

RESEARCH COMMUNICATION

Houttuynia cordata Thunb Fraction Induces Human Leukemic Molt-4 Cell Apoptosis through the Endoplasmic Reticulum Stress Pathway

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Abstract

Houttuynia cordata Thunb (HCT) is a native herb found in Southeast Asia which features various pharmacological activities against allergy, inflammation, viral and bacterial infection, and cancer. The aims of this study were to determine the cytotoxic effect of 6 fractions obtained from silica gel column chromatography of alcoholic HCT extract on human leukemic Molt-4 cells and demonstrate mechanisms of cell death. Six HCT fractions were cytotoxic to human lymphoblastic leukemic Molt-4 cells in a dose-dependent manner by MTT assay, fraction 4 exerting the greatest effects. Treatment with IC₅₀ of HCT fraction 4 significantly induced Molt-4 apoptosis detected by annexinV-FITC/propidium iodide for externalization of phosphatidylserine to the outer layer of cell membrane. The mitochondrial transmembrane potential was reduced in HCT fraction 4-treated Molt-4 cells. Moreover, decreased expression of Bcl-xl and increased levels of Smac/Diablo, Bax and GRP78 proteins were noted on immunoblotting. In conclusion, HCT fraction 4 induces Molt-4 apoptosis cell through an endoplasmic reticulum stress pathway.

Keywords: *Houttuynia cordata* Thunb - apoptosis - human leukemic Molt-4 cells - endoplasmic reticulum stress

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Introduction

Apoptosis is a programmed cell death found in both the physiological and pathological processes such as sloughing off of the gastrointestinal epithelium, neurological degenerative diseases, autoimmune diseases and infection. The mechanism of apoptosis can be divided into two main pathways, viz. death receptor and mitochondrial pathways. Fas or tumor necrosis factor receptors are bound to the ligands and become trimerization. FADD or TRADD form complex with the receptors and activate procaspase-8 or -10 to its active form. Ionizing radiation, oxidative stress and DNA damage can trigger mitochondrial pathway. Bax and Bak are pivotal effectors of the mitochondrial apoptosis pathway, as either Bax or Bak is needed to permeabilize the mitochondrial outer membrane. Bax and Bak, proapoptotic protein in the Bcl-2 family, form homodimers at the mitochondrial membrane and cause the release of cytochrome c, Smac/Diablo, and apoptosis inducing factor (AIF). The expressions of anti-apoptotic protein in Bcl-2 family, such as Bcl-xl, Mcl-1, Bcl-2, and Bcl-w, are reduced in apoptotic cells. Cytochrome c forms complex with Apaf-1, procaspase-9, called apoptosome, and activate procaspase-9 to be active caspase-9, which then activates the executioner caspases-3, -6, and -7 to cause apoptotic cell death (Hengartner, 2000; Galluzzi et

al., 2012).

Endoplasmic reticulum (ER) is an organelle responsible to the posttranslational modification of proteins. It controls protein folding by chaperones and the process of protein glycation. The response of ER stress is the accumulation of misfolded proteins. ER stress leads cells to activate self protective mechanisms: (1) transcriptional up-regulation of ER chaperones and folding enzymes; (2) translational attenuation to limit further accumulation and aggregation of misfolded proteins; and (3) ER-associated degradation (ERAD) which eliminates misfolded proteins from the ER (Shiraishi et al., 2006; Hussain & Ramaiah, 2007). Three types of ER membrane receptors, ATF6, IRE1 and PERK, sense the stress in the ER, and eventually activate transcription factors for induction of ER chaperones, such as GRP78, for inhibition of synthesis of new proteins. These processes are called unfolded protein response (UPR) (Imaizumi et al., 2001). It has been found that ER stress pathway involves the mechanism of apoptotic cell death. Apaf-1 and the mitochondrial pathway of apoptosis play significant roles in ER stress-induced apoptosis (Shiraishi et al., 2006).

