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ORIGINAL ARTICLE

Flavonoid Component Determination and Apoptotic Induction Evaluation of *Houttuynia cordata* Thunb Extract on Human Acute Leukemic Cells

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ABSTRAK

Kejadian ketahanan dadah masa kini dan kesan samping beberapa dadah alopati telah meningkatkan penekanan ke atas penggunaan bahan tumbuhan sebagai sumber perubatan ke atas pelbagai penyakit termasuk leukemia. Houttuynia cordata Thunb (H.cordata) ialah tumbuhan setempat di utara Thailand yang dikenali sebagai Plucao, mempunyai pelbagai aktiviti biologi seperti anti-inflamasi, anti-kanser dan anti-leukemia. Tujuan kami ialah untuk menentukan komponen aktif flavonoid H. cordata dan menyiasat kesan ekstrak etanol H.cordata ke atas induksi apoptosis pada sel leukemia limfoblastik akut manusia. Dalam kajian ini, kami mendapati ekstrak etanol H. cordata mempunyai jumlah flavonoid sebanyak 231.21 ± 4.19 mg QE/g H.cordata kering. Analisa LC-MS kuantitatif ke atas ekstrak menunjukkan ia mempunyai beberapa komponen flavonoid termasuk hiperin 6.35 ± 0.41, kuarsetin 0.34 ± 0.02, isokuarsetin 1.10 ± 0.03, and rutin 0.88 ± 0.04 (%w/w). Hasil toksisiti menunjukkan penurunan bergantung dos pada tumbesaran sel leukemia Jurkat. Corak 'blebbing' pada sel apotosis ditemui pada sel yang dirawat ekstrak etanol H.cordata selama 24 dan 48 jam. Tambahan lagi, kami mendapati ekstrak tersebut boleh menginduksi kematian sel Jurkat melalui apoptosis pada 12 dan 24 jam. Kesimpulannya, hasil kajian menunjukkan bahawa ekstrak etanol H.cordata yang terdiri dari beberapa flavonoid mempunyai aktiviti anti-leukemik melalui induksi apoptosis pada sel leukemia Jurkat.

Kata kunci: leukemia limfoblastik akut, apoptosis, flavonoid, Houttuynia cordata Thunb, jurkat

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ABSTRACT

Development of resistance to currently used drugs and side effects of several allopathic drugs have led to increased emphasis on plant materials uses as a source of medicines for a wide variety of human illnesses including leukemia. *Houttuynia cordata* Thunb (*H.cordata*), a Northern Thailand local plant and commonly known as Plucao, has various biological activities such as anti-inflammatory, anti-cancer and anti-leukemic activities. We aimed to determine active flavonoid components of *H.cordata* and to investigate the effect of *H.cordata* ethanolic extract on apoptotic induction on human acute lymphoblastic leukemia cells. In this study, we found that *H.cordata* ethanolic extract had total flavonoid of 231.21 ± 4.19 mg QE/g dried *H.cordata*. The extract analyzed by quantitative LC-MS consists of several flavonoid components including hyperin 6.35 ± 0.41, quercetin 0.34 ± 0.02, isoquercetin 1.10 ± 0.03, and rutin 0.88 ± 0.04 (%w/w). The cytotoxicity results showed dose dependent decrease in growth of Jurkat leukemic cells. Blebbing pattern of cell apoptosis was found in cells treated with *H.cordata* ethanolic extract for 24 and 48 hrs. Moreover, we found that the extract could substantially induce Jurkat cell death through apoptosis at both 12 and 24 hrs. In conclusion, these results indicated that *H.cordata* ethanolic extract which is composed of several flavonoids, possesses anti-leukemic activity through apoptotic induction in Jurkat leukemia cells.

Keywords: acute lymphoblastic leukemia, apoptosis, flavonoid, *Houttuynia cordata* thunb, jurkat

INTRODUCTION

Acute lymphoblastic leukemia (ALL), a clonal malignancy disease of bone marrow, is a life threatening neoplasm characterized by uncontrolled growth and leukemic expansion of immature lymphoblastic progenitor cells (Inaba et al. 2013). ALL is most commonly found in children and has poor prognosis in adults. The main treatment regimen of ALL is a combination of chemotherapy, but it has many side effects. Moreover, there are more relapse and chemo resistant incidence (Winter et al. 2014). Therefore, screening and investigating anti-leukemic compounds in plants for reducing the treatment side effects are significant and needed (Russo et al. 2011).

