# Induction of Apoptosis by Xanthones from Mangosteen in Human Leukemia Cell Lines

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We examined the effects of six xanthones from the pericarps of mangosteen, *Garcinia mangostana*, on the cell growth inhibition of human leukemia cell line HL60. All xanthones displayed growth inhibitory effects. Among them, α-mangostin showed complete inhibition at 10 $\mu$M through the induction of apoptosis.

The pericarps of mangosteen, *Garcinia mangostana* Linn., have been used as a traditional medicine for treatment of skin infection and wounds in Southeast Asia for many years. Phytochemical studies have shown that it contains a variety of secondary metabolites, such as oxygenated and prenylated xanthones.[1,2] These xanthones have demonstrated various biological activities, such as antibacterial,[3] antiinflammatory,[4] and antifungal.[5] These observations led to further investigation of the biological activity of the compounds. In the present study, we examined the in vitro cytotoxic effects of xanthones isolated from the pericarps of mangosteen.

The chemical structures of six xanthones isolated from the pericarps of mangosteen are illustrated in Figure 1A. These xanthones were obtained from the benzene extract, as previously described.[1] We examined in vitro cytotoxic effects of these xanthones in HL60 cells at 72 h after the start of treatment of concentrations from 5 to 40 $\mu$M (Figure 1B). Although all xanthones exhibited significant growth inhibition, α-, β-, and γ-mangostin were particularly effective even at the low dose of 10 $\mu$M. The 50% inhibitory concentration ($IC_{50}$) values in HL60 cells were calculated from Figure 1B and are shown in Table 1. Since α-mangostin, a major component of the extract,[1] showed the strongest activity (complete inhibition at 10 $\mu$M), we further examined the cell growth inhibitory activity of α-mangostin against other leukemia cell lines, namely, K562, NB4, and U937. Figure 2 shows cell growth curves at the various concentrations of α-mangostin. α-Mangostin showed no significant effect on the cell growth at concentrations lower than 2 $\mu$M, but was inhibitory to all cell lines at 5 $\mu$M. A 10 $\mu$M α-mangostin concentration markedly inhibited growth of all cell lines tested, especially HL60, NB4, and U937, with complete suppression of growth at 72 h after the treatment (Figure 2A,C,D). Among the cell lines tested, K562 cells seemed to be most resistant to α-mangostin (Figure 2B). Thus, α-mangostin exhibited strong growth inhibition against leukemia cell lines at concentrations of 5–10 $\mu$M. Furthermore, α-mangostin appears to preferentially inhibit leukemia cells, since no cytotoxic effect (at 10 $\mu$M) was observed against concanavalin-A-stimulated normal peripheral blood lymphocytes (Figure 3).
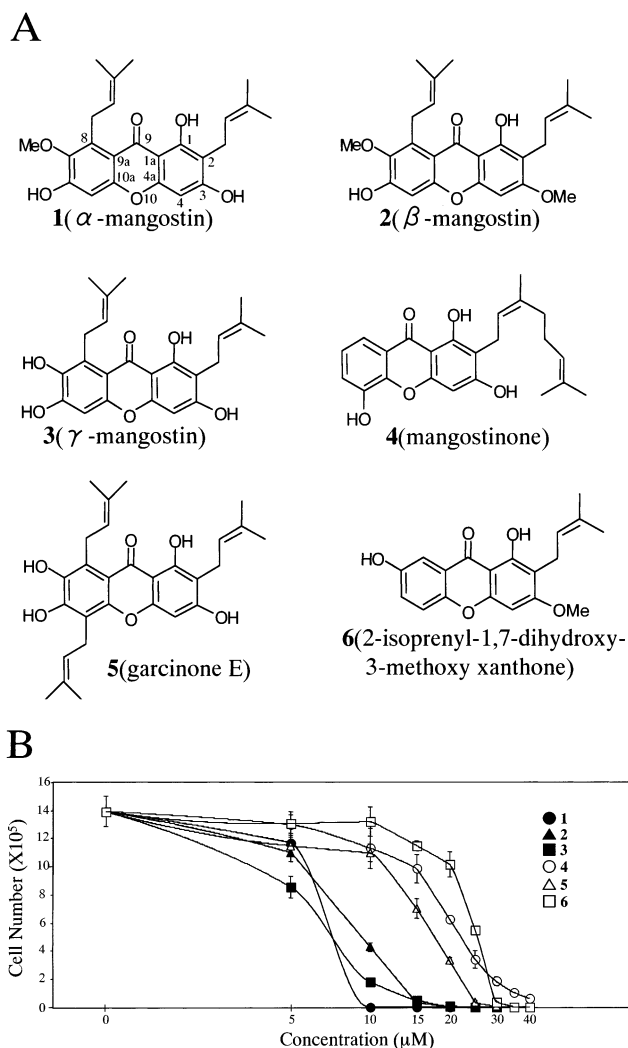
A



