

RESEARCH COMMUNICATION

Ethanollic Extract of Fermented Thunb Induces Human Leukemic HL-60 and Molt-4 Cell Apoptosis Via Oxidative Stress and a Mitochondrial Pathway

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Abstract

Houttuynia cordata Thunb (HCT) is a medicinal plant of the Saururaceae family which features antimutagenic and antiviral properties. For extraction, the whole plants were fermented or non-fermented with yeast and ethanol then the whole plants were dried, ground and extracted with 95% ethanol or water. The aims of this study were to compare cytotoxic effects, apoptosis induction, and mechanism(s) with the ethanollic and water extracts of fermented and non-fermented HCT. Cytotoxicity was assessed using the MTT assay in human leukemic HL-60, Molt-4 and peripheral blood mononuclear cells (PBMCs). Apoptotic death was characterized by staining with propidium iodide and examined under a fluorescence microscope. Peroxide radical production and reduction of mitochondrial transmembrane potential (MTP) were determined using 2',7'-dichlorohydrofluorescein diacetate and 3,3'-dihexyloxycarbocyanine iodide and flow cytometry, respectively. The expression of caspase-9 was identified by immunoblotting. The ethanollic extract of fermented HCT was cytotoxic to HL-60 > Molt-4 > PBMCs, to a greater extent than the non-fermented preparation and the number of apoptotic cells was higher. The alcoholic (fermented) extract produced more radicals than the non-fermented in HL-60 cells but the converse was observed in Molt-4 cells. Reduction of MTP was found in HL-60 and Molt-4 cells treated with the alcoholic (fermented) extract and caspase-9 was cleaved dose-dependently in both cells. In conclusion, the alcoholic extract of fermented HCT was more toxic to human leukemic cells than the non-fermented and both cell lines underwent apoptosis via oxidative stress and a mitochondrial pathway.

Keywords: *Houttuynia cordata* Thunb - apoptosis - fermentation - human leukemic cells – oxidative stress - mitochondria

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Introduction

Houttuynia cordata Thunb (HCT) is a perennial herb that is native to Japan, Korea, southern China and Southeast Asia. It has been reported that HCT has many biological properties such as anti-mutagenic (Chen et al., 2003), antioxidant (Kusirisin et al., 2009), anti-allergic (Han et al., 2009), antiviral (Hayashi et al., 1995) and antibacterial activities (Lu et al., 2006). There are recent reports of HCT extract induces apoptosis in human colon adenocarcinoma HT-29 cells (Tang et al., 2009) and human primary colorectal cancer cells (Lai et al., 2010) via the mitochondrial pathway and ROS production. Furthermore, it was demonstrated antileukemic activities of HCT in various leukemic cell lines including K562, L1210, U937, Raji and P3HR1 (Chang et al., 2001). However, the process of preparing the HCT is modified by a Thai company as the whole plants were fermented by yeast and alcohol. The aims of this study were to compare the cytotoxic effects of water and alcoholic extracts of both fermented and non-fermented HCT and to identify type of cell death and the molecular mechanism of cell

death. It was found that alcoholic extract of fermented HCT was cytotoxic to HL-60 > Molt-4 > peripheral blood mononuclear cells (PBMCs) whereas water or alcoholic extract of non-fermented HCT and water extract of fermented HCT were less toxic to both cell lines and PBMCs. The ethanollic extract of fermented HCT induced both cell lines to undergo apoptosis via oxidative stress and mitochondrial pathway.

Materials and Methods

Chemicals

Histopaque, MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide, propidium iodide (PI), 3,3'-dihexyloxycarbocyanine iodide (DiOC₆) and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium was obtained from Invitrogen, USA. Rabbit polyclonal antibody to caspase-8 and rabbit monoclonal antibody to caspase-9 and horseradish peroxidase (HRP) conjugated secondary antibody were purchased from Abcam, Cambridge, UK. SuperSignal West Pico

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Chemiluminescent Substrate was obtained from Pierce, Rockford, IL, USA. Complete mini protease inhibitor cocktail was from Roche, Basel, Switzerland.

