-2 cells, the same as tumor cells [6]. Recently, **1** was reported to induce G1/S cell cycle arrest in human colon cancer cells via nitric oxide- and p53-mediated pathways [7].

In conclusion, 245 extracts were screened against MT-1 and MT-2 cells. Two of these extracts showed the strongest inhibitory activity (EC_{50} values 0.1–1 μg/mL; indicated as +++) against both cells, whereas 7 extracts showed moderate inhibitory activity (EC_{50} values 1–10 μg/mL; indicated as ++), 43 extracts showed weak inhibitory activity (EC_{50} values 10-100 μg/mL; indicated as +), and the remaining extracts did not show any activity (EC_{50} values over 100 μg/mL; indicated as –) against MT-1 cells. On the other hand, in addition to the 2 extracts above, 10 extracts showed moderate inhibitory activity, 48 extracts showed weak inhibitory activity, and the remaining extracts did not show any activity against MT-2 cells.

Three aporphine alkaloids were isolated as the active principles from the leaves of the two annonaceous plants, *Annona reticulata* and *A. squamosa*.

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