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Apoptosis-Inducing Activity of Galloylglucoses from *Juglans mandshurica* in Human Promyeloid Leukemic HL-60 Cells

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Abstract – Two galloyl monosaccharides, 1,2,6-trigalloylglucose (**1**, TRgG) and 1,2,3,6-tetragalloylglucose (**2**, TEgG), were isolated from the stem-bark of *Juglans mandshurica.* Two galloylglucoses showed cytotoxic effects on human promyelocytic leukemia HL-60 cells. In order to elucidate their mechanism of action, we have investigated the flow cytometric analysis after Annexin V-FITC and PI staining, caspase-3 activity, and internucleosomal DNA fragmentation in HL-60 cells. HL-60 cells treated with both compounds **1** and **2** at 150 and 100 μM, respectively, led to a morphological features of apoptosis, such as plasma membrane blebbing and cell shrinkage. TRgG (**1**) and TEgG (**2**) increased the percentage of $FITC^+$ and $FITC^+PI^+$ cells in flow cytometry after Annexin V-FITC and PI staining. The increase of apoptotic cells was preceded by the activation of caspase-3 reported to play a central role in apoptotic process and inducing internucleosomal DNA fragmentation. TEgG (**2**) showed to have stronger apoptosis inducing activity in HL-60 cell lines as compared with TRgG (**1**).
Keywords – *Juglans mandshurica*, apoptosis, 1,2,6-trigalloylglucose, 1,2,3,6-tetragalloylglucose, HL-60 cells, morphological feature, flow cytometry, caspase-3, DNA fragmentation

Introduction

Several chemotherapeutic compounds have been found to induce apoptosis, and apoptosis may be the primary mechanism for their anticancer, anti-inflammatory, and anti-virus activity (Gunji *et al.*, 1991; Simon and Blaser, 1995; Uchide *et al.*, 2002). Recently plant compounds were widely studied the abilities to induce apoptosis of cancer cells because of their low toxicity and great medicinal value (Cai *et al.*, 2002). Apoptosis is an active process of cell death and plays important roles in maintaining homeostasis in multicellular organism. It was originally defined in terms of characteristic structural changes, such as a reduction in cell volume, condensation of chromatin in the nucleus, the digestion of chromatin into fragments of DNA by an endogenous endonuclease, and a relatively high degree of preservation of the plasma membrane and cytoplasmic organelles (Masuda *et al.*, 1997). Caspases make up of a family of cysteine proteases that cleave substrates after aspartic residue, and play important roles in apoptosis. Caspases family members are expressed as proenzymes, which are cleaved to be activated during the apoptotic process. There are two major apoptotic pathways in mammalian cells, such as the death domain receptor and the mitochondrial pathways. Caspases-8 and -9 are activated in these two pathways respectively and converge at the level of caspase-3 activation (Cai *et al.*, 2002; Ikeda and Nagase, 2002).

Juglans mandshurica Maximowicz (Juglandaceae) grows in Northeast Asia (Bae, 2000). Its roots are used as a folk medicine for treatment of cancer in Korea (Son, 1995). A literature survey showed that this plant was found to be rich in phenolic compounds, i.e. naphthoquinones, naphthalenyl glucosides, α-tetralonyl glucopyranosides, diarylheptanoyl glucopyranosides, and flavonoids, and these compounds have been shown to have cytotoxic activity against human cancer cell lines (Joe and Son, 1996; Kim *et al.*, 1998; Lee *et al.*, 2000), inhibitory effects on DNA polymerase and RNase H activities of HIV-1 reverse transcriptase (Min *et al.*, 2000), and Anti-HIV-1 activity in MT-4 cells (Min *et al.*, 2002).

In the present study, the apoptosis-inducing activities of two galloyl glucoses, 1,2,6-trigalloyl glucose (**1**, TRgG) and 1,2,3,6-tetragalloyl glucose (**2**, TEgG) isolated from

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J. mandshurica, were investigated in human leukemia HL-60 cells.