Currently, herbal and natural product compounds are used as alternative medicine. *Houttuynia cordata* Thunb or Plucao in Thai, is in the Saururaceae family, and mostly found in Northern of Thailand and Asia. It has many bioactivities including anti-inflammation, anti-oxidative, anti-diabetes and anti-cancer (Yang & Jiang 2009; Cho et al. 2003; Wangchauy & Chanprasert 2012). Major active components of *H.cordata* are flavonoids, alkaloids and volatile oils (Xu et al. 2006; Wu

et al. 2009). Previously, several studies suggested that flavonoids played a prominent role in cancer prevention via ROS production, inflammatory regulation, cell cycle arrest and growth inhibitory pathways, cell signal transduction pathways related to cell proliferation and cell apoptosis (Wu et al. 2009; Okwu & Nnamdi 2011). In the present study, we aimed to determine active flavonoid components of *H. cordata* and to investigate the effect of *H.cordata* ethanolic extract on apoptotic induction on human acute lymphoblastic leukemia cells.

MATERIALS AND METHODS

CHEMICALS

In-vitro Toxicology assay kit, XTT Based (TOX_2), Vincristine and Aluminium chloride ($AlCl_3$) were obtained from Sigma-Aldrich (USA). Sodium nitrite ($NaNO_2$), ethyl alcohol (EtOH), and sodium hydroxide (NaOH) were purchased from MERCK (Germany). RPMI medium 1640, fetal bovine serum (FBS), penicillin/streptomycin, trypan blue and apoptosis kit were obtained from GIBCO™ Invitrogen (USA).

PLANT MATERIAL, EXTRACTION AND LC-MS

Whole plants of *Houttuynia cordata* Thunb were collected by our group in March, 2012 from Lamphun province, Northern Thailand. The plant was authenticated and a voucher specimen (BCU013515) was deposited at the Plants of Thailand Research Unit, Department of Botany, Faculty of Sciences, Chulalongkorn University, Bangkok, Thailand. *H.cordata* leaves were cleaned and air-dried at room temperature then dried for 3 days at 45˚C. *H.cordata* dried leaves were extracted with 95% EtOH and lyophilized to dry as described previously (Wangchauy & Chanprasert 2012). The lyophilized extract and flavonoid standards were dissolved in dimethyl sulfoxide (DMSO) and diluted in RPMI Medium 1640 to give final concentration of 5 mg/ml for *H.cordata* extract and 1 mg/ml for flavonoid standards, respectively. The diluted extract was analyzed for flavonoid components (%w/w) by LC-MS system with a Symmetry C18-column (Gemini C18, 250 x 4.6 mm) using a Dionex Ultimate 3000 in combination with an electrospray ionization (ESI)/ quadrupole ion trap mass spectrometer (Bruker Daltonik, Germany) at the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok, Thailand.

TOTAL FLAVONOID CONTENT ASSAY

Total flavonoid content of *H.cordata* extract was measured by $AlCl_3$ colorimetric assay as described previously (Kim et al. 2003). One ml of 500 µg/ml *H.cordata* extracts or standard Quercetin solution (7.8125-1000 µg/ml) was added to 4 ml distilled deionized water and the 0.3 ml of 5% $NaNO_2$was added to the solution. Then, after 5 min., 0.3 ml of 5% $AlCl_3$ was added. Next, at 6

min, 2 ml of 1M NaOH and 2.4 ml of distilled deionized water were added and the solution was thoroughly mixed. Afterwards, the absorbance of the mixture was measured at 510 nm versus prepared water as a blank. Total flavonoid of *H.cordata* extracts were expressed as mg quercetin equivalents (QE)/g of dried *H.cordata* material.

CELL LINE AND CELL CULTURE

Jurkat (TIB-152), human T cell leukemia cells obtained from American Type Culture Collection were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% Penicillin/ Streptomycin (final concentration of 100 units/ml penicillin and 100 mg/ml streptomycin). The leukemic cells were maintained at 37˚C in humidified 5% CO_2 environment.

