

Xanthorrhizol Induces Apoptotic Cell Death through Molecular Cross Talks between Mitochondria-dependent and Death Receptor-mediated Signaling in Human Promyelocytic Leukemia Cells

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Xanthorrhizol, isolated from *Curcuma xanthorrhiza*, has been shown to induce cell cycle arrest and apoptotic cell death by mainly mitochondrial-dependent signaling pathway in several human cancer cells. We investigated whether xanthorrhizol could apoptosis in HL-60 human promyelocytic leukemia cells. Xanthorrhizol treatment inhibited cell viability and induced apoptotic cell death. The levels of procaspase-3, -8 and -9 were reduced and the cleavage of full length PARP was clearly observed in xanthorrhizol-treated cells. Xanthorrhizol treatment did not affect Bcl-2 protein level but increased Bax protein level, resulting in the diminished Bcl-2/Bax ratio. The levels of cleaved Bid, Fas death receptor and p53, not FasL, were also increased by xanthorrhizol treatment. In conclusion, molecular cross talks between the intrinsic and extrinsic apoptotic pathway via Bid may play an important role for induction of apoptosis in xanthorrhizol-treated HL-60 cells. Therefore, xanthorrhizol has the chemopreventive and anti-cancer potential against human promyelocytic leukemia cells. (*Cancer Prev Res* 18, 41-47, 2013)

Key Words: Xanthorrhizol, HL-60 human promyelocytic leukemia cells, Apoptotic cell death, Apoptotic pathway, Bid

INTRODUCTION

Acute promyelocytic leukemia (APL) with its specific morphology and abnormality on chromosomes 15 and 17 is highly malignant form of acute myeloid leukemia (AML), and characterized by numerous promyelocytes in the blood and bleeding tendencies from a low fibrinogen level and platelet count. It requires prompt treatment as a medical emergency and its treatment may last one to two years. Thus, commonly used drugs for the treatment of APL have been known to cause diverse side effects or complications, including bleeding related

to coagulopathy, differentiation syndrome, hyperleukocytosis, myelosuppression, and hepatic or cardiac toxicity.¹⁾

One of the attractive strategies considered in current cancer chemoprevention/chemotherapy is dietary or pharmaceutical manipulation to induce apoptotic cell death in preneoplastic or malignant cells with chromosomal aberration.²⁾ There is accumulated evidence that phytochemicals in medicinal herbs and dietary plants exert cancer chemopreventive or anticancer activity by inducing apoptosis in cancer cells.^{3~7)} *Curcuma xanthorrhiza* Roxb. (Zingiberaceae), known as temulawak or Javanese tumeric, has been traditionally used in Indonesia for dietary and medicinal purposes.⁸⁾ Xanthorrhizol (Fig. 1A) is a sesqui-