Materials and Methods

Chemicals – RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco Laboratories. Ac-Asp-Glu-Val-Asp 7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC) was obtained from Enzyme Systems Products (Livermore, CA, U.S.A.). Annexin V-FITC was purchased from BD Biosciences (San Diego, CA, U.S.A.). Camptothecin, penicillin, streptomycin, trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), propidium iodide (PI), and HEPES were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Plant Material – The stem-bark of *J. mandshurica* was collected during September 1998 at a mountain area of Kimchun, Kyungbook, Korea, and dried at room temperature for 3 weeks. A voucher specimen is deposited at the herbarium of the College of Pharmacy, Chungnam National University, Korea.

Extraction and isolation – Stem-bark of *J. mandshurica* (3.0 kg) was extracted with MeOH at room temperature for 24 h to give a dark-brown extract (390 g). The MeOH extract (300 g) was suspended in H_2O (2500 ml) and extracted with hexane (2500 × 3) to give a hexane-soluble fraction (48 g). The resulting H_2O layer was extracted with CH_2Cl_2 (3000 ml × 3), EtOAc (3000 ml × 3), and BuOH (3000 ml × 3), successively. The EtOAc-soluble fraction (90 g) was chromatographed on a column of silica gel (1 kg). The column was eluted using a gradient of $CHCl_3$, MeOH, and H_2O to give six fractions (Fr. A–F: 2.7 g, 15.9 g, 23.9 g, 7.5 g, 5.4 g, and 10.2 g, respectively). Repeated column chromatography of Fr. C on Sephadex LH-20 (MeOH and $CHCl_3$-MeOH, 1:9), silica gel ($CHCl_3$-MeOH, 8:2), and ODS column (40 % aq. MeOH), followed by MPLC on RP-18 (40% aq. MeOH and 80% aq. CH_3CN) afforded compounds **1** and **2** (Fig. 1) (Min *et al.*, 2000).

1,2,6-Trigalloylglucose (1, TRgG) – White amorphous powder, $[\alpha]_D$ −94° (*c* 0.1, MeOH). IR ν_{max} cm^{-1}: 3420, 1710, 1610, 1540, 1525, 1450, 1355. UV λ_{max} nm (log ε): 216 (4.6), 278 (4.4).

1,2,3,6-Tetragalloyl glucose (2, TEgG) – White amorphous powder, $[\alpha]_D$ +39° (*c* 0.1, MeOH). IR ν_{max} cm^{-1}: 3400, 1700, 1610, 1540, 1455, 1355. UV λ_{max} nm (log ε): 216 (4.9), 278 (4.5).

Cell Culture – Human promyelocytic leukemia HL-60 cells were obtained from American Type Cell Culture (ATCC). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. The cell culture was maintained at 37°C in 5% CO_2 humidified incubator.

Cell Viability – Cell viability was processed by the standard MTT techniques. Cells were seeded to a concentrations of $5{\times}10^5$ cells/ml. Then compounds **1** and **2** were added to the suspension. Two hundreds μl of these cells suspensions were then transferred to 96 well microplate. Twenty μl MTT (0.01 M) was added to every well. After 4 h incubation (37°C) and 5 min centrifugation (3000 rpm), the resulting formazan precipitate was dissolved with 200 μl DMSO and the absorption was measured at 570 nm on a microplate reader (Hercules, CA, U.S.A.) (Wang *et al.*, 2001). The growth inhibition was determined using;

Growth inhibition = (control's OD – samples OD)/controls OD

Morphological Assessment of Apoptosis – Cells were treated with 150 μM TRgG (**1**) or 100 μM TEgG (**2**) for 3 h, 6 h, 12 h, 24 h, or 48 h. The cell morphology was defined under a light microscope (CETI Versus, Belgium).