CYTOTOXICITY EFFECT BY XTT-BASED COLORIMETRIC ASSAY

H.cordata extract cytotoxic effect were determined by XTT assay. Jurkat cells were prepared at 5.0 x 10^5 cells/ ml and seeded 90 µl/well to 96-well culture plate. Ten µl of serial concentration of *H.cordata* extracts (5000, 2500, 1250, 625, 312.5, 156.25 and 78.125 µg/ ml) were applied onto culture wells. Vincristine (10 ng/ml) and RPMI-1640 with 0.1% dimethyl sulfoxide were used as positive control and negative control, respectively. Then, cells were determined for cytotoxicity with TOX_2 after 48 hrs incubation at 37˚C in humidified 5% CO_2 atmosphere. The absorbance was measured at 450 nm with a reference wavelength of 690 nm using microtiter plate reader. Finally, number of viable cells was calculated and data was showed as % cell viability.

CYTOTOXICITY EFFECT BY MICROSCOPIC ANALYSIS

Concentration of 1.0 x 10^5 cells/ml of Jurkat was prepared and seeded 900 µl/well to 24–well culture plate. A hundred µl/ well of *H.cordata* extract (3000 µg/ml) was applied onto culture wells. Ten ng/ml Vincristine and RPMI-1640 with 0.1% DMSO were used as positive and negative controls, respectively. After incubation for 24 and 48 hrs at 37˚C in humidified 5% CO_2 environment, cells were assayed with an inverted light microscope.

APOPTOSIS ASSAY BY FLOW CYTOMETRY

Jurkat leukemic cells were prepared at a final concentration of 1.0 x 10^6 cells/ well. Cells were cultured in 6-well culture plates and treated with fresh RPMI medium containing *H.cordata* extract at final concentration of 300 µg/ml. Ten ng/ml Vincristine and RPMI medium 1640 with 0.1% DMSO were used as positive and negative controls, respectively. Cultured cells were incubated for 12 and 24 hrs at 37˚C in a humidified 5% CO_2 environment. Jurkat cells were harvested and incubated with Alexa Flour 488 annexin V and propidium iodide (PI) in the dark. Apoptotic cells were analyzed by FACS Calibur flow cytometer (Becton Dickinson, USA).

Table 1: Flavonoid components of *Houttuynia cordata* Thunb ethanolic extract analyzed by LC-MS.

Flavonoid compositions	%w/w
Quercetin	0.34 ± 0.02
Isoquercetin	1.10 ± 0.03
Hyperin	6.35 ± 0.41
Rutin	0.88 ± 0.04

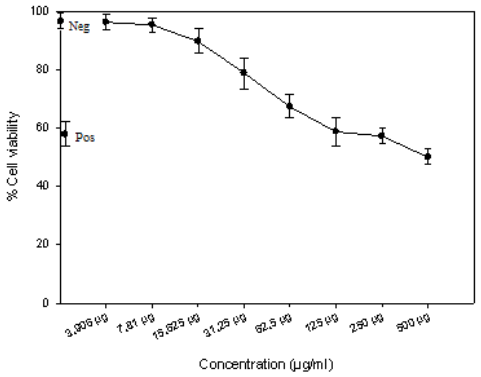


Figure 1: Effect of H. cordata ethanolic extract on Jurkat cell viability. Treated Jurkat with various concentrations of H. cordata extract. Vincristine (10 ng/ml) and RPMI-1640 with 0.1% DMSO were used as positive control and negative control, respectively. Data are shown as mean ± S.D. (n=3).

STATISTICAL ANALYSIS

Data were expressed as mean ± SEM. Differences were evaluated statistically using one-way analysis of variance (ANOVA) test. *p*-value < 0.05 was considered statistically significant.

RESULTS

H.CORDATA ETHANOLIC EXTRACT'S TOTAL FLAVONOID DETERMINATION

Total flavonoids content of *H.cordata* ethanolic extract was 231.21 ± 4.19 mg QE/g dried *H.cordata.*

H.CORDATA EXTRACT FLAVONOID COMPONENT ANALYSIS BY LC-MS

H.cordata ethanolic extract was analyzed by reverse phase-high performance liquid chromatography and mass spectrometry. We found that flavonoid components of *H.cordata* crude extract were quercetin, isoquercetin, hyperin and rutin as shown in Table 1.

CYTOTOXICITY OF *H.CORDATA* ETHANOLIC EXTRACT

Jurkat cells were treated with *H.cordata* extract at various concentrations. The *H.cordata* extract cytotoxicity was assessed as cell viability using the XTT assay as shown data in Figure 1. The concentration required for inhibiting growth by 50% (IC_{50}) for 48 hrs on Jurkat cells was 469.01 ± 6.32 µg/ml of *H.cordata* extract. The data showed that *H.cordata* extract can inhibit leukemic cell growth.