Plant material and extraction

Houttuynia cordata Thunb whole plants were collected in June, 2009 from Chiang Mai province, Thailand authenticated and a voucher specimen (QBG42697) deposited at the Queen Sirikit Botanic Garden, Chiang Mai, Thailand. The whole plants were non-fermented or fermented with yeast and ethanol, dried, powdered and extracted with 95% ethanol or water, which was then evaporated in vacuo at 45-50 °C and freeze-dried to obtain dry extracts. Percent yields of the ethanolic fermented, non-fermented; water extract of fermented and non-fermented HCT were 2.3%, 4.6%; 8.3% and 5.6%, respectively. The dry ethanolic-extract was dissolved maximally in dimethylsulfoxide (DMSO) (10 mg/ml), diluted in RPMI-1640 medium and added to the cells at the indicated concentrations. Both fermented and non-fermented dry water-extracts were maximally dissolved at 25 mg/ml in DMSO, i.e., the cells exposed to water extracts received a higher range of concentrations.

Cell culture

Human promyelocytic leukemic HL-60 and human acute T lymphoblastic leukemic Molt-4 cells were gifts from Dr. Sukhathida Ubol and 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 extracts at indicated concentration and duration.

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood from adult volunteers by density gradient centrifugation using Histopaque following 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 extracts at 10, 20, 40, 80 µg/ml for 24h.

Cytotoxicity test

Following the extract treatments for 24 h, cell viability was assessed by MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide) assay (Su et al., 2002). 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 a 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.

Fluorescence microscopy

Human leukemic cells were treated with each extract

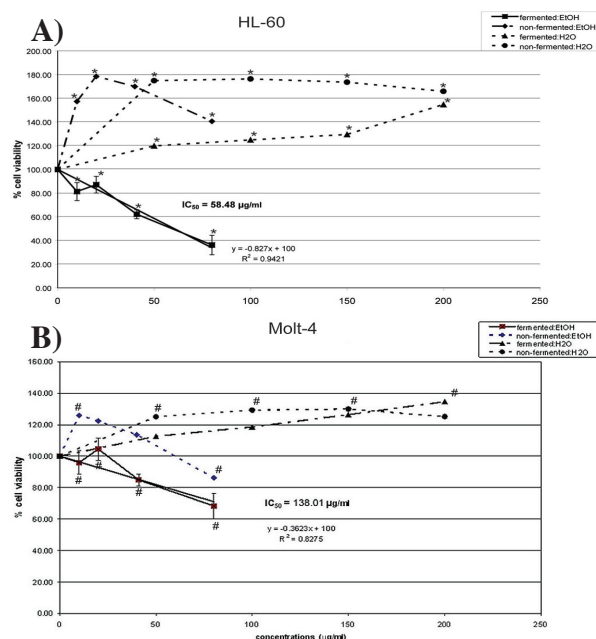


Figure 1. Cell Viability of Human Leukemic Cells after Treatment with the Ethanolic (EtOH) and Water (H₂O) Extracts of the Fermented and Non-fermented HCT. Cytotoxicity of the extracts on HL-60 (A) and Molt-4 (B) cells for 24 h was determined by MTT assay. Data are shown as mean \pm S.D., obtained from 3 independent experiments conducted in triplicate. *, #, $p < 0.05$, compared to untreated HL-60 and Molt-4 cells, respectively

(0, 20, 40, 80 µg/ml) for 24 h, and then cytospun onto glass slides. After air drying, cells were fixed with absolute methanol for 10 min at -20 °C, washed twice with phosphate-buffered saline (PBS) and air-dried. Propidium iodide (200 µg/ml) was applied to the fixed cells for 10 min at room temperature. After washing with PBS and drying, slides were mounted with 90% glycerol and examined under fluorescence microscope (Olympus, Japan). Apoptotic cells (condensed nuclei and fragmented cells) were recorded from a total of 200 cells per slide. Three independent experiments were performed in triplicate.