B



**Figure 1.** Chemical structure of xanthones (A) and effects of the xanthones on concentration-dependent cell growth inhibition in HL60 cells at 72 h after treatment (B). Starting cell number is $1 \times 10^5$/mL. Data are expressed as means ± SD of three different experiments.

To examine the participation of apoptosis in the growth inhibition, we have carried out Hoechst 33342 nuclear staining and nucleosomal DNA gel electrophoresis at 24 h after the treatment with 10 $\mu$M α-mangostin. As shown in Figure 4A–D, Hoechst 33342 nuclear staining revealed

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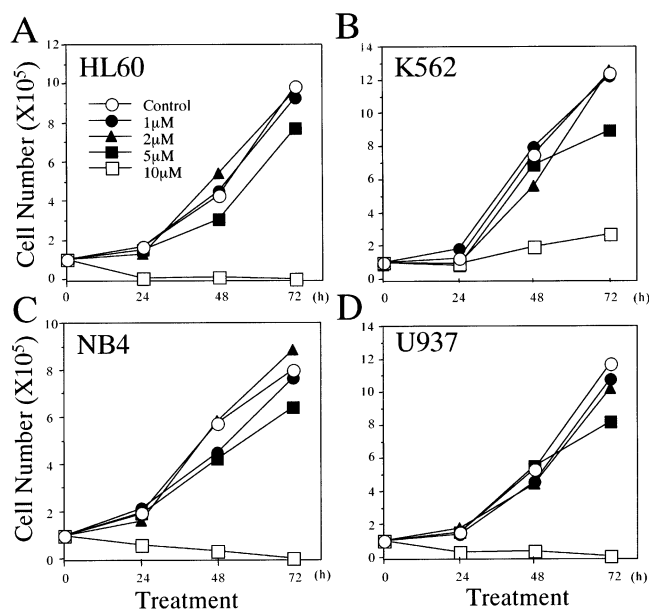[†] Gifu International Institute of Biotechnology.
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**Table 1.** $IC_{50}$ Values of Xanthones from Mangosteen

| | α-mangostin | β-mangostin | γ-mangostin | mangostinone | garcinone E | 2-isoprenyl-1,4-dihydroxy-3-methoxyxanthone |
|---|---|---|---|---|---|---|
| $IC_{50}$ (μM) | 6.8 | 7.6 | 6.1 | 19.0 | 15.0 | 23.6 |



**Figure 2.** Effect of α-mangostin on cell growth of human leukemia cell lines HL60 (A), K562 (B), NB4 (C), and U937 (D): ○, control; ●, 1 μM; ■, 2 μM; ▲, 5 μM; □, 10 μM.
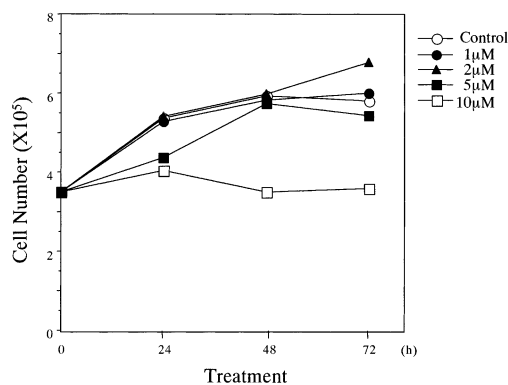