CYTOTOXICITY EFFECTS OF *H.CORDATA* EXTRACT BY MICROSCOPIC ANALYSIS

After incubation for 24 and 48 hrs of Jurkat cells with 300 µg/ml *H.cordata* extract, microscopic analysis showed

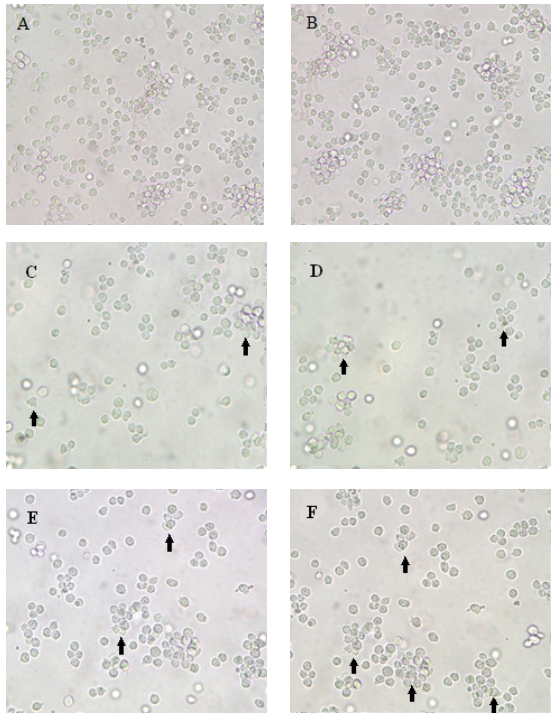


Figure 2: Cytotoxic effect of H.cordataextract on Jurkat cells by microscopic analysis.Microscopic analysis showed membrane blebbing (arrow) of apoptotic cell(A) Negative control-24 h, (B) Negative control-48 h, (C) Positive control-24 h, (D) Positive control-48 h, (E) H.cordataextract (300 g/ml)-24 h, (F) H.cordataextract (300 g/ml)-48 h.

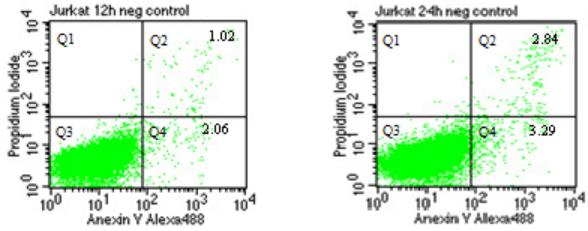
the blebbing pattern of apoptotic cell as shown in Figure 2 suggesting that *H.cordata* extract might involve the apoptotic process of leukemic cells. Interestingly, the number of Jurkat cells after treatment with *H.cordata* extract for 24 and 48 hrs were significantly lower than negative control, which means that *H.cordata* extract can inhibit leukemic cell growth that is consistent with the cytotoxicity test (XTT assay) as described above.

EFFECT OF *H.CORDATA* EXTRACT ON APOPTOTIC INDUCTION BY FLOW CYTOMETRY ANALYSIS

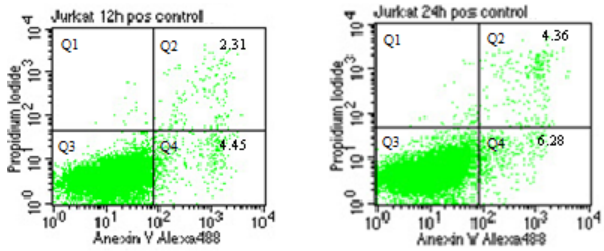
After incubation for 12 and 24 hrs of Jurkat cells with *H.cordata* extract (300 µg/ml), cells were stained with AnxV and PI. The apoptotic cells were analyzed by flow cytometer. The frequency of early apoptosis (Anx V-/PI+) and late apoptosis (Anx V+/PI+) cells are indicated by numbers in the corresponding quadrants. As shown in Figure 3, the data demonstrated that the ethanolic extract of *H.cordata* can induce a significant increment of Jurkat cell death through apoptosis at both 12 and 24 hrs compared with negative control.