Determination of reactive oxygen species production and mitochondrial transmembrane potential

For flow cytometric assessment of ROS production, 1x10⁶ cells were treated with the ethanolic extracts of fermented or non-fermented HCT at 10, 20, 40, 80 µg/ml for 4 h, harvested and re-suspended in a PBS containing 5 µM of DCFH-DA. For MTP determination, 5x10⁵ cells were treated with the ethanolic extracts of fermented or non-fermented HCT at 10, 20, 40, 80 µg/ml for 24 h, harvested and re-suspended in a PBS containing 40 nM of DiOC₆. 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 ethanolic fermented-extract-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

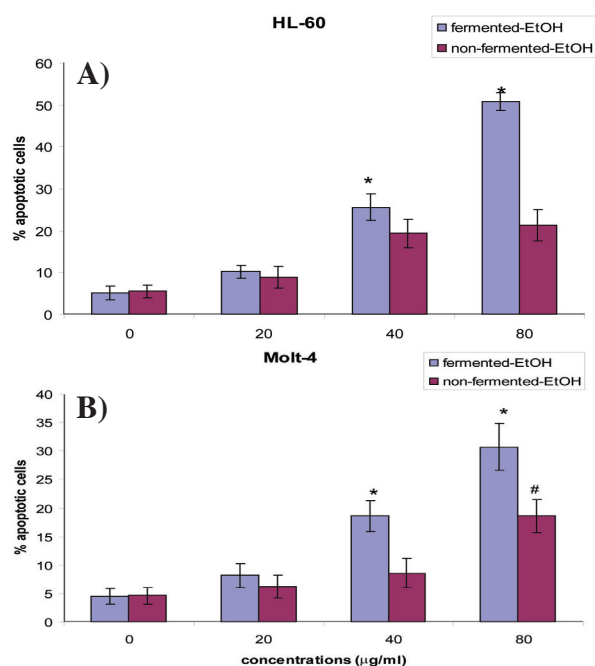


Figure 2. The Extract-induced Apoptosis of Human Leukemic Cells. HL-60 (A) and Molt-4 (B) cells were treated with the ethanolic (EtOH) extracts of fermented and non-fermented HCT at indicated concentrations for 24 h and apoptotic cells were examined by treatment with PI staining and visualization under a fluorescence microscope. Percentage of apoptotic cells is expressed as mean and S.D. of 200 cells from 3 independent experiments conducted in triplicate. *, #, $p < 0.05$, compared to untreated HL-60 and Molt-4 cells, respectively

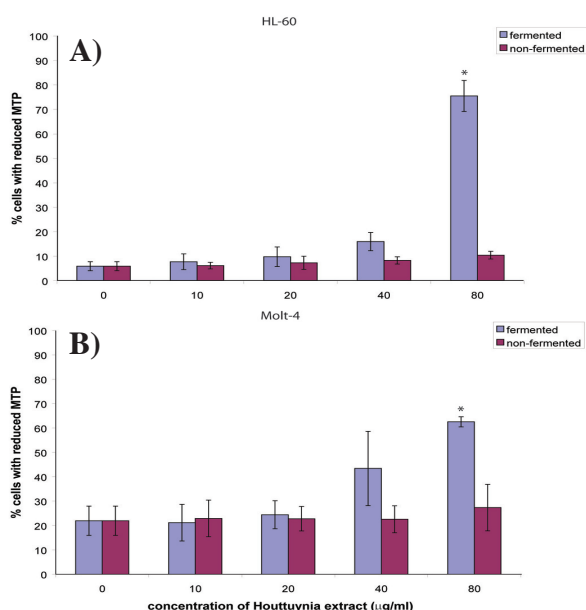


Figure 4. Reduction of Mitochondrial Transmembrane Potential in the extract-treated HL-60 and Molt-4 Cells. Percent cells with decreased MTP of HL-60 (A) and Molt-4 cells (B) are shown as mean \pm S.D., obtained from 3 independent experiments conducted in triplicate. *, #, $p < 0.05$, compared to untreated HL-60 and Molt-4 cells respectively

centrifugation at 20,000 \times g for 20 min, supernatant (50 μ g, 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 caspase-8 (1:1,500)

