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Ellagitannins from *Terminalia calamansanai* induced apoptosis in HL-60 cells

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ABSTRACT

Terminalia calamansanai (Blanco) Rolf. (Combretaceae) is used medicinally as lithontriptic in Philippines. The 70% acetone extracts of *T. calamansanai* leaves inhibited the viability of human promyelocytic leukemia HL-60 cells. 1-α-*O*-Galloylpunicalagin, punicalagin, 2-*O*-galloylpunicalin, sanguiin H-4, and methyl gallate were the main components isolated from *T. calamansanai* with the IC_{50} values of 65.2, 74.8, 42.2, 38.0 and >100 μM, respectively, for HL-60 cells. Apoptosis of HL-60 cells treated with 1-α-*O*-galloylpunicalagin, punicalagin, 2-*O*-galloylpunicalin, and sanguiin H-4 was noted by the appearance of a sub-G_1 peak in flow cytometric analysis and DNA fragmentation by gel electrophoresis. 2-*O*-Galloylpunicalin and sanguiin H-4 induced a decrease of the human poly(ADP-ribose)polymerase (PARP) cleavage-related procaspase-3 and elevated activity of caspase-3 in HL-60 cells, but not normal human peripheral blood mononuclear cells (PBMCs), suggesting that both compounds may be new candidates for drug development in the prevention and treatment of cancer.

1. Introduction

Genus *Terminalia*, comprising 250 species, are widely distributed in tropical areas of the world. Several species have been used as traditional medicine in Asia, such as *Terminalia catappa* L. and *T. chebula* L. in China and Taiwan for diarrhea (Huang, 1993) and *T. calamansanai* (Blanco) Rolf. in Philippines as a lithontriptic (Tanaka et al., 1991). The medical functions depend on the species and its organs. For example, the fruit of *T. chebula* Retzius is used for the treatment of diarrhea and collapsed anus and as an antispasmodic (Huang, 1993), and the fallen leaves of *T. catappa* L. for preventing hepatoma and treating hepatitis in Taiwan (Chen et al., 2000). Early studies reported that *Terminalia* species contain a high abundance of tannins (Lin et al., 2000). Further, calamanins A, B, and C, together with 10 tannins, have been isolated from the leaves of *T. calamansanai* (Tanaka et al., 1991).

Ellagitannins have been reported in leaves of *T. calamansanai*, with a high level of 1-α-*O*-galloylpunicalagin and a low level of punicalagin (Tanaka et al., 1991), and in the bark and heartwood of *Punica granatum* L. (pomegranate) (Tanaka et al., 1986; El-Toumy and Rauwald, 2002) and the leaves of *T. trifolia* (Griseb.) Lillo (Martino et al., 2004) with 2-*O*-galloylpunicalin. Punicalagin is the major constituent of folk medicine found in the leaves of *T. catappa* (Chen et al., 2000) and pericarp of pomegranate, and demonstrated antioxidant activity in cultured Chinese hamster ovary cells and protective effects against bleomycin-induced genotoxicity (Chen et al., 2000; Gil et al., 2000; Chen and Li, 2004). In addition, punicalagin induced apoptosis in Caco-2 cells through the activation of caspase 3 (Larrosa et al., 2006). Nevertheless, long-term oral administration of punicalagin is not toxic to the rat (Cerda et al., 2003); other structure-related components from *T. calamansanai* leaves might own functions differing from punicalagin. However, the anticancer activities of compounds from this species have seldom been surveyed thoroughly (Ko et al., 2003; Saleem et al., 2002; Conrad et al., 2001; Kandil and Nassar, 1998; Pettit et al., 1996). Therefore, because of the higher mortality from cancer in Taiwanese, we used carcinoma cell lines of different origins, including human promyelocytic leukemia (HL-60), human gastric carcinoma (AGS), human cervix epithelioid carcinoma (HeLa), human hepatoma (Hep G2), human colon adenocarcinoma (HT 29), and human bladder carcinoma (T24) to explore the cytotoxicity of polyphenols isolated from *T. calamansanai*.

With reexamination and characterization of new compounds purified from 70% acetone extract of *T. calamansanai* leaves, the biological functions of these new compounds in relation to different tumor cell lines were determined by measuring cell viability with MTT assay and apoptosis of tumor cells with nuclear DNA fragmentation. Five main polyphenols were identified. Four of them inhibited the growth of tumor cells. Especially, 2-*O*-galloylpunicalin and sanguiin H-4 are suitable for use as bioactivity marker substances in *T. calamansanai*. The purpose of this study was to present

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scientific evidence for the use of *T. calamansanai* as a candidate for new anticancer drugs.