Houttuynia cordata Thunb (HCT), which is in the family of Saururaceae, is a commonly used herb in traditional Asian medicine. It has been reported to have various bioactivities to counteract with oxidative stress

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(Kusirisin et al., 2009), cancer (Tang et al., 2009; Lai et al., 2010), allergy (Han et al., 2009) and inflammation (Li et al., 2011). The water extract of HCT protects rat primary cortical cells from Abeta25-35-induced neurotoxicity via regulation of calcium influx and mitochondria-mediated apoptosis (Park & Oh, 2012). We have previously reported that fermented HCT extract induces human leukemic HL-60 and Molt-4 cell apoptosis via oxidative stress and mitochondrial pathway (Banjerdpongchai & Kongtawelert, 2011). However, in the process of identifying active compound(s) in HCT, silica gel column chromatography was performed and six fractions were obtained. The aims of this study were to determine the cytotoxic effect of six HCT fractions on Molt-4 cells, the mode of cell death and the mechanism involved. In the present study, HCT fraction 4 could induce human leukemic Molt-4 cell apoptosis via the ER stress and co-activated through the mitochondrial pathway indicated by the increase expression of GRP78, Bax and Smac/Diablo, mitochondrial transmembrane permeability alteration, and the reduction in protein expression of Bcl-xl. Further study is to purify the active compound(s) in fraction 4, which is (are) responsible for the apoptotic inducing property of HCT. It will provide new drug development from this medicinal herb.

Materials and Methods

Chemicals

Histopaque, MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide, propidium iodide (PI) and 3, 3'-dihexyloxycarbocyanine iodide (DiOC₆) were obtained from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium was obtained from Invitrogen, USA. Annexin V-FITC kit was obtained from Roche, Indianapolis, IN, USA. Rabbit polyclonal antibody to GRP78 and Bcl-xl, mouse monoclonal antibody to Bax, and rabbit monoclonal antibody to Smac/Diablo and horseradish peroxidase (HRP) conjugated secondary antibody were purchased from Abcam, Cambridge, UK. SuperSignal West Pico Chemiluminescent Substrate was obtained from Pierce, Rockford, IL, USA. Complete mini protease inhibitor cocktail was from Roche, Basel, Switzerland.

Plant material, extraction and isolation

The *Houttuynia cordata* Thunb whole plants were collected in June, 2009 from Chiang Mai province, Thailand. The plant was authenticated and a voucher specimen (QBG42697) has been deposited at the Queen Sirikit Botanic Garden, Chiang Mai, Thailand. The whole plants were fermented with yeast and ethanol. One kilogram air-dried and finely powdered of HCT had been percolated 5 times with 10 liters of ethanol for 4 days at room temperature. The extracts were combined and evaporated to dryness under reduced pressure to afford a crude ethanolic extract.

The ethanolic extract was separated by column chromatography over silica gel (Merck No. 7734, Mesh 70-230 ASTM). Elution started with hexane, gradually enriched with ethylacetate in hexane up to 20% ethylacetate and methanol. Fractions (300 ml each) were

collected, monitoring by TLC behavior and combined. The solvent was evaporated to dryness to afford six fractions (F1-F6). Fraction 4 was further processed through high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) to obtain the chromatogram and NMR spectrum, respectively.

Cell culture

Human acute T lymphoblastic leukemic Molt-4 cells were gifts from Dr. Watchara Kasinroek. The cells were cultured in 10% fetal bovine serum in RPMI-1640 medium supplemented with penicillin G (100 units/ml) and streptomycin (100 µg/ml) at 37 °C in a humidified atmosphere containing 5% CO₂. The human leukemic cells (1x10⁶) were treated with the fractions at indicated concentrations and durations.

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood obtained from adult volunteers by density gradient centrifugation using Histopaque according to standard protocols. Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. PBMCs (3x10⁶) were treated with the fractions at 10, 20, 40, 80 µg/ml for indicated times.