Flow cytometric analysis – For flow cytometric analysis, $2{\times}10^5$ HL-60 cells in exponential growth were treated with test compounds (**1** and **2**) at time course, and then washed

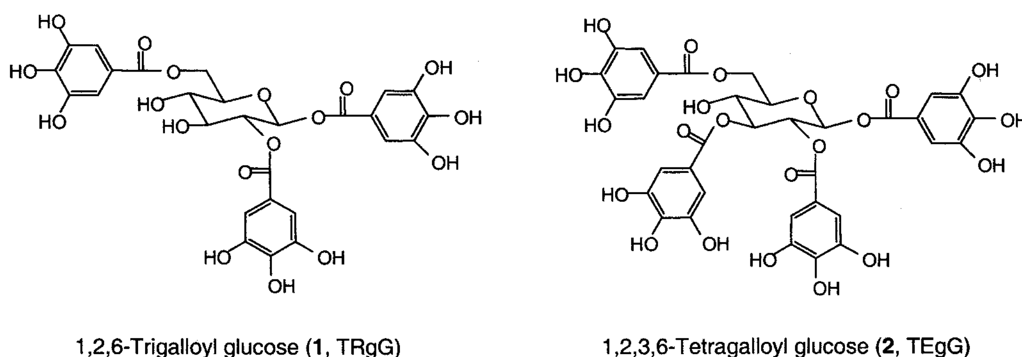

Fig. 1. Structures of 1,2,6-Trigalloylglucose (**1**, TRgG) and 1,2,3,6-Tetragalloylglucose (**2**, TEgG) Isolated from the Stem-bark of *J. mandshurica*.

twice with PBS. The cell pellet was re-suspended with Annexin binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$; pH 7.4) and stained with 5 μl of Annexin V-FITC and 10 μl of propidium iodide (PI) at room temperature for 15 min in the dark. The reaction mixture was measured by FACScalibur (BDscience, CA, U.S.A.) and stained cells analyzed by CellQuest (Lansiaux *et al.*, 2002).

Assay of caspase-3 activity – Caspase-3 like enzyme activity was measured by proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AFC counting a microplate fluorometer (Berthold, Bad Wildbad, Germany). After incubation with 150 μM TRgG (**1**) or 100 μM TEgG (**2**) for 3 h, 6 h, 12 h, 24 h, or 48 h, cells were harvested and washed once with cold PBS. The pellets were lysed using 15 μl of lysis buffer containing 10 mM EDTA, 0.5% Triton X-100, and 10 mM Tris-HCl (pH 8.0) at room temperature for 10 min, and then added 100 μl of assay buffer (100 mM HEPES; pH 7.5, 10 mM dithiothreitol, 10% sucrose, 0.1% CHAPS, 0.1% BSA). Fluoroscene at 390 nm (excitation) and 505 nm (emission) was measured after incubation at 37°C for 1 h (McFarlane *et al.*, 1997).

DNA fragmentation – After incubation with 150 μM TRgG (**1**) or 100 μM TEgG (**2**) at time course, cells were harvested and washed with PBS. Apoptotic DNA was purified using Apoptotic DNA ladder kit (Roche, Mannleim, Germany). Then cell pellets were lysed in 200 μl of lysis buffer (10 mM Tris-HCl; pH 8.0, 10 mM Urea, 6 M guanidine-HCl, and 20% Triton X-100; pH 4.4) at room temperature for 30 min. The lysate flowed to the filter and washed the filter with 4 mM NaCl, 0.4 mM Tris-HCl (pH 7.5), and 80% EtOH. The DNA binded at the filter was eluted with 200 μl elution buffer (10 nM Tris-HCl, pH 8.5). The isolated DNA fragments were separated by 1% agarose gel containing 0.5 μg/ml of ethidium bromide and analyzed under ultraviolet illuminator (Vogelstein & Gillespie, 1979).