2. Materials and methods

2.1. General

^{1}H-(500 MHz) and ^{13}C-NMR (125 MHz) spectra were measured by a Bruker DRX 500 instrument and chemical shifts were given in δ (ppm) values. ESI-MS were taken on a Waters ZQ-4000 mass spectrometer with direct injection of the MeOH solution of a sample. Normal phase HPLC was conducted with YMC-pack SIL-A003 (4.6 mm × 250 mm) by using the following solvent systems: *n*-hexane–MeOH–THF–HCOOH (60:45:15:1) containing oxalic acid (500 mg/1.2 L) with a flow rate of 1.5 mL/min and 280 nm for detection at room temperature. Reversed-phase HPLC was performed with a LiChrospher RP-18e (4.0 mm × 250 mm) column by using the following solvent systems: 0.05% trifluoroacetic acid–CH_3CN (88: 12; RP1), and 0.05% trifluoroacetic acid–CH_3CN (92: 8; RP2) with a flow rate of 1.0 mL/min and 280 nm for detection at 40 °C. Column chromatography was carried out with different columns, including Toyopearl HW-40C (Tosoh Corp., Tokyo, Japan), Diaion HP-20 (Mitsubishi Chemical Industry, Tokyo, Japan) and LiChroprep RP-18 (40–63 μm, Merck KGaA, Darmstadt, Germany).

Dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), adriamycin (ADR), trypan blue, Tris–HCl, M EDTA, Sarkosyl and other chemicals were purchased from Sigma Industry (St. Louis, MO, USA). RPMI-1640, fetal bovine serum (FBS), antibiotics, and glutamine were purchased from Gibco (Grand Island, NY, USA). Western blotting was performed using an antibody specific to human poly-(ADP-ribose) polymerase (PARP, sc-7150), caspase 3 (sc-7148), -tubulin (sc-8035), anti-rabbit IgG-AP (sc-2007), and anti-mouse IgG-AP (sc-2008), which were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents and chemicals used were of the highest purity grade available.

2.2. Plant materials

The leaves of the *T. calamansanai,* identified by Prof. Fu-Yuen Lu, Department of Forestry, National Chiayi University, were collected at the campus of the same university in July 2002. A voucher specimen (#NCYU-H0101) has been deposited in the Graduate Institute of Biomedical and Biopharmaceutical Sciences, National Chiayi University.

2.3. Extraction and isolation of polyphenols

The dried leaves of *T. calamansanai* (1.0 kg) were homogenized with 70% aqueous acetone (20 L × 3) and the homogenate was then filtered. The filtrate was concentrated by evaporation and further freeze-dried to yield the 70% acetone extract (320 g). Most (310 g) of the 70% aqueous acetone extract was chromatographed over a Diaion HP-20 column (9.5 cm i.d. × 45 cm) with H_2O, and H_2O–MeOH (20% MeOH → 40% → 60% → 100%). Among these fractions, 20% and 40% MeOH elutes were further characterized.

The 40% MeOH eluate (13 g) was chromatographed over a Toyopearl HW-40C column (2.5 cm i.d. × 51 cm), developing with H_2O → 60% MeOH → 70% MeOH → MeOH–H_2O–acetone (7:2:1) → MeOH–H_2O–acetone (8:1:1). The MeOH–H_2O–acetone (7:2:1) eluate was rechromatographed over a LiChroprep RP-18 column (2.5 cm i.d. × 48 cm) with RP1 to yield 1-α-*O*-galloylpunicalagin (**1**) (900 mg, 0.235%). The 70% MeOH eluate was rechromatographed over LiChroprep RP-18 (2.5 cm i.d. × 48 cm) with RP2 to yield punicalagin (**2**) (189 mg, 0.049%). The 60% MeOH eluate was rechromatographed over LiChroprep RP-18 (2.5 cm i.d. × 48 cm) with 0.05% trifluoroacetic acid–CH_3CN (95:5) to yield sanguiin H-4 (**4**) (137 mg, 0.036%) and methyl gallate (**5**) (31 mg, 0.008%).

The 20% MeOH eluate (11 g) obtained from the column chromatography of the Diaion HP-20 was also rechromatographed over a Toyopearl HW-40C column (2.5 cm i.d. × 51 cm), developing with H_2O → 60% MeOH → 70% MeOH → MeOH–H_2O–acetone (7:2:1) → MeOH–H_2O–acetone (8:1:1). The 60% MeOH eluate (2 g) was rechromatographed with a LiChroprep RP-18 column (2.5 cm i.d. × 48 cm) with 0.05% trifluoroacetic acid–CH_3CN (94: 6) to give 2-*O*-galloylpunicalin (**3**) (98 mg, 0.037%). All structures were estimated by ^{1}H and ^{13}C NMR, including 2D-NMR techniques, and also by comparison of those data with authentic compounds (Tanaka et al., 1991; Tanaka et al., 1986; Yoshida et al., 1989; Yoshida and Okuda, 1985). The purity of each compound was determined by HPLC and was shown to exceed 95%.