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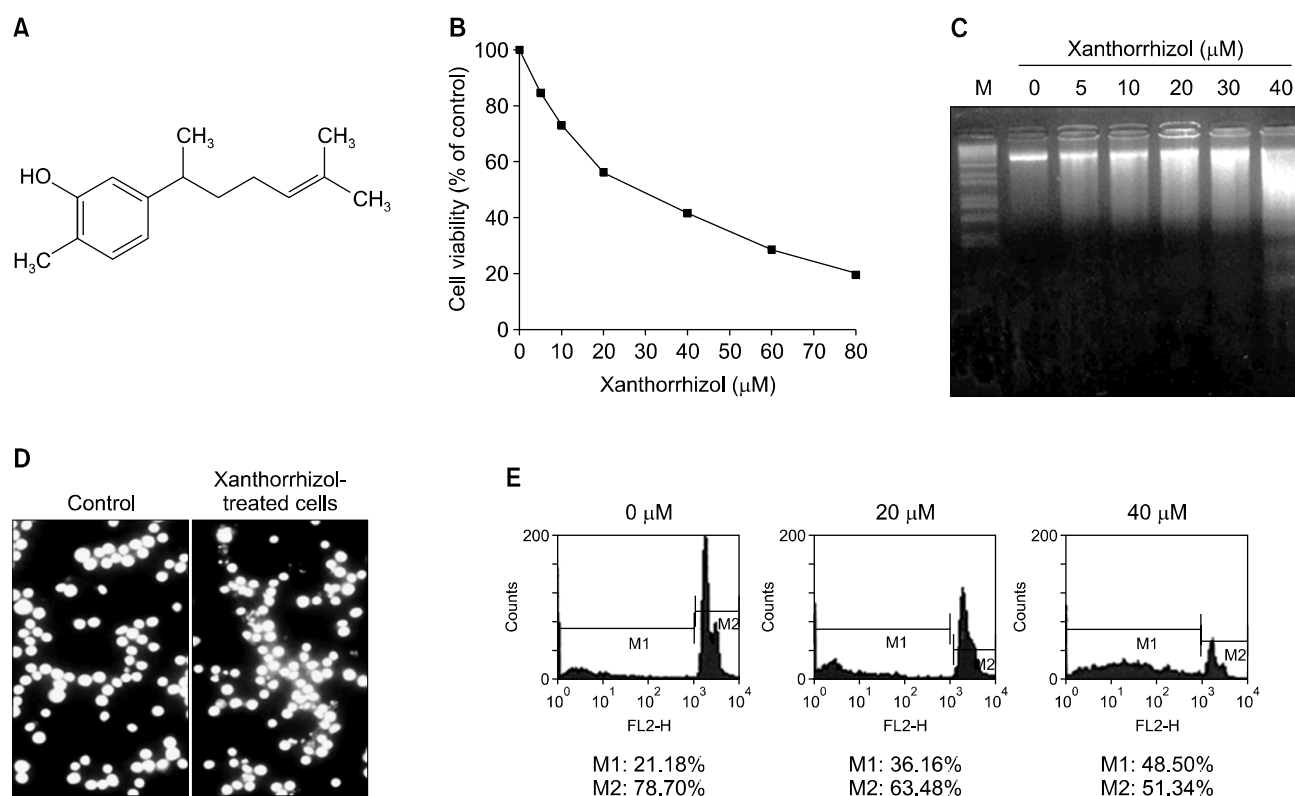


Fig. 1. The induction of apoptosis in xanthorrhizol-treated HL-60 cells. (A) Chemical structure of xanthorrhizol. (B) HL-60 human promyelocytic leukemia cells were cultured in serum-free RPMI 1640 medium with various concentrations of xanthorrhizol for 24 h. Cell viability was measured by a MTT assay. (C) The extracted DNA was electrophoresed on 1.8% agarose gel with ethidium bromide. M: 1 kb DNA ladder size marker. (D) The cells treated with 40 μM xanthorrhizol were stained with Hoechst 33258 and observed with fluorescence microscopy ($\times 400$). (E) The cellular DNA was stained with propidium iodide and the stained cells were analyzed with a flow cytometer. Data were the representative of three-independent experiments.

terpenoid isolated from *C. xanthorrhiza* that has been known to possess diverse pharmacological activity, including anti-bacterial against oral pathogens,⁹ anti-inflammatory, and antioxidant.¹⁰ In addition, xanthorrhizol showed antiproliferative and apoptosis-inducing activity in human colon, breast, liver, and cervical cancer cells,^{11~15} as well as anticarcinogenic and antimetastatic activity in mice.^{16,17} Xanthorrhizol induced apoptosis by modulating protein levels of anti-apoptotic Bcl-2, tumor suppressor p53, and poly-(ADP-ribose) polymerase (PARP) for DNA repair in MCF-7 estrogen-receptor positive breast cancer and HepG2 hepatoma cells. Pro-apoptotic Bax protein level was not changed by xanthorrhizol treatment in these cells.^{11,12} In contrast, xanthorrhizol upregulated Bax protein and did not affect Bcl-2 expression in HeLa cervical cancer cells.¹³ Xanthorrhizol also induced apoptosis through the mitochondrial-mediated pathway, closely related with caspase-3 and caspase-9 but not caspase-6 or caspase-8, in MDA-MB-

231 estrogen-receptor negative breast cancer cells.¹⁴ In HCT116 human colon cancer cells, xanthorrhizol arrested cell cycle progression in the G0/G1 and G2/M and induced apoptosis via release of cytochrome c, activation of caspases, cleavage of PARP, and upregulation of pro-apoptotic non-steroidal anti-inflammatory drug-activated gene-1.¹⁵ In addition, xanthorrhizol showed potent neuroprotective effects on glutamate-induced neurotoxicity and reactive oxygen species (ROS) generation in the murine hippocampal HT22 cell line.¹⁸