DISCUSSION

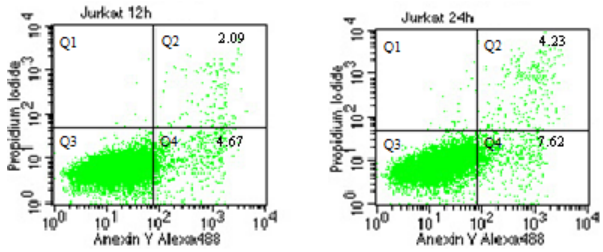
In general, most treatment plans for acute lymphoblastic leukemia have three steps. These are induction,



A) Negative control (0.1% DMSO)



B) Positve control (vincristine 10 ng/ml)



C) H. cordata extract (300 µg/ml)

Figure 3: H. cordata extract induces apoptosis in human acute lymphoblastic leukemia cells. Flow cytometry analysis of Jurkat cells treated with DMSO (A), vincristine (B) and H. cordata extract (C) for 12 and 24 hours. Representative figures showing population of viable (Q3), early apoptotic (Q4), late apoptotic (Q2) and necrotic (Q1) cells.

consolidation, and maintenance. For the purpose of improving the survival rate and preventing remission and relapse of leukemia, leukemic treatment have several methods including chemotherapy, radiotherapy and bone marrow or stem cell transplantation. Nevertheless, many side-effects such as headache, vomiting, nausea and weight-loss occurred after treatment. As a result, herbal therapy is an alternative method for leukemia treatment which diminishes side effects. Currently, there are several leukemic treatments from plant-derived compounds used in clinical including vincristine, vinblastine, camptothecin and paclitaxel (Riccardi et al. 1995; Myrick et al. 1999; Goossens et al. 2000; Liu et al. 2007). Our previous studies demonstrated that *H.cordata* Thunb extract can inhibit growth and induce apoptosis in human leukemic cells (Pawinwongchai & Chanprasert 2011; Wangchauy & Chanprasert 2012).

In present study, we found that

quercetin, isoquercetin, hyperin and rutin are the major flavonoid components in *H.cordata* Thunb extract from Northern Thailand. These results are consistent with the findings of previous study in China (Wu et al. 2009). However, flavonoid components from different sites show the different proportion of flavonoids according to the geographical origin.

In the present study, we investigated the effect of *H.cordata* extract in Jurkat leukemia cells which were the representative of ALL. We found that the extract can inhibit cell proliferation in dose dependent manner. By microscopic analysis, we found that the number of Jurkat cells after treatment with *H.cordata* extract and positive control for 24 hrs and 48 hrs were significantly lower than negative control, which means *H.cordata* extract can inhibit leukemic cell growth that consistent with the cytotoxicity test (XTT assay). Interestingly, membrane blebbing is the morphological feature of apoptosis which is found in Jurkat treated with *H.cordata* extract for both 24 hrs and 48 hrs that is similar to the cells treated with vincristine. Late apoptotic cell exhibited cytoplasmic blebbing and irregularity in shape apoptotic bodies. In the same way, *Pupalialappacea,* containing flavonoid rutin as the main component, caused the morphological changes in K562 cell by cell shrinkage and membrane blebbing when treated with the extract for 24 h (Ravi et al. 2012).

In order to confirm the above result, we investigated the apoptotic effect of *H.cordata* extract in Jurkat cells. Apoptotic death cells were analyzed in early apoptosis and late apoptosis by flow cytometry analysis. Our data once again confirmed the effect of *H.cordata* extract on induction of apoptosis in Jurkat leukemic cells, consistent with previous study of Chang et al. (2001) which demonstrated that *H.cordata* extract could inhibit 5 leukemic cells including K562, U937, L1210, Raji and P3HR1cells at IC_{50} between 478 to 662 µg/ml but was well tolerated by healthy human cell at IC_{50} greater than 1,000 µg/ml. Nevertheless, the molecular mechanisms of cell death induction by *H.cordata* extract remained unclear. The possible molecular signaling pathway of cell death in response to *H.cordata* may be via intrinsic pathway. However, intrinsic and extrinsic pathways or autophagy-associated cell death pathway still need further studies to identify in deep.

CONCLUSION

In summary, our study demonstrated that *H.cordata* ethanolic extract composed of active flavonoids and can inhibit cell proliferation and induce apoptosis in Jurkat leukemia cells. *H.cordata* may be useful in the combination of leukemia treatment in the future. However, besides the use of cell model, animal experiments and human critical trials should be employed to explore the possible applications of *H.cordata* and its flavonoids for the treatment.

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