2.4. Cell cultures

Peripheral blood mononuclear cells (PBMCs) were obtained from normal volunteers and purified by Ficol-Hypaque™ Plus (Amersham Pharmacia Biotech AB, Sweden) density gradient centrifugation, as described previously (Wang et al., 2002). Different tumor cell lines, including HL-60, AGS, HeLa, Hep G2, HT 29, and T24 were purchased from American Type Cell Culture (ATCC) (Rockville, MD, USA). All cell lines and PBMCs were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 mg/L streptomycin, and 100 IU/mL penicillin at 37 °C and 5% CO_2.

2.5. Cytotoxicity assay

Cells were counted in a suitable number (1×10^4 cells/well for the adhesion-type, 1×10^6 cells/well for the suspension type) and seeded in 96-well plates overnight. The second day, the culture medium was removed from the content cells-plate and treated with ellagitannins. Ellagitannins were prepared as a 20 mM stock solution by dissolution in H_2O, and then stored at 4 °C until use. Further, working solutions of 12.5, 25, 50 and 100 μM were added into the suspension type (HL-60 cells and PBMCs) for 12 h and the adhesion-type (AGS, HeLa, Hep G2, HT 29 and T24) for 24 h without renewal of the medium. However, the levels of the ellagitannins were up to 200 and 400 μM in PBMCs. Cell viability was measured by using the tetrazolium (MTT) assay (Wang et al., 2002). The cytotoxicity index (CI%) was calculated according to the following equation:

$$CI = [1 - (T/C)] \times 100\%,$$

where *T* and *C* represent the mean optical density of the treated and vehicle control groups, respectively. In accordance with the CI% of the dose–response curve, the concentration of each ellagitannin that inhibited 50% cell growth (IC_{50} value) was calculated. Doxorubicin® is a widely used clinical anticancer drug, and has the chemical name of adriamycin (ADR). ADR was used as a positive control for CI measurement.

2.6. DNA fragmentation assay

After being treated separately with each ellagitannin for 12 h, HL 60 cells (1×10^6 cells/well) were harvested by centrifugation and washed with PBS. The cells were incubated for 10 min in 200 μl lysis buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.5% Sarkosyl) at room temperature, then centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant was incubated overnight at 56 °C

with 250 μg/ml proteinase K. Cell lysates were then treated with 2 mg/ml RNase A and incubated at 56 °C for 1.5 h. DNA was extracted with 1 volume of chloroform/phenol/isoamyl alcohol (25:24:1), and precipitated from the aqueous phase by centrifugation at 14,000*g* for 30 min at 0 °C. The extent of DNA fragmentation of HL-60 cells was assessed by 1.5% agarose gel electrophoresis (Wang et al., 2002).

2.7. Flow cytometric analysis

After incubation with 2-*O*-galloylpunicalin, HL-60 cells (5×10^5 cells/well) were centrifuged and then washed with PBS. Further, these cells were fixed by Ice-cold 80% ethanol, treated with 1.0 mg/mL RNase A, and stained with 50 μg/mL propidium iodide. Samples were run through a FACScan (Becton Dickinson, San Jose, CA, USA). DNA fragmentation at different levels of 2-*O*-galloylpunicalin was determined by the change of fluorescence intensity (Wang et al., 2002).

2.8. Western blotting

To understand the change of human poly-(ADP-ribose) polymerase (PARP) and pro-caspase 3, HL-60 cells (5×10^5 cells/well) were treated with ellagitannins at different levels for 12 h. Protein samples of these cells were collected and separated by denaturing SDS–PAGE methods (Wang et al., 2001). The proteins were transferred onto a nitrocellulose membrane. Western blotting with specific anti-goat and anti-mouse antibodies conjugated to alkaline phosphatase and BCIP/NBT (Sigma Industry) were used to visualize the protein bands. α-tubulin was used as an internal control.

2.9. Caspase-3 activity assay

After incubation of HL-60 cells (1×10^6 cells/well) with ellagitannins at 50 and 100 μM separately for 12 h, the change of caspase-3 expression, one of the main proapoptotic proteins, was determined using the CaspSELECT™ Caspase-3 Immunoassay kit (BioVision, CA, USA). Rh-caspase-3 (1 unit) was used as an internal standard. The fluorescent densities of test samples were read at Ex/Em = 400 nm/505 nm in a fluorescent microtiter plate reader (Synergy™ HT, Bio-TEK, USA). Chemical ADR was used as positive control.