Cytotoxicity test

Following six fraction treatments for indicated times, cell viability was assessed by MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide) assay (Su et al., 2000). This method is based on the ability of viable cells to reduce MTT and form a blue formazan product. MTT solution (sterile stock solution of 5 mg/ml) was added to cell suspension at final concentration of 100 µg/ml and the solution incubated for 4 h at 37 °C in a humidified 5% CO₂ atmosphere. The medium was then removed and cells were treated with DMSO for 30 min. The optical density of the cell lysate was measured at 540 nm with reference wavelength of 630 nm using microtiter plate reader (Biotek, USA). Number of viable cells was calculated from untreated cells, and the data were expressed as percent cell viability.

Determination of phosphatidylserine externalization in apoptotic cells

Treated cells were washed once in phosphate-buffered saline solution, centrifuged at 200 x g and the cell pellet was suspended in 100 µl of binding buffer provided by the annexin V-FITC reagent kit. Annexin V-FITC (2 µl) and PI (2 µl) were added and the cell suspension was left at room temperature for 15 min in the dark. Finally 900 µl of binding buffer were added. Analysis was conducted using FACScan (Becton Dickinson, USA). Cells that were stained with annexin V-FITC, and annexin V-FITC together with PI, were designated as early and late apoptotic cells, respectively.

Determination of mitochondrial transmembrane potential (MTP)

For MTP determination, 5x10⁵ cells were treated with the HCT fraction 4 at IC₁₀, IC₂₀ and IC₅₀ for indicated

times, harvested and re-suspended in a PBS containing 40 nM of DiOC₆ (Li et al., 2007). Then the cells were incubated for 15 min at 37 °C before cells were subjected to flow cytometer (Becton Dickinson, USA).

Western blot analysis

The fraction 4-treated cells were washed once in ice cold PBS and incubated at 4 °C for 10 min with ice-cold cell lysis buffer (250 mM sucrose, 70 mM KCl, 0.25% Triton X-100 in PBS containing complete mini protease inhibitor cocktail). Following centrifugation at 20,000 x g for 20 min, supernatant (50 µg, determined by Bradford method) was separated by 17% SDS-PAGE and transferred onto nitrocellulose membrane. After treating with 5% non-fat milk in PBS containing 0.2% Tween-20, membrane was incubated with rabbit polyclonal antibody to GRP78 or Bcl-x1, mouse monoclonal antibody to Bax, or rabbit monoclonal antibody to Smac/Diablo, followed by appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:20,000). Protein bands were visualized on X-ray film with SuperSignal West Pico Chemiluminescent Substrate.

Statistical analysis

Results are expressed as mean ± S.D. Statistical difference between control and treated group was determined by one-way ANOVA (Kruskal Wallis analysis) at limit of $p < 0.05$ from 3 independent experiments conducted in triplicate. For comparison between two groups, data were analyzed using Student's *t*-test.

Results and Discussion

Cytotoxicity of HCT fractions

Six HCT fractions were cytotoxic to human leukemic Molt-4 cells dose dependently as shown in Figure 1. The fraction 4 was the most toxic to Molt-4 cells with IC₅₀ value of 15.5 µg/ml. Therefore HCT fraction 4 was selected to study further for the effect on normal human PBMCs and determine the mode of cell death by using IC₁₀, IC₂₀ and IC₅₀ concentrations of 5, 8.5 and 15.5 µg/ml, respectively. The HCT fraction 4 was less toxic to PBMCs with the IC₅₀ more than 70 µg/ml (data not shown).

HCT fraction 4-induced Molt-4 cell apoptosis

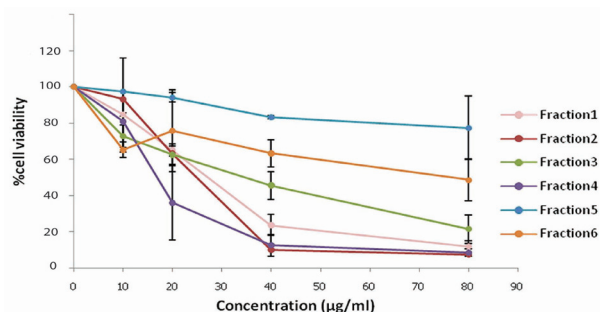


Figure 1. Cell Viability of Human Leukemic Lymphoblast T Molt-4 Cells after Treatment with the HCT Fractions. Cytotoxicity of the six fractions on Molt-4 cells was determined by MTT assay. Data are shown as mean ± S.D., obtained from 3 independent experiments conducted in triplicate