Results and Discussion

We isolated two galloyl glucoses, 1,2,6-trigalloylglucose (**1**, TRgG) and 1,2,3,6-tetragalloylglucose (**2**, TEgG), from the stem-bark of *J. mandshurica* (Fig. 1). TRgG (**1**) and TEgG (**2**) showed potent inhibitory activities against DNA polymerase activity of HIV-1 RT with IC_{50} values of 67 and 40 nM, as well as inhibition of RNase H activity in this enzyme with IC_{50} values of 310 and 39 μM, respectively (Min *et al.*, 2000). TEgG (**2**) also exhibited cytotoxic activity against MT-4 cells (Min *et al.*, 2002). Accordingly, orally administered green tea and (–)-epigallocatechin gallate, a major constituent of green tea, reported to have some antitumor activity. Green tea inhibited *in vivo* metastasis and *in vitro* invasion of mouse Lewis lung carcinoma LL2-Lu3. These results suggested that a polyphenolic structure with a galloyl group might be involved in antitumor activity (Saeki *et al.*, 1999). This study detected the apoptotic effect of two compounds from the stem-bark of *J. mandsurica* in HL-60 cells and studies the pathway involved in apoptosis.

To evaluate the apoptotic activity of TRgG (**1**) and TEgG (**2**), we examined several effects to detect the apoptotic features in HL-60 cells. The proliferation of HL-60 cells treated with 150 and 200 μM TRgG, or 100 and 150 μM TEgG showed a time-dependent inhibitory effect (Fig. 2). The IC_{50} values of TRgG (1) and TEgG (2) against human HL-60 leukemia cells were 155 and 134 μM, respectively. As shown in Fig. 3, cells exhibited typical morphological changes of apoptosis after treatment with two compounds. The cells shrank and had a relative smaller volume than control cells. Flow cytometric analysis of phosphatidylserine (PS) exposure by Annexin V staining quantified the apoptotic cells. Concomitant staining with PI identified cells with loss of membrane integrity that is characteristic of late apoptosis (Vermes *et al.*, 1995). However, a small percentage below

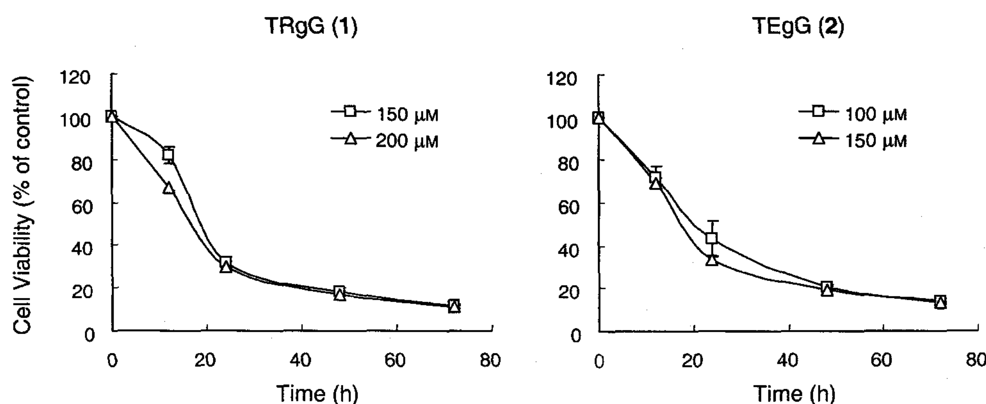

Fig. 2. Cell Viability of TRgG (**1**) and TEgG (**2**) in HL-60 Cells Measured by MTT Assay. Data points represent the mean values of six replications with bars indicating SD.