2.10. Statistical analysis

Each experiment was performed at least in triplicate. Results are expressed as the mean ± standard deviation (SD). Statistical

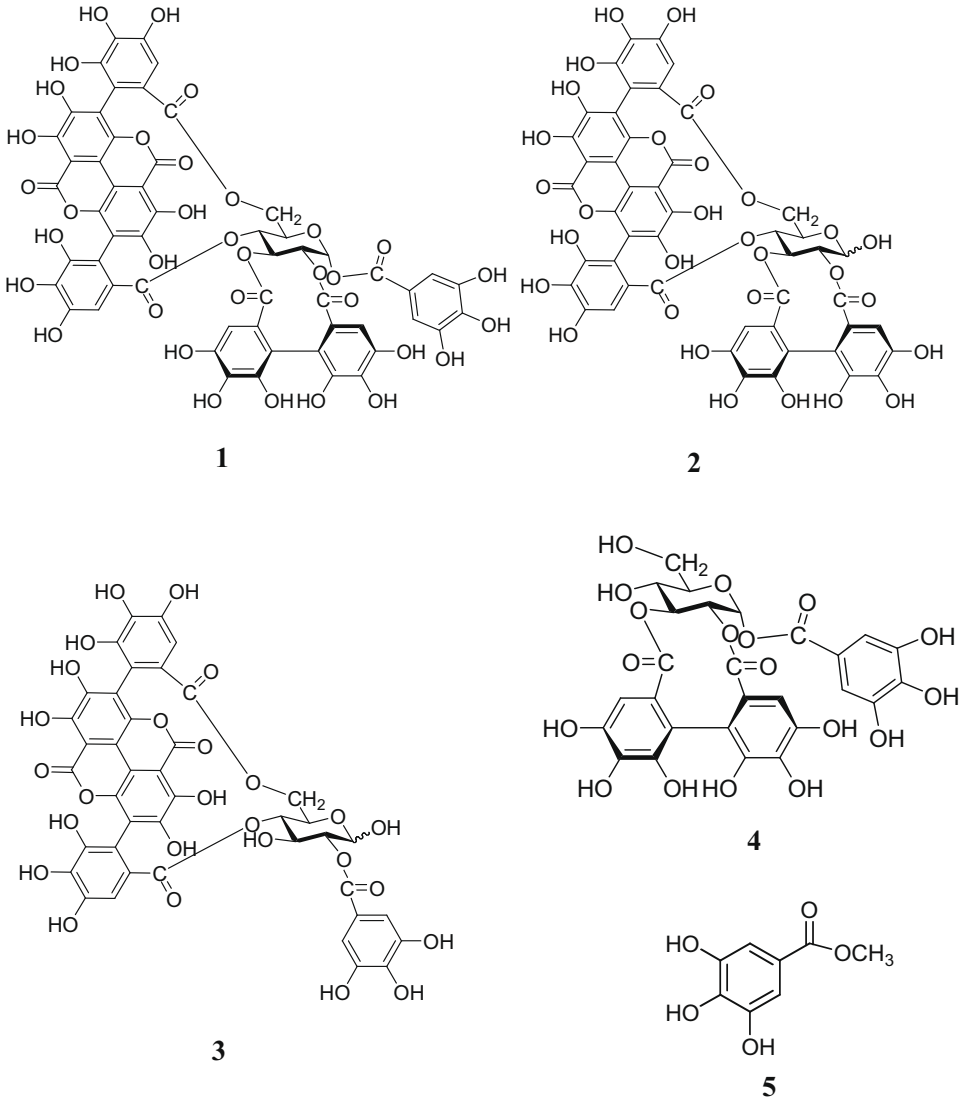

Fig. 1. Chemical structures of 1-α-*O*-galloylpunicalagin (1), punicalagin (2), 2-*O*-galloylpunicalin (3), sanguiin H-4 (4) and methyl gallate (5) of *Terminalia calamansanai*.

Table 1
IC_{50} values and CI of four ellagitannins in various tumor cell lines of the adhesion-type after 24 h treatment.

Compounds	CI (%)[a]					IC_{50} (μM)		
	AGS	HeLa	Hep G2	HT 29	T 24	HeLa	Hep G2	T 24
1-α-*O*-Galloylpunicalagin	9.34 ± 6.68	23.13 ± 5.36	15.38 ± 6.69	16.10 ± 10.88	22.61 ± 13.02	141.08	–	–
Punicalagin	14.69 ± 1.89	26.60 ± 1.80	25.54 ± 8.41	14.56 ± 3.91	28.92 ± 8.04	130.04	–	–
2-*O*-Galloylpunicalin	20.28 ± 5.20	37.30 ± 1.34	15.20 ± 1.36	5.54 ± 4.92	48.38 ± 3.07	152.20	–	106.68
Sanguiin H-4	2.69 ± 2.44	24.34 ± 4.73	38.99 ± 2.19	8.10 ± 6.37	80.58 ± 5.98	142.64	152.65	61.25
ADR[b]	71.52 ± 3.53	72.04 ± 5.30	52.02 ± 3.48	51.07 ± 5.28	69.68 ± 3.28	0.30	1.02	0.33

Data were calculated from three separate experiments.
[a] CI values of tumor cells were after treatment with ellagitannins at 100 μM.
[b] ADR values of tumor cells were after treatment with adriamycin (ADR) at 1.72 μM.

analysis was performed using the student's *t*-test. *p*-Values < 0.05 were considered significant.