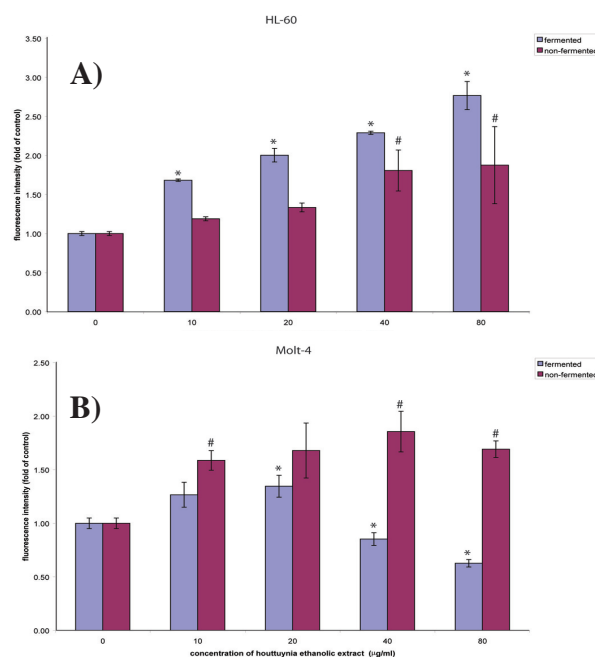


Figure 3. ROS Production in the Extract-treated HL-60 and Molt-4 Cells. HL-60 (A) and Molt-4 (B) cells were treated with the ethanolic (EtOH) extracts of fermented and non-fermented HCT at indicated concentrations for 4 h and ROS production was determined by using 2',7'-dichlorodihydrofluorescein diacetate and flow cytometry. Data are shown as mean \pm S.D., obtained from 3 independent experiments conducted in triplicate. *, #, $p < 0.05$, compared to untreated HL-60 and Molt-4 cells respectively

and rabbit monoclonal antibody to caspase-9 (1:1,000), 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 \pm SD. 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

Cell cytotoxicity and apoptotic induction

Ethanolic extract of fermented HCT was toxic to the HL-60 > Molt-4 cells as the IC_{50} levels were 58.5 and 138.0 μ g/ml, respectively (Figure 1A and 1B). At the same time, the ethanolic extract of non-fermented HCT, the water extracts of both fermented and non-fermented was less cytotoxic to both cell lines. For PBMCs, all four extracts did not inhibit cell viability and the levels of IC_{50} were > 200 μ g/ml for all extracts (data not shown).

When the leukemic cell lines were stained with PI and examined under fluorescence microscope, both HL-60 and Molt-4 cells were induced to die via apoptosis with the morphology of condensed nuclei and apoptotic bodies. The number of apoptotic cells was increased in a dose response manner as shown in Figure 2.

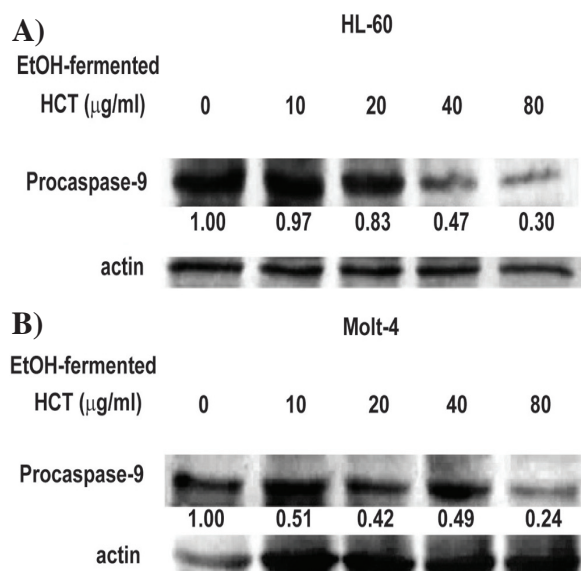


Figure 5. Caspase-9 Protein Expression in the ethanolic extract of fermented HCT-treated Human Leukemic Cells. HL-60 (A) and Molt-4 (B) cells were exposed to the alcoholic extract of fermented HCT at 10, 20, 40 and 80 µg/ml for 24 h. Levels of the pro-form of caspase-9 protein were examined by Western blot as described in Materials and Methods. Representative results are shown of 3 independent experiments. The number beneath the bands represents the folds of intensity compared to control and normalized with band intensity of actin

ROS production and reduction of mitochondrial transmembrane potential

5,7-Dimethoxyflavone sensitizes hepatocellular carcinoma cells to TRAIL-induced apoptosis and induce the death receptor 5 (DR5) expression level, accompanying the generation of reactive oxygen species (ROS) and the upregulation of CHOP, GRP78, and ATF4 protein expression (Yang et al., 2011). In the present study, alcoholic extract of fermented and non-fermented HCT induced ROS production in a dose dependent manner (Figure 3A and 3B), however, at the higher doses of ethanolic extract of fermented HCT in Molt-4 cells, the ROS induction was decreased, indicating the anti-oxidative status at 40 and 80 µg/ml (Figure 3B). Furthermore, the reduction of MTP occurred in higher concentrations of the ethanolic extracts of fermented HCT (80 µg/ml), whereas the ethanolic extract of non-fermented HCT did not alter the MTP as shown in Figure 4A and 4B.