**Figure 3.** Effect of α-mangostin on concanavalin-A-stimulated normal peripheral lymphocytes: ○, control; ●, 1 μM; ■, 2 μM; ▲, 5 μM; □, 10 μM.

nuclear condensation and fragmentation, and DNA ladder formation was observed in all the cell lines, suggesting that growth inhibition by α-mangostin in the leukemia cell lines was due to apoptosis.

Apoptosis of tumor cells can be triggered by a diversity of extracellular and intracellular factors including cytokines, tumor suppressor genes, oncogenes, radiation, and anticancer drugs.[6] Apoptotic signal transduction and execution require activation of a series of caspases (cysteinyl aspartate-specific protease).[7] Caspases are all expressed as proenzymes that contain three domains, an $NH_2$-terminal domain, a large subunit, and small subunit. Their activation involves proteolytic processing between domains. Caspase-3 has been considered as the principal effector caspase in the apoptotic process. Its activated form cleaves numerous substrates to cause the characteristics of apoptosis, such as DNA ladder formation[8] and chromatin condensation.[9] To assess caspase-3 activation in α-mangostin-treated HL60 cells, we carried out Western blot analysis in the time course after treatment with 10 μM α-mangostin. Figure 5A shows that the nontreated control

sample expresses only the pro-caspase-3 (32 kD), but the active forms of caspase-3 (19 kD: $NH_2$-terminal domain and large subunit complex, and 17 kD: large subunit) are discernible after 3 h of treatment. This result indicated that α-mangostin triggers apoptotic signaling earlier than 3 h. On the other hand, DNA ladder formation was also detected at 3 h and was distinct at 6 h after treatment (Figure 5B). These data demonstrate the caspase-3 activation to be concurrent with DNA ladder formation in α-mangostin-induced apoptosis and also suggest that this apoptotic signaling proceeds rapidly. There are some reports describing the in vitro biological activities of α-mangostin related to apoptosis, such as the inhibitory effect of topoisomerases,[10] acidic sphingomyelinase,[11,12] and $Ca^{2+}$-ATPase.[13] These observations should help to disclose the mechanism underlying α-mangostin-induced apoptosis, because these enzymes relate to apoptotic signaling.[14–17] We are currently investigating the detailed signaling pathway in the in vitro system using cultured cells.

In summary, the present study showed that the pericarps of mangosteen contain a variety of xanthones which exhibit antiproliferative activity against human leukemia HL60 cells and α-mangostin-induced apoptosis in leukemia cell lines.

## Experimental Section

**Reagents.** Pericarps of *Garcinia mangostana* Linn. were collected in Indonesia in April 1993 by one of the coauthors (I.M.), and a voucher specimen is deposited in Gifu Pharmaceutical University. Dried and ground pericarps (2.7 kg) of *G. mangostana* was extracted successively with *n*-hexane (5l × 3), benzene (5l × 3), acetone (5l × 3), and 70% methanol (5l × 3) under reflux. A series of xanthones (α-mangostin, β-mangostin, γ-mangostin, mangostinone, garcinone E, and 2-isoprenyl-1,7-dihydroxy-3-methoxyxanthone) were obtained from the benzene extract after purification by recrystallization and chromatography on silica gel and Sephadex LH-20 (Amersham Bioscience, Piscataway, NJ). Their structures were determined in our previous study.[1]

**Cell Culture and Treatments.** Human leukemia cell lines, HL60, K562, NB4, and U937, provided by RIKEN Cell Bank (Tsukuba, Ibaraki, Japan), and human peripheral blood lymphocytes from healthy donors were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum. Human peripheral blood lymphocytes were stimulated with concanavalin-A (75 μg/mL) (Sigma, St. Louis, MO) for 48 h and used for a growth suppression study. Six xanthone samples were dissolved in DMSO and added to the cell cultures with a final DMSO concentration of 0.1% v/v. This concentration of DMSO had no significant effect on the growth and differentiation of all leukemia cell lines tested (data not shown). Viable cell number was measured by trypanblue dye exclusion test using a Bürker-Türk type cell count chamber.