3. Results

3.1. Five polyphenols from T. calamansanai

Five main polyphenols were identified from 70% acetone extracts of dried leaves of *T. calamansanai*, using column chromatography, and included four ellagitannins, 1-α-*O*-galloylpunicalagin, punicalagin, 2-*O*-galloylpunicalin, sanguiin H-4, and methyl gallate (Fig. 1). Among these, 2-*O*-galloylpunicalin and sanguiin H-4 were the first time isolated for this species.

In the structural analysis of these ellagitannins, 1-α-*O*-galloylpunicalagin, punicalagin, and 2-*O*-galloylpunicalin consisted of the same tetragalloyl (gallagyl) ester group that is attached to the glucopyranose C_4, C_6-positions, and lacked an acyl group at the anomeric center of hydrolyzable tannins, as determined by examining the duplicated peaks in a reversed-phase HPLC chromatogram (data not shown). These chromatographic phenomena and ^1H and ^{13}C NMR spectra characters were consistent with previous reports (Hatano et al., 1988; Tanaka et al., 1986). In the presence of the gallagyl group, an increase in the content of α-glucopyranose anomer revealed a peak area ratio of ^1H NMR of α- and β-anomer of about 2:1 and 3:1 for punicalagin and 2-*O*-galloylpunicalin, respectively. Due to the low concentration and signal overlap with α-anomer, the NMR assign of the β-anomer could not be resolved.

3.2. Inhibition of cell growth

The five adhesion-type carcinoma cell lines (AGS, HeLa, Hep G2, HT 29 and T 24) each responded differently to the five polyphenols from *T. calamansanai* at 100 μM for 24 h, and the four ellagitannins exhibited higher cytotoxic effects than methyl gallate (Table 1). Therefore, the methyl gallate data is not shown and was not analyzed in the other experiments. Sanguiin H-4 (**4**) with the IC_{50} value of 61.25 μM caused more cytotoxicity in the T24 cells. In general, T24 cells were more sensitive to all ellagitannins and showed more severe cytotoxicity than other cells (Table 1). In contrast to PBMCs, the CI% values of 1-α-*O*-galloylpunicalagin, punicalagin, 2-*O*-galloylpunicalin, and sanguiin H-4 were higher for HL-60 cells (Table 2), suggesting that HL-60 cells were more susceptible to these four ellagitannins. In addition, the antiproliferative effects of 2-*O*-galloylpunicalin and sanguiin H-4 were more pronounced in the leukemia HL-60 cells than in the normal PBMCs (Fig. 2).

3.3. Ellagitannin-inducing apoptosis

To characterize the factors that influence the cell death of HL-60 cells, several apoptotic bodies were observed, firstly in HL-60 cells treated with 50 μM of 2-*O*-galloylpunicalin for 12 h (data not shown). Further, DNA fragmentation shown in agarose gel electrophoresis revealed an increase of DNA fragmentation in HL-60 cells treated with 100 μM of 2-*O*-galloylpunicalin and sanguiin H-4, respectively, for 12 h (Fig. 3A and C) and flow cytometric analysis (Fig. 3B and D). Apoptotic cells with degraded DNA were mostly located below the G_1 peak in the DNA histogram (M1) (Fig. 3B and D). In the apoptotic process, a catalytically active band of intact PARP at 116 kDa is cleaved to form an active 85-kDa PARP. Treatment of HL-60 cells with 1-α-*O*-galloylpunicalagin, punicalagin, 2-*O*-galloylpunicalin and sanguiin H-4 for 12 h, respectively, showed a decrease in the 116-kDa PARP and a dose-dependent increase of inactive PARP (Fig. 4).

3.4. Caspase-3 activity assay

Activation of caspase 3 plays a key role in the initiation of apoptosis. Reduction of pro-caspase 3 was also associated with an increasing level of ellagitannin (Fig. 4). Indeed, all four polyphenols significantly activated the expression of caspase 3 ($p < 0.05$) in a dose-dependent manner (Fig. 5). Especially, the efficacy of 2-*O*-galloylpunicalin and sanguiin H-4 was as good as that of adriamycin, a clinical anticancer drug, at a high dose (100 μM). The results suggest an ellagitannin-inducing apoptosis of HL-60 cells *via* caspase-3 activation.

4. Discussion

The results of the present study demonstrate that four ellagitannins, 1-α-*O*-galloylpunicalagin, punicalagin, 2-*O*-galloylpunicalin, and sanguiin H-4, isolated from *T. calamansanai,* exert cytotoxic effects on human promyelocytic leukemia HL-60 cells in a dose-dependent manner. The differential sensitivities of HL-60 cells and PBMCs to cell death by the four ellagitannins were also observed. As shown in Fig. 2, the cytotoxic effects of ellagitannins from *T. calamansanai* were greater on HL-60 cells than in other adhesion-type carcinoma cell lines. Apoptotic bodies were observed

Table 2
IC_{50} values and CI of ellagitannins on HL-60 and PBMCs after 12 h treatment.