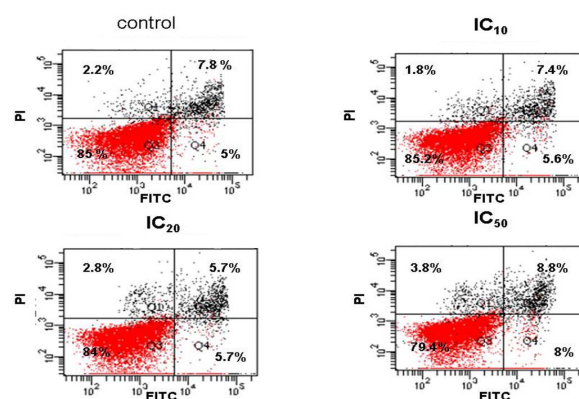


Figure 2. The HCT Fraction 4-induced Apoptosis of Human Leukemic Cells. Molt-4 cells were treated with the HCT fraction 4 at indicated concentrations (IC₁₀, IC₂₀ and IC₅₀) for indicated times and apoptotic cells were examined by annexin V-FITC/PI staining and processed through flow cytometer. Percentage of viable, early and late apoptotic cells are expressed as mean values in left lower, right lower and right upper quadrants, respectively, from 3 independent experiments

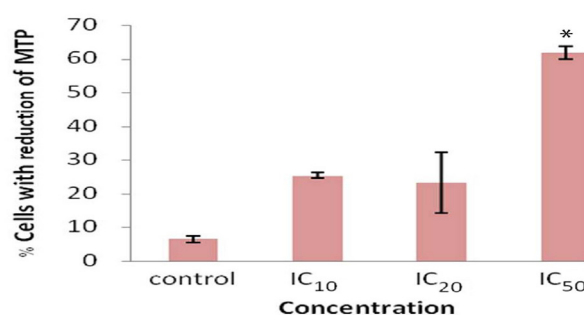


Figure 3. Reduction of Mitochondrial Transmembrane Potential in the HCT Fraction 4-treated Molt-4 Cells. Percent cells with decreased MTP of Molt-4 cells are shown as mean ± S.D., obtained from 3 independent experiments conducted in triplicate. * $p < 0.05$, compared to untreated Molt-4 cells

There are various modes of cell death, which includes apoptosis, necrosis, and autophagic cell death. Molt-4 cells were induced to undergo apoptosis as shown in Figure 2, by the flip-flop out of the phosphatidylserine to the outer layer of cell membrane. Percentage of Molt-4 cells with early apoptosis (right lower quadrant) was significantly different in the HCT fraction 4 at the dose of IC₅₀ for 4 hours of incubation compared to without treatment ($p < 0.05$).

Mitochondrial transmembrane potential

HCT fraction 4 reduced mitochondrial transmembrane potential (MTP) as shown in Figure 3. DiOC₆ is cationic lipophilic fluorochrome specific for mitochondrial membrane. In apoptotic cells, DiOC₆ leaks into cytoplasm compared to viable cells, in which accumulation in the mitochondria occurs. Cells with reduction of MTP increased significantly at the dose of IC₅₀ ($p < 0.05$).