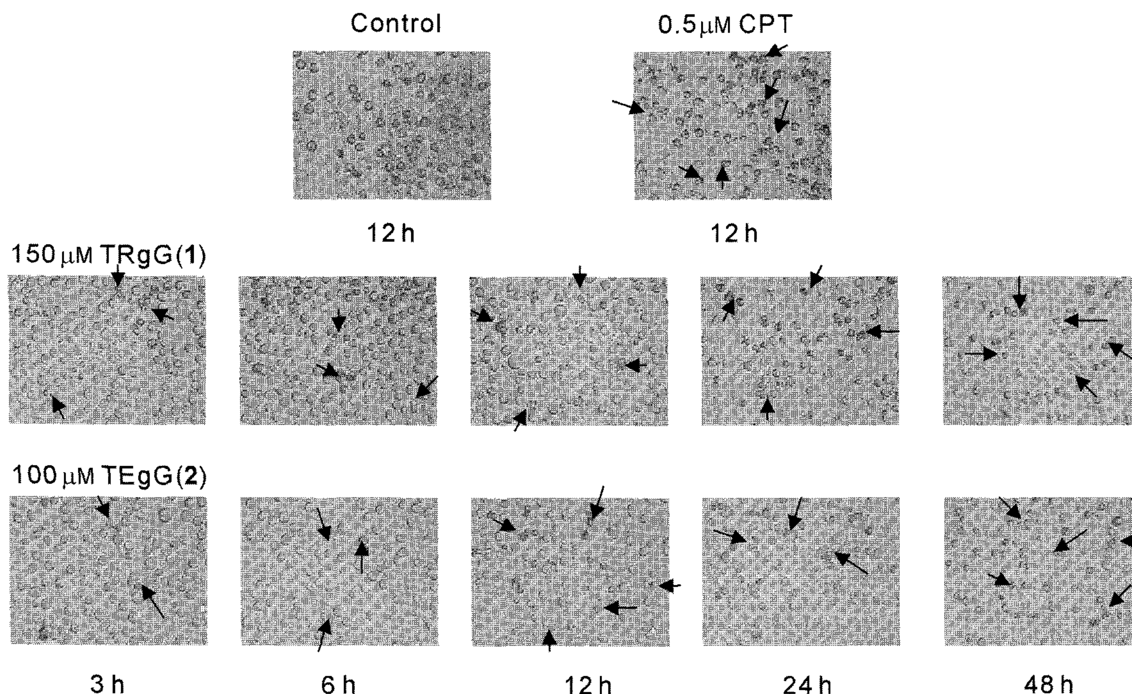

Fig. 3. Microscope Observation of Morphological Change in HL-60 Cells. 0.5 μM Camptothecin and control cells incubated for 12 h, cells were treated with 150 μM TRgG (**1**) or 100 μM TEgG (**2**) cultured for 3 h to 48 h.

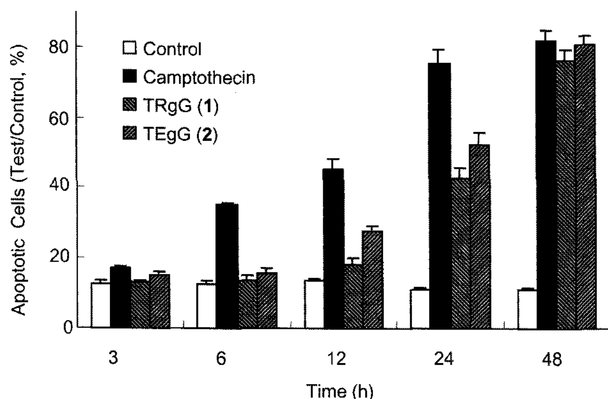

Fig. 4. Flow Cytometric Analysis of Apoptosis in HL-60 Cells. Cells were treated with 150 μM TRgG or 100 μM TEgG for up to 48 h, analyzed by CellQuest software. Data points represent the mean values of three replications with bars indicating SD.

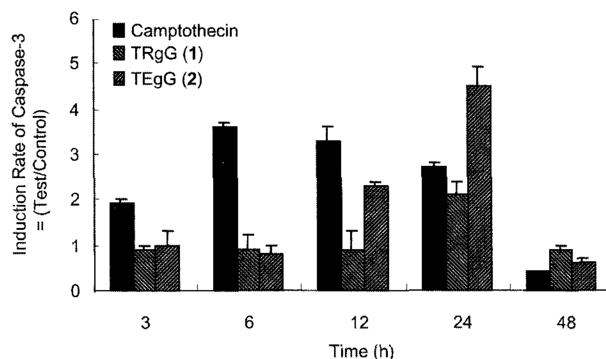

Fig. 5. Effect of TRgG (**1**) and TEgG (**2**) on Caspase-3 Activation. HL-60 cells were treated with 150 μM TRgG or 100 μM TEgG for 3, 6, 12, 24, or 48 h, then collected and washed with PBS. Cell lysate was incubated at 37°C with Ac-DEVD-AFC for 1 h. After incubation, the fluorescence intensity was measured. Data points represent the mean values of six replications with bars indicating SD.