Given that xanthorrhizol substantially restricts the proliferative activity of some cancer cell types, the present studies were undertaken to determine whether xanthorrhizol could inhibit the growth of the human HL-60 promyelocytic leukemia cells. We found that xanthorrhizol induced apoptosis in human myeloid leukemia cells through both mitochondrial- and death receptor-mediated pathways. Xanthorrhizol induces apoptotic cell death via differential signaling pathway in different cancer

types, thereby it has the high potential to develop as a beneficial agent for cancer prevention and anti-cancer.

MATERIALS AND METHODS

1. Materials

Xanthorrhizol was provided by Professor Jae-Kwan Hwang, a coauthor.¹⁹⁾ It was dissolved with dimethyl sulfoxide (DMSO) and diluted with cell culture medium for experiments. RPMI-1640 medium, phosphate-buffered saline (PBS) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium dodesyl sulfate (SDS), RNase, proteinase K, Hoechst 33258, were obtained from Sigma-Aldrich (St. Louis, MO, USA). The following primary antibodies were purchased from their respective sources: procaspase-8, procaspase-9, cytochrome *c*, Bax, Bcl-2, Fas and p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), β -actin (Sigma-Aldrich Chemicals), procaspase-3 (Transduction Laboratories, Lexington, KY, USA), PARP (New England BioLab, Beverly, MA, USA), Bid (R&D Systems, Minneapolis, MN, USA), Smac/DIABLO (Upstate Biotechnology, Lake Placid, NY, USA), FasL (BD Biosciences, Bedford, UK). Secondary antibodies conjugated to horseradish peroxidase were obtained from Santa Cruz Biotechnology. All other chemicals and reagents were the highest grade commercially available.

2. Cell culture and *in vitro* cytotoxicity assay

HL-60 human promyelocytic leukemia cells were maintained in RPMI 1640 supplemented with 10% FBS in a humidified atmosphere of 5% CO₂ at 37°C. Cells (1×10^4 cells) were seeded into each well of a 96-well plate with RPMI 1640 m and cultured overnight. The cells were exposed to serum-free media with various concentrations of xanthorrhizol for 24 h. Then, a MTT solution (1 mg/ml) was added and the plates were incubated for 4 h at 37°C. The cellular formazan product was dissolved with DMSO and the absorbance was measured at 570 nm using a microplate reader (BIO-RAD, Hercules, CA, USA).

3. DNA fragmentation

HL-60 cells (5×10^5 cells/ml) were cultured in serum-free media with different concentrations of xanthorrhizol for 24 h and washed twice with ice-cold PBS. The cells were lysed with 500 μ l lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5,

20 mM EDTA) for 1 h on ice. Lysates were centrifuged at $1,000 \times g$ for 10 min and the supernatants were incubated for 4 h at 55°C with 50 μ g/ml RNase A, 120 μ g/ml proteinase K and 0.5% SDS. DNA was extracted with phenol/chloroform/isoamylalcohol (25 : 24 : 1) and precipitated with ice-cold absolute ethanol. After the precipitates were resuspended with 30 μ l TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), each DNA sample was electrophoresed on 1.8% agarose gel with 0.5 μ l/ml ethidium bromide, and then visualized under UV light.

4. Hoechst 33258 nuclear staining

HL-60 cells (1×10^6 cells/ml) at 70~80% confluence were treated with 40 μ M xanthorrhizol for 24 h, fixed with 4% paraformaldehyde for 30 min at room temperature, and washed with PBS. The fixed cells were treated with 50 ng/ml Hoechst 33258 for 30 min at room temperature and washed with PBS again. The cells were mounted and their nuclear morphology was examined by fluorescence microscopy.