Caspase-8 and -9 expression

Since extrinsic (death receptor-mediated) and intrinsic (mitochondria-mediated) pathways were conveyed the signaling through caspase-8 and -9, respectively (Hengartner, 2000). The immunoblot of both caspases were performed in the HL-60 and Molt-4 cells to demonstrate the signaling pathway. It was found that the pro-form of caspase-9 was decreased dose-dependently in both cells (Figure 5), whereas the caspase-8 expression did not alter (data not shown). The data indicated that the ethanolic extract of fermented HCT induced apoptosis via the mitochondrial pathway.

Taken together, this study demonstrated that the ethanolic extract of fermented HCT could induce both human leukemic HL-60 and Molt-4 cells to undergo

apoptosis more than the non-fermented one. The signaling was via the oxidative stress and mitochondrial pathway. The process of fermentation could increase the cytotoxic and apoptotic induction effect of the ethanolic extract compared to the non-fermented HCT. However, the mechanism of fermentation in increasing the apoptotic effect needs further experiments to identify the purified compound(s) and amount of compounds occur in the process of fermentation, which is in progress.

Acknowledgements

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References

- Chang JS, Chiang LC, Chen CC, et al (2001). Antileukemic activity of *Bidens pilosa* L. var. minor (Blume) Sherff and *Houttuynia cordata* Thunb. *Am J Chin Med*, **29**, 303-12.
- Chen YY, Liu JF, Chen CM, Chao PY, Chang TJ (2003). A study of the antioxidative and antimutagenic effects of *Houttuynia cordata* Thunb. using an oxidized frying oil-fed model. *J Nutr Sci Vitaminol*, **49**, 327-33.
- Han EH, Park JH, Kim JY, Jeong HG (2009). *Houttuynia cordata* water extract suppresses anaphylactic reaction and IgE-mediated allergic response by inhibiting multiple steps of FcεRI signaling in mast cells. *Food Chem Toxicol*, **47**, 1659-66.
- Hayashi K, Kamiya M, Hayashi T (1995). Virucidal effects of the steam distillate from *Houttuynia cordata* and its components on HSV-1, influenza virus, and HIV. *Planta Med*, **61**, 237-41.
- Hengartner MO (2000). The biochemistry of apoptosis. *Nature*, **407**, 770-6.
- Kusirisin W, Srichairatanakool S, Lerttrakarnnon P, et al (2009). Antioxidative activity, polyphenolic content and anti-glycation effect of some Thai medicinal plants traditionally used in diabetic patients. *Med Chem*, **5**, 139-47.
- Lai KC, Chiu YJ, Tang YJ, et al (2010). *Houttuynia cordata* Thunb extract inhibits cell growth and induces apoptosis in human primary colorectal cancer cells. *Anticancer Res*, **30**, 3549-56.
- Lu H, Wu X, Liang Y, Zhang J (2006). Variation in chemical composition and antibacterial activities of essential oils from two species of *Houttuynia* THUNB. *Chem Pharm Bull (Tokyo)*, **54**, 936-40.
- Su WC, Chang SL, Chen TY, Chen JS, Tsao CJ (2002). Comparison in in vitro growth-inhibitory activity of carboplatin and cisplatin on leukemic cells and hematopoietic progenitors: the myelosuppressive activity of carboplatin may be greater than its antileukemic effect. *Jpn J Clin Oncol*, **30**, 562-7.
- Tang YJ, Yang JS, Lin CF, et al (2009). *Houttuynia cordata* Thunb extract induces apoptosis through mitochondrial-dependent pathway in HT-29 human colon adenocarcinoma cells. *Oncol Rep*, **22**, 1051-6.
- Yang JF, Cao JG, Tian L, Liu F (2011). 5, 7-Dimethoxyflavone sensitizes TRAIL-induced apoptosis through DR5 upregulation in hepatocellular carcinoma cells. *Cancer Chemother Pharmacol*, **69**, 195-206.