5% of cells showed necrotic character stained by PI only at 48 h. This fact demonstrated that TRgG (**1**) and TEgG (**2**) had potential to introduce apoptosis in HL-60 cells (Fig. 4).

Another evidence of apoptosis was provided by activation of caspase-3, a central executioner of apoptosis in many mammalian cells, in HL-60 cells after being treated with TRgG (**1**) or TEgG (**2**) (Fig. 5). The intracellular caspase-3 activity at 150 μM TRgG or 100 μM TEgG increased linearly to a level approximately 2.1 or 4.5-fold at 24 h that of non-treated control. Furthermore, the biological hallmark of apoptosis is the DNA fragmentation, generating the typical DNA ladder pattern on DNA electrophoresis (Fig. 6). DNA ladder was observed in a time-dependently at 150 μM TRgG (**1**) or 100 μM TEgG (**2**).

Our study found that TRgG (**1**) and TEgG (**2**) induced apoptotic cell death with the activation of caspase-3 and suppressed tumor cell growth *in vitro*. TRgG (**1**) showed a mild apoptotic activity in HL-60 cells, whereas TEgG (**2**) confirmed to have stronger apoptosis inducing activity in this cell lines as compared with TRgG (**1**). That is, TEgG

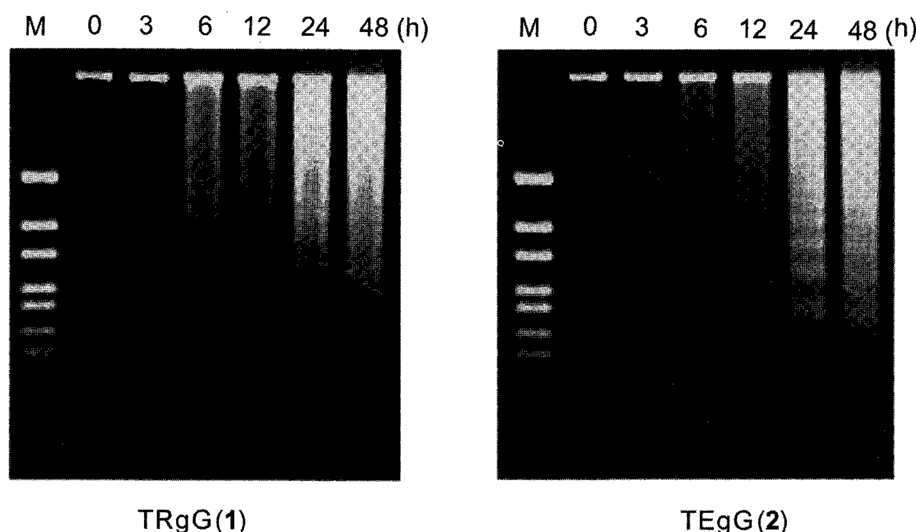

Fig. 6. DNA Fragmentation Induced by TRgG (**1**) and TEgG (**2**). HL-60 cells were treated with TRgG (1) or TEgG (2) for 0, 3, 6, 12, 24, or 48 h and then collected. DNA fragmentation of treated cells was detected 1% agarose gel electrophoresis. M is a DNA size marker.

(**1**) was showed to inhibit cell proliferation at a lower concentration that TRgG (**2**), which has a relatively more potent antitumor activity (Nishida *et al.*, 2003). This result was consistent with data of Saeki *et al.* (1999), which described tetragalloyl glucose induced strongly apoptosis as found by chromatin condensation, DNA ladder formation and inhibition by a caspase inhibitor, while monogalloyl glucose was only marginally active. Therefore, the number of their phenolic groups is important for apoptosis induction. Based on the experimental results, TEgG (**2**) and TRgG (**1**) had an apoptotic activity in human leukemia cells as well, it may be considering as a good candidate for a cancer chemopreventive agent, furthermore other mechanism studies are needed to evaluate the respective apoptotic activities of TRgG (**1**) and TEgG (**2**).

Acknowledgments

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