Compounds	CI (%)		IC_{50} (μM)
	HL-60[a]	PBMCs[b]	HL-60
1-α-*O*-Galloylpunicalagin	76.8 ± 0.47	20.8 ± 2.02	65.2
Punicalagin	70.7 ± 1.67	39.4 ± 1.13	74.8
2-*O*-Galloylpunicalin	95.2 ± 0.57	39.4 ± 1.10	42.2
Sanguiin H-4	93.0 ± 0.42	45.6 ± 0.30	38.0
ADR[c]	92.9 ± 3.45	–	0.27

Data were calculated from three separate experiments.
[a] CI values of HL-60 cells were after treatment with ellagitannins at 100 μM.
[b] CI values of PBMCs were after treatment with ellagitannins at 400 μM.
[c] CI values of HL-60 cell were after treatment with adriamycin (ADR) at 1.72 μM.

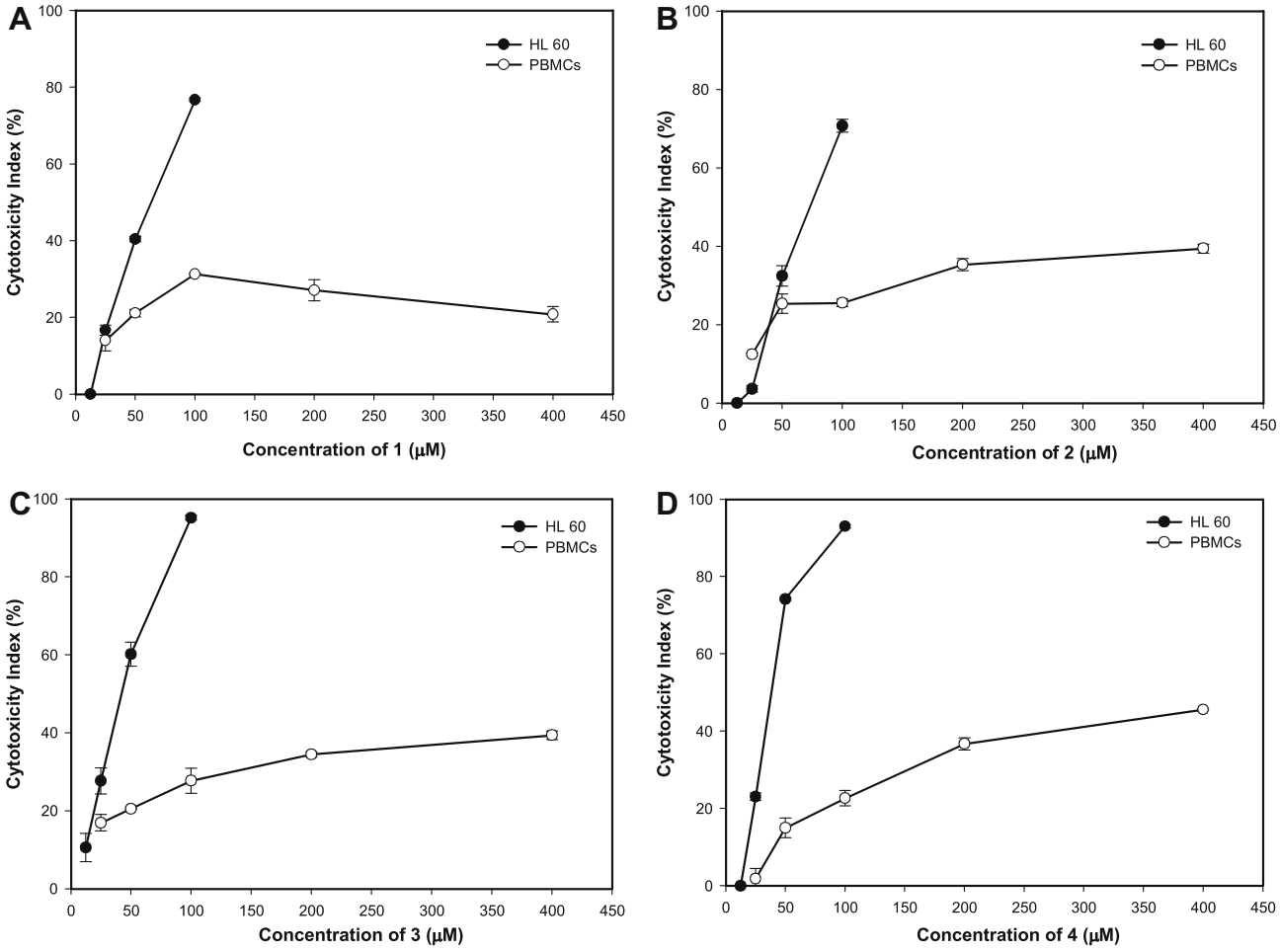

Fig. 2. The cytotoxicity effects of 1-α-*O*-galloylpunicalagin (A), punicalagin (B), 2-*O*-galloylpunicalin (C) and sanguiin H-4 (D) between HL-60 cells and PBMCs treated with serial dilution concentrations for 12 h. Data from three separate experiments were calculated. PBMCs: human peripheral blood mononuclear cells.