5. Flow cytometric analysis

HL-60 cells were treated with 20 or 40 μ M xanthorrhizol for 24 h. The cells (1×10^5 cells) in 100 μ l of PBS were treated with 200 μ l of 95% ethanol, incubated at 4°C for 1 h, washed with PBS, and resuspended with 250 μ l of 1.12% sodium citrate buffer (pH 8.4) with 12.5 μ g of RNase. Additional incubation was continued for 30 min at 37°C. The cellular DNA was then stained with 250 μ l of propidium iodide (50 μ g/ml) for 30 min at room temperature. The stained cells were detected by a FACScan flow cytometer (BD Biosciences) and the cytometric data were analyzed using a commercially available software package (Winlist Version 5.0; Verify, Topsham, ME, USA). Assessment of apoptosis was determined by monitoring the sub-diploid population.

6. Immunoblot analysis

Total protein was extracted from HL-60 cells (1×10^6 cells/ml) with lysis buffer containing 150 mM NaCl, 1 mM sodium EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin and 20 mM Tris-HCl, pH 7.5 for 90 min. The lysates were centrifuged at $2,000 \times g$ for 15 min. To obtain the mitochondrial and cytosolic fractions, HL-60 cells were resus-

pended in 3 volumes of lysis buffer containing 20 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, and 10 mM phenylmethylsulfonyl fluoride, 10 μ M leupeptin, 10 μ M aprotinin, and 250 mM sucrose. After chilling on ice for 3 min, the cells were disrupted with a glass homogenizer. The homogenate was centrifuged twice at 2,500 \times g at 4°C to remove unbroken cells and nuclei. The mitochondria were then pelleted by centrifugation at 12,000 \times g for 30 min and suspended with lysis buffer. The supernatant was filtered through 0.2 μ m and then 0.1 μ m Ultrafree MC filters (Millipore) to collect cytosolic proteins. Protein concentration was measured using a BCA assay kit (Pierce).

The protein extracts (30 μ g) were subjected to SDS-polyacrylamide gel electrophoresis and the blots on the gel were electrotransferred onto polyvinylidene difluoride membrane. The membranes were blocked with 10% skim milk and 0.1% Tween 20 in PBS and then were incubated with the specific primary antibodies against target proteins at a 1 : 1,000 dilution in PBS for 1 h at room temperature. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse, anti-goat or anti-rabbit IgG antibodies at a 1 : 5,000 dilution for 1 h at room temperature. The target proteins were detected with an enhanced chemiluminescence detection kit (Amersham Life Science, Little Chalfont, UK) according to manufacturer's protocol.

RESULTS AND DISCUSSION

The search for new chemopreventive and antitumor agents that are more effective and less toxic has kindled great interest

in phytochemicals. In particular, apoptosis-inducing phytochemicals have high potential for suppression of carcinogenesis and cancer progression because apoptosis is considered as a defense strategy against tumorigenesis. Xanthorrhizol, isolated from the rhizome of the plant *Curcuma xanthorrhiza*, has been shown to induce cell cycle arrest and apoptotic cell death by mainly mitochondrial-dependent signaling pathway in several human cancer cells.^{11~15)} In this study, we investigated whether xanthorrhizol could apoptosis in HL-60 human promyelocytic leukemia cells. As a result of a MTT assay, xanthorrhizol treatment for 24 h inhibited cell viability in a dose-related manner (IC₅₀ = 28.2 μ M) (Fig. 1B). Xanthorrhizol treatment at 40 μ M resulted in internucleosomal DNA fragmentation (Fig. 1C) and the generation of apoptotic bodies (Fig. 1D). The sub-G1 (sub-1N, M1) fraction was dose-dependently increased in HL-60 cells treated with xanthorrhizol for 24 h (Fig. 1E).

Next, we determined the molecular mechanism by which xanthorrhizol induces apoptosis in HL-60 cells. The characteristic biochemical and morphological changes displayed by apoptotic cells in response to a variety of stimuli have been recognized to orchestrate by caspases, which are synthesized in cells as inactive zymogens procaspases and can be activated by proteolytic cleavage.^{20,21)} Apoptosis proceeds via two distinct caspase cascades designated as intrinsic and extrinsic pathways.^{22,23)} Mitochondrial-mediated (intrinsic) pathway via caspase-9 and death receptor-activated (extrinsic) pathway via caspase-8 converge at caspase-3 and finally induce cell death via caspase-3-mediated cleavage of PARP.^{24,25)} In this study, we found that the levels of procaspase-3, -8 and -9 was reduced in a time-dependent and dose-dependent fashion in HL-60 cells exposed to xanthorrhizol. The cleavage of full length PARP (116 kDa) to