**Morphological Assay.** For morphological examination of apoptotic changes, cells were stained with Hoechst 33342 (Calbiochem, San Diego, CA). Hoechst 33342 was added to cultured medium at a concentration of 5 μg/mL. After incubation for 30 min, cells were collected and washed with phosphate-buffered saline (PBS) and then observed under a fluorescence microscope, Olympus BX-50 (Olympus, Tokyo, Japan).

**DNA Extraction and Agarose Gel Electrophoresis.** Cultured cells treated with 10 μM α-mangostin or untreated were collected and washed with PBS. Cells were incubated at 37 °C overnight with 100 mM Tris-HCl (pH 7.4), 5 mM EDTA, 200 mM NaCl, 0.2% SDS, and 200 μg/mL proteinase K
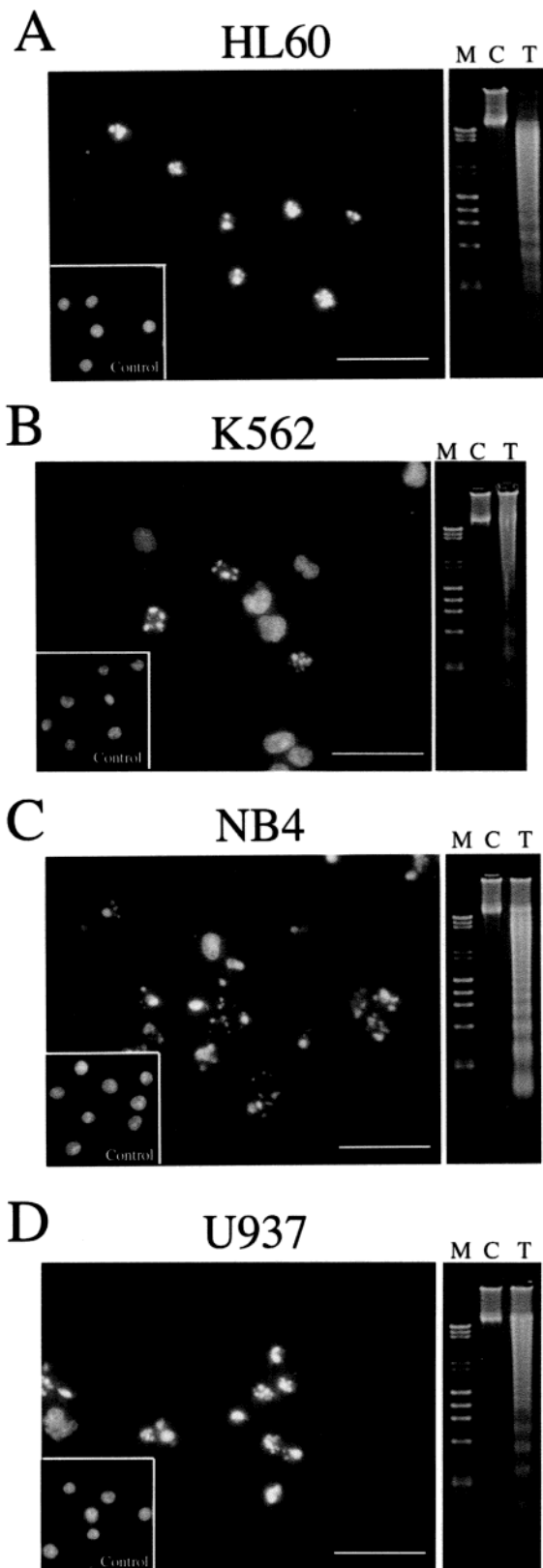
**Figure 4.** α-Mangostin-induced apoptosis in human leukemia cell lines HL60 (A), K562 (B), NB4 (C), and U937 (D) treated at 10 μM for 24 h. Morphological examination was performed by Hoechst 33342 staining (× 400, bar = 50 μm). The films of untreated control cells were inserted into the left corners in contrast with treated cells. Nucleosomal DNA fragmentation detected by agarose gel electrophoresis in each cell line was also demonstrated (M, DNA size marker; C, control; T, α-mangostin-treated cells).