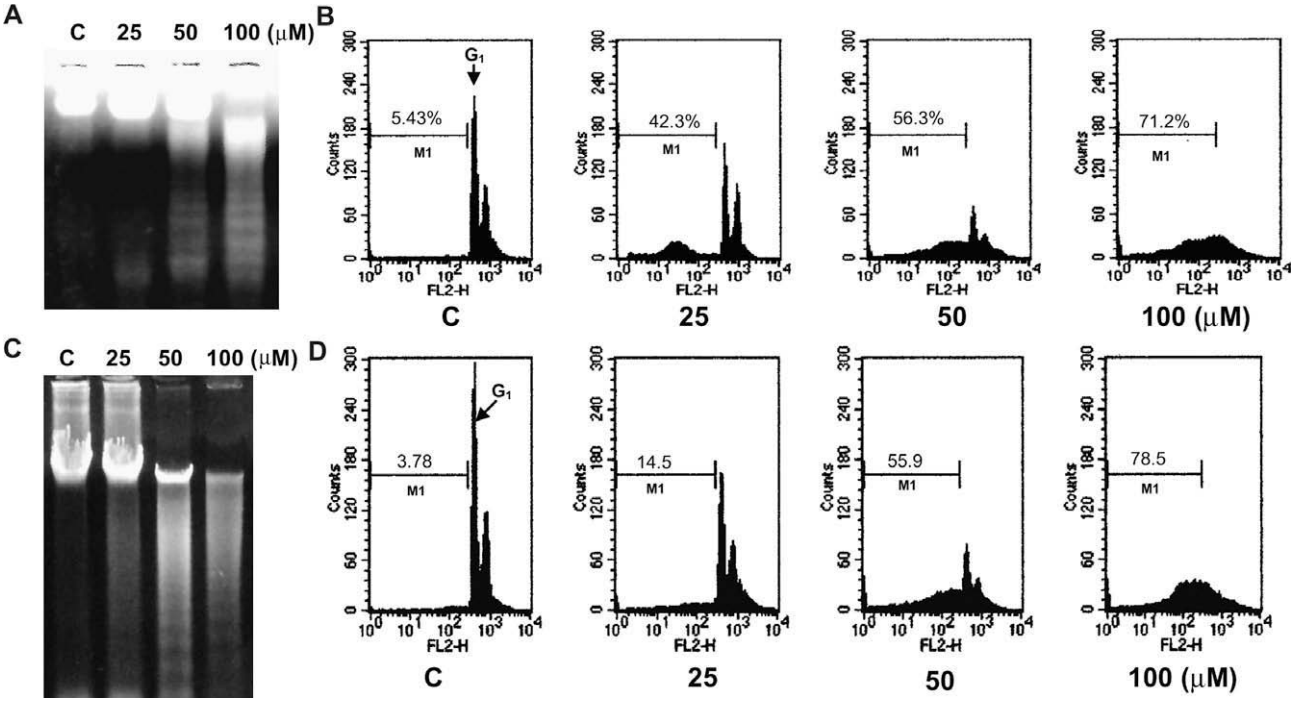

Fig. 3. DNA fragmentation of HL-60 cells after treatment with 2-*O*-galloylpunicalin (A and B) and sanguiin H-4 (C and D) for 12 h. DNA fragmentation was estimated by agarose gel electrophoresis (A and C) and flow cytometric analysis (B and D). The degraded DNA (M1) was estimated below the G_1 peak in the DNA histogram and the percentage of M1 is given in (B). (C) indicated control (H_2O). Data from three separate experiments were calculated.

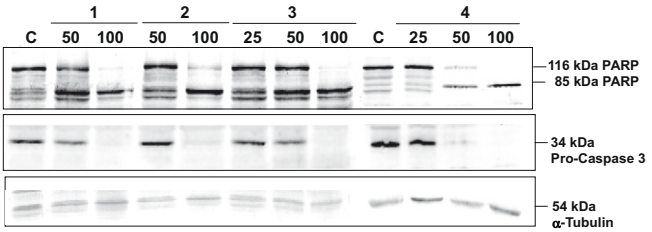

Fig. 4. Western blot analysis of PARP, pro-caspase 3 and α-tubulin proteins in 1-α-*O*-galloylpunicalagin, punicalagin, 2-*O*-galloylpunicalin, and sanguiin H-4 -treated HL-60 cells for 12 h. C means solvent control (H_2O). α-Tubulin was used as an internal control to ensure equal amounts of protein loading in each lane. Data from three separate experiments were used.