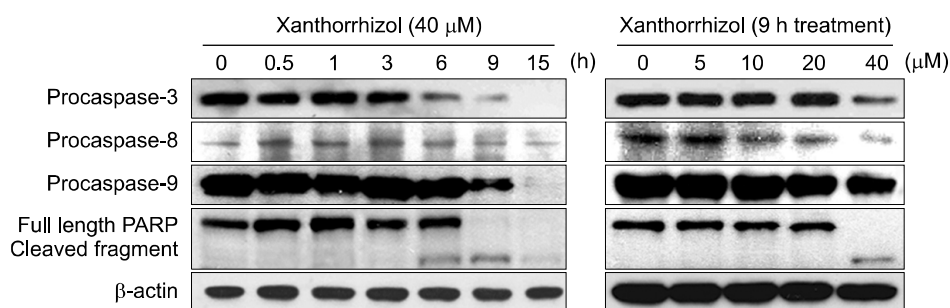


Fig. 2. Activation of caspases and PARP cleavage in xanthorrhizol-treated HL-60 cells. HL-60 cells were treated with 40 μ M xanthorrhizol at the indicated time points or with various concentrations of xanthorrhizol for 9 h. The levels of target proteins in the extracted lysates were detected by immunoblotting with the specific primary antibodies. Data were the representative of three-independent experiments.

fragment (85 kDa) was clearly observed when the cells were treated with 40 μ M xanthorrhizol for 6 h and more than 6h (Fig. 2). These results suggest that xanthorrhizol may induce apoptosis via both death receptor and mitochondrial pathway.

We further detected the levels of a Bcl-2 family, cytochrome *c*, and Smac in xanthorrhizol-treated HL-60 cells. The activation of caspase-9 and caspase-3 relies on the release of cytochrome *c* from the intermembrane space of mitochondria to cytosol, which occurs in response to several apoptotic stimuli including serum deprivation, DNA damage, and activation of cell surface death receptors.²⁶⁾ Recently, second mitochondria-derived activator of caspases (Smac), released together with cytochrome *c* from the mitochondria in response to apoptotic stimuli, was found to promote caspase activation by binding and neutralizing the inhibitor of apoptosis proteins (IAPs), which blocks caspase-9 and caspase-3 activity.^{27,28)} In this study, xanthorrhizol treatment increased cytosolic levels of cytochrome *c* and Smac and decreased mitochondrial level of these in a time and dose-dependent manner (Fig. 3A). Mitochondrial functions during apoptosis are controlled by the Bcl-2 family localized at the outer mitochondrial membranes.²⁹⁾ The antiapoptotic Bcl-2 interrupt cytochrome *c* release from mitochondria, whereas the pro-apoptotic Bax with three multi-domains plays a major role in initiating cytochrome *c* release. Thus, Bcl-2/Bax ratio within the cytosol has been eventually accepted to decide

whether the cell takes into apoptotic pathway.^{30,31)} Bax can be caspase-independently activated by cytosolic p53 tumor suppressor protein in intrinsic pathway. The translocated p53 into the mitochondria physically interacts with Bcl-2 and Bcl-xL and antagonizes their anti-apoptotic stabilization of the outer mitochondrial membrane, leading the release of Bax.³²⁾ Extrinsic pathway can also activate Bax with Bid, which is the BH3-only protein that links death receptor to pro-apoptotic events at mitochondria and cleaved to truncated Bid (tBid) by caspase-8. The translocated tBid to mitochondrial membrane triggers mitochondrial outer membrane permeabilization by promoting oligomerization of the pro-apoptotic Bcl-2 family proteins Bak and/or Bax, resulting in egress of cytochrome *c* and Smac/DIABLO from the mitochondrial intermembrane space.^{33,34)} Our data indicated that xanthorrhizol treatment did not affect Bcl-2 protein level but increased Bax protein level, resulting in the diminished Bcl-2/Bax ratio. The level of cleaved Bid was also increased by xanthorrhizol treatment (Fig. 3B). In addition, we detected that FasL expression was not changed but Fas death receptor and p53 was upregulated in xanthorrhizol-treated HL-60 cells (Fig. 4). These results demonstrate that xanthorrhizol induced apoptotic death of HL-60 cells by releasing mitochondrial cytochrome *c* and Smac into cytoplasm by p53-mediated Bax upregulation, which triggered the proteolytic activation of a series of caspases and subsequent cleavage