The expression of Bcl-2 family and Smac/Diablo proteins by Western blot

In the incubation of Molt-4 cells with HCT fraction 4 for various times, the expression of anti-apoptotic Bcl-x1 protein decreased whereas that of Bax protein increased (Figure 4). It indicates the involvement of

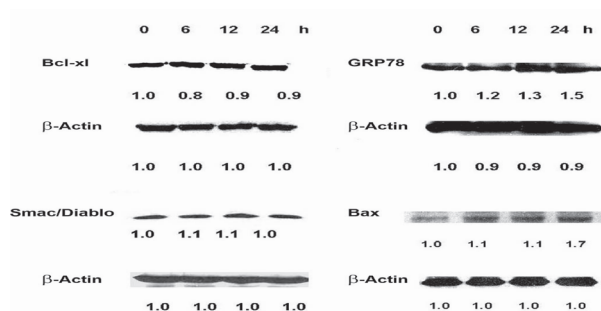


Figure 4. Apoptotic Protein and ER Stress Protein Expressions in the HCT Fraction 4-treated Human Leukemic Cells. Molt-4 cells were exposed to the HCT fraction 4 at IC₂₀ for indicated times. Levels of the proteins were examined by Western blot as described in Materials and Methods. Representative results are shown of 3 independent experiments. The number beneath the bands represents the folds of band intensity compared to control and normalized with band intensity of actin

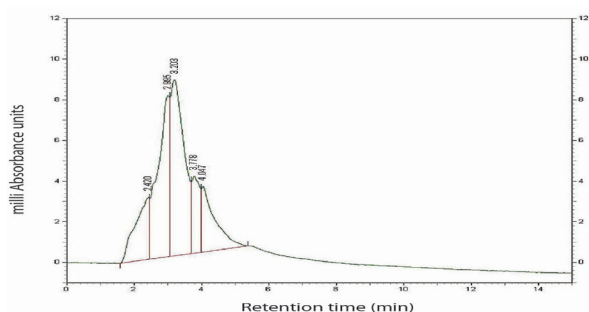


Figure 5. High Performance Liquid Chromatogram of HCT Fraction 4. The chromatogram is composed of several peaks at different retention times as depicted in the top of each peak in unit of minutes

mitochondrial pathway. Therefore, the Smac/Diablo protein, which is released from mitochondria and inhibits the inhibitors of apoptotic proteins (IAPs), was determined by immunoblot. Since Smac/Diablo inhibits the IAPs, which blocks caspase-3 activity, the high cytosolic Smac/Diablo level indicates the mitochondrial pathway activation (Hengartner, 2000). As shown in Figure 4, Smac levels slightly increased at 6 and 12 h. Altogether, HCT fraction 4 induced human leukemic cell apoptosis via the mitochondrial or intrinsic pathway.

ER stress protein expression

Glucose regulated kinase/immunoglobulin heavy chain binding protein (GRP78/Bip) acts as an ER chaperone in stress response. This protein expression increases in ER stress-induced late apoptosis in HL-60 cells treated with curcumin (Pae et al., 2007). HCT fraction 4 treatment in human leukemic Molt-4 cells induced a time dependent increase of GRP78 expression as shown in Figure 4. A molecular chaperone inducer, such as Bip inducer X (BIX) protects neurons from ER stress in the treatment of cerebral disorders associated ischemia (Kudo, 2008). Unfolded protein response (UPR) has dual roles on cell survival and death, depending on the type of tumor. Compounds either inducing ER stress and cell death, or blocking the cytoprotective function of the altered UPR of cancer cells, could be used either alone or in combination

with conventional chemotherapeutic agents (Verfaillie et al., 2010). HCT fraction 4 induced human leukemic cell apoptosis via the increased expression of transcription factor Bip/GRP78, which reduces transcription and translation.

High performance liquid chromatogram of fraction 4

Even though fraction 4 was pooled from several continuing individual isolates appearing as a single band on thin layer chromatography, HCT fraction 4 from the silica gel column chromatography and thin layer chromatography (TLC) was not pure. Since various small peaks appeared in high performance liquid chromatogram (Figure 5), fraction 4 is composed of several compounds confirmed by nuclear magnetic resonance (NMR) spectrum (data not shown). Further purification of fraction 4 is required to obtain the active compound(s) and assess for the cytotoxic effect on cancer cells.

Taken together, HCT fraction 4 induced human lymphoblastic T leukemic Molt-4 cells to undergo apoptosis via the intrinsic and endoplasmic reticulum stress pathways.

Acknowledgements

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