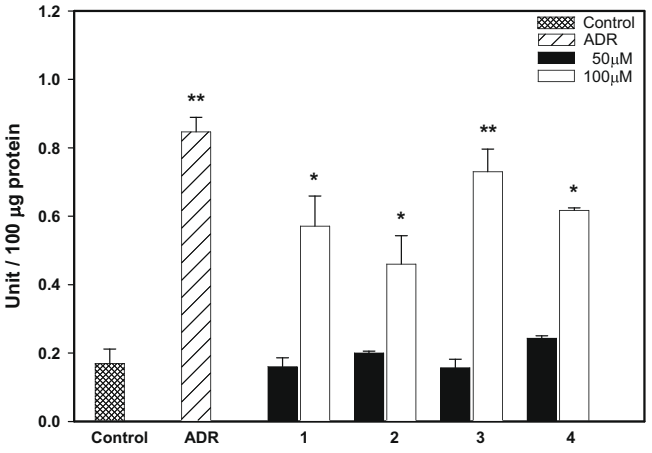

Fig. 5. Significant activation of caspase 3 in HL-60 cells associated with high levels of 1-α-*O*-galloylpunicalagin, punicalagin, 2-*O*-galloylpunicalin (3), and sanguiin H-4 treated for 12 h. C means use of H_2O instead of other compounds. ADR: adriamycin was 1.72 μM as a positive control. Data from three separate experiments were used. $^{*}p < 0.05$; $^{**}p < 0.005$.

in HL-60 cells 12 h after treatment with 25 μM of 2-*O*-galloylpunicalin and sanguiin H-4. These results indicated that 1-α-*O*-galloylpunicalagin, punicalagin, 2-*O*-galloylpunicalin, and sanguiin H-4 could all induce apoptosis on HL-60 cells through a caspase-3 activation pathway.

Several reports have indicated that ellagitannins inhibit the proliferation of cells by inhibiting cell cycle progression and inducing apoptosis. Ellagitannins, such as punicalagin, can metabolize the ellagic acid released by human microflora and colon adenocarcinoma Caco-2 cells (Larrosa et al., 2006). Moreover, ellagic acid has been demonstrated to have antitumor effects in animal models and induced-G1 arrest, induce apoptosis on tumor cells, and be a topoisomerases inhibitor (Narayanan et al., 1999; Constantinou et al., 1995; Mertens-Talcott and Percival, 2005). On the other hand, ellagitannins containing galloyl groups, such as woodfordin I and rugosin E, induced apoptosis through the activation of caspase 3 (Liu et al., 2004; Kuo et al., 2007), and woodfordin C, an ellagitannin, induced tumor cell death and inhibited DNA topoisomerase II (Kuramochi-Motegi et al., 1992). Macrocyclic ellagitannins also showed antitumor effects in S-180 tumor-bearing mice (Wang et al, 1999), and induced apoptosis in tumor cells (Kuramochi-Motegi et al., 1992; Wang et al, 2000). Moreover, gallagic acid that is a hydrolyzed product of 1-α-*O*-punicalagin, and was frequently found in the liver and kidney of rats after oral administration of high doses of the pomegranate ellagitannin (Cerda et al., 2003). Gallic acid also revealed antioxidation activity (Reddy et al., 2007) and cytotoxicity (Sakagami et al., 1995). Therefore, we suggested that ellagic acid and gallic acid hydrolyzed from ellagitannin may be important products of cytotoxicity in tumor cells.

The adhesion-type tumor cell lines were treated with 1-α-*O*-galloylpunicalagin, punicalagin, 2-*O*-galloylpunicalin, and sanguiin H-4, respectively. Only 2-*O*-galloylpunicalin and sanguiin H-4 had stronger cytotoxicity and more sensitivity in T 24 cells. The two compounds both induced DNA fragmentation in HL-60 cells. DNA fragmentation may be due to the proteolytic cleavage of PARP by caspase-3 (Tewari et al., 1995), which results in a reduction of PARPs enzymatic activity (Surh, 1999), and thereby inhibits DNA repair. Our results confirmed that DNA fragmentation of HL-60 cells was due to the proteolytic cleavage of PARP by these two compounds, in a dose-dependent manner (Fig. 4), and active caspase-3 (Fig. 5). The other two compounds, 1-α-*O*-galloylpunicalagin and punicalagin also could inhibit the growth of HL-60 cells and the cleavage of PARP, and induce apoptosis (Figs. 4 and 5). Despite ellagitannins being able to induce cell death, they have several kinds of bioactivities, such as the inhibitory activity of 2-*O*-galloylpunicalin on HIV-1 reverse transcriptase (Martino et al., 2004) and the antioxidant activity of punicalagin (Chen et al., 2000; Gil et al., 2000; Chen and Li, 2004).

In conclusion, our study found that the four ellagitannins in *T. calamansani* leaves could inhibit HL-60 tumor cell population growth and induce apoptosis, and that the potential cytotoxic agents 2-*O*-galloylpunicalin and sanguiin H-4 are suitable for use as novel anticancer agent candidates. Further investigation of the antitumor effects using animal models is being planned.

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