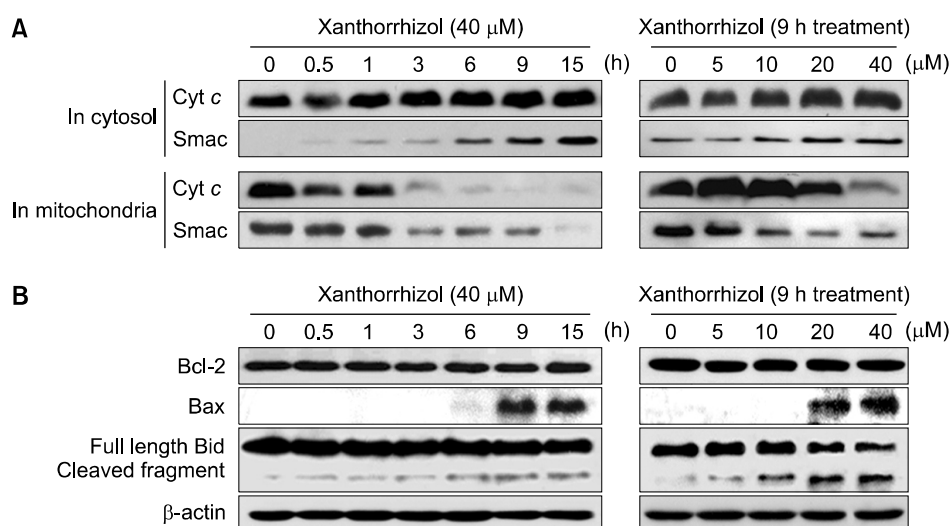


Fig. 3. The levels of cytosolic cytochrome *c*, Smac, and proapoptotic and antiapoptotic Bcl-2 family. HL-60 cells were treated with 40 μ M xanthorrhizol at the indicated time points or with various concentrations of xanthorrhizol for 9 h. The cells were extracted with lysis buffer and cytosolic and nuclear fractions were obtained the extracted cell lysates as described in materials and methods. The levels of target proteins in total lysates or cytosolic and nuclear fractions were detected by immunoblotting with the specific primary antibodies. Data were the representative of three-independent experiments.

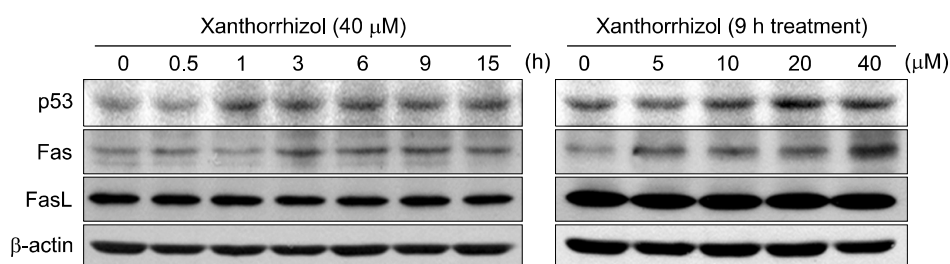


Fig. 4. The levels of p53, Fas death receptor and FasL in xanthorrhizol-treated HL-60 cells. HL-60 cells were treated with 40 μ M xanthorrhizol at the indicated time points or with various concentrations of xanthorrhizol for 9 h. The levels of target proteins in total lysates were detected by immunoblotting with the specific primary antibodies. Data were the representative of three-independent experiments.

of the DNA repair enzyme PARP leading to internucleosomal DNA fragmentation. In addition, xanthorrhizol treatment may result in tBid-dependent Bax activation by upregulating Fas death receptor and activating caspase-8.

CONCLUSION

In conclusion, xanthorrhizol treatment induces apoptosis of HL-60 cells via both intrinsic and extrinsic apoptotic pathway. Molecular cross talks between the two pathways via Bid may play an important role for induction of apoptosis in xanthorrhizol-treated HL-60 cells. Therefore, xanthorrhizol has the chemopreventive and anti-cancer potential against human promyelocytic leukemia cells.

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