(Takara, Ohtsu, Shiga, Japan) and then extracted with phenol/ chloroform. DNA was precipitated with ethanol and treated
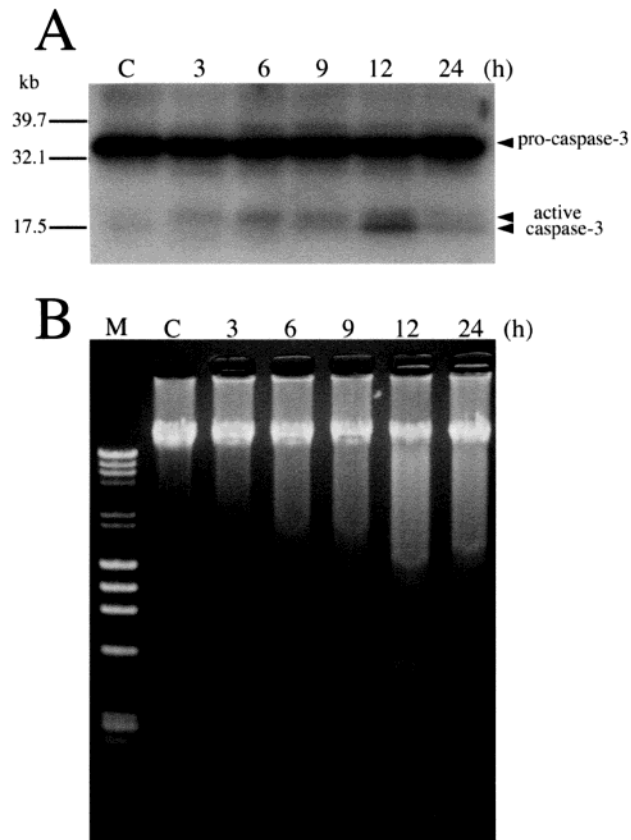


**Figure 5.** Caspase-3 activation examined by Western blot analysis (A) and induction of nucleosomal DNA fragmentation (B) after treatment with 10 μM α-mangostin in HL60 cells. Lane M is a DNA size marker. C, control. The periods of treatment are indicated in each lane.

with 0.1 mg/mL RNaseA (Sigma) and analyzed by electrophoresis on 2% agarose gel.

**Western Blot Analysis.** Cultured cells prepared as described in DNA extraction were mixed with lysis buffer containing 10 mM Tris-HCl pH 7.4, 1% NP-40, 0.1% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, and 1% Protease Inhibitor Cocktail (Sigma) and stood for 30 min on ice. After centrifugation at 10 000 rpm for 10 min, supernatants were collected as protein samples and protein content was measured with a DC protein assay kit (BIORAD, Hercules, CA). Ten micrograms of protein of each protein sample was mixed with loading buffer (250 mM Tris-HCl pH 7.2, 500 mM DTT, 10% SDS, 0.02% Bromophenol Blue, 20% glycerol) and boiled for 5 min. After cooling on ice, the samples were separated by 5−20% SDS gradient gel (BIORAD) and then blotted on a PVDF membrane (Roche, Mannheim, Germany). The membranes were blocked with blocking solution containing 1% BSA and 50% Block Ace (Dainihon-seiyaku, Osaka, Japan) in PBS and then incubated with rabbit polyclonal anticaspase-3 antibody H-277 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. Following incubation, the membrane was further incubated with HRP-conjugated goat anti-rabbit antibody (Amersham Bioscience) for 1 h at room temperature. Proteins were visualized using an ECL plus detection system (Amersham Bioscience).

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