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Journal of Chemical and Pharmaceutical Research

ISSN No: 0975-7384
CODEN(USA): JCPRC5

J. Chem. Pharm. Res., 2011, 3(4):204-212

Antileukemic activity of *Houttuynia cordata* Thunb. extracts in Jurkat and U937 human leukemic cells

Jaturawat Pawinwongchai[1], Supantitra Chanprasert[2]

[1]Graduate Program in Clinical Hematology Sciences, Department of Clinical Microscopy, Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok, Thailand
[2]Molecular Hematology Research Unit, Department of Clinical Microscopy, Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok, Thailand

ABSTRACT

Leukemia is a hematopoietic cancer and the treatment has many side effects. For this reason, herbal therapy is an alternative to reduce the side effects. Plucao or Houttuynia cordata Thunb., a local plant in Northern Thailand, has many biological activities including anti-cancer, anti-leukemic, anti-microbial, anti-oxidant, anti-inflammatory and immunomodulatory effects. The active components in Plucao are alkaloids and flavonoids. In this study, we extracted Plucao by distilled deionized water and alcohol and found that H.cordata ethanolic extract had total flavonoid content more than H.cordata water extract. Total flavonoid of H.cordata ethanolic extract was 224.90±2.80 mg QE/g dried H.cordata while total flavonoid of H.cordata water extract was 130.79±4.89 mg QE/g dried H.cordata. Moreover, H.cordata extracts could inhibit the proliferation of either Jurkat or U937 leukemic cell lines. The half maximal inhibitory concentration (IC_{50}) values of H.cordata extracts were between 403.3 to 445.1 μg/ml and 386.2 to 416.4 μg/ml, respectively. In addition, our cytokine study found that H.cordata extract especially H.cordata ethanolic extract substantially reduced the Tumor Necrosis Factor-alpha (TNF-α). Therefore, these finding highlight the fact that H.cordata has anti-leukemic activity and suggest its use as an alternative medicinal plant in the treatment of leukemia.

Keywords: *Houttynia cordata*; Leukemia; Anti-leukemic activity.

INTRODUCTION

Leukemia is a common hematopoietic cancer worldwide characterized by abnormal proliferation, differentiation and overproduction of white blood cells and their precursors [1-2]. The treatment of leukemia includes several methods such as chemotherapy, radiotherapy and bone marrow or stem cell transplantation, for the purpose of improving the survival rate and

preventing remission and relapse of leukemia. However, there are many side-effects such as headache, nausea, vomiting and weight-loss after treatment. As a result, herbal therapy is an alternative method of leukemia treatment which reduced side effects.

Houttuynia cordata thunb. is a local plant in Northern Thailand which has vary biological activities including anti-cancer, anti-leukemic, anti-microbial, anti-oxidant, anti-inflammatory and immunomodulatory effects [3-5]. It consists of several components such as alkaloids and flavonoids [6-7]. Flavonoids are polyphenolic compounds in plant that have biological activities such as carcinogen inactivation, antiproliferation, cell cycle arrest, induction of apoptosis, inhibition of angiogenesis and antioxidation, resulting in anti-cancer and anti-leukemic properties [8-9]. Major flavonoid components that found in *H.cordata* are quercetin, quercitrin and hyperin [4].

Cytokines are small proteins that involve in the communication and interaction between cells, which include immunomodulatory effects and control of hematopoiesis. Tumor Necrosis Factor-alpha (TNF-α) is an inflammatory cytokine associated with several human diseases including leukemia. Oster *et al.*, demonstrated that TNF-α can induce colony stimulating factor (CSF) production by endothelial cells and may provide a paracrine loop to support leukemia growth [10].

In present study, we attempt to examine the cytotoxicity effects of *H.cordata* extracts in leukemic cell lines. And we also explore *H.cordata* extracts effects in reducing the TNF-α production.

EXPERIMENTAL SECTION

Chemicals

Vincristine, *In Vitro* Toxicology assay kit, XTT Based (TOX2) and aluminium chloride ($AlCl_3$) were purchased from Sigma-Aldrich (St. louis, MO, USA). RPMI-1640, fetal bovine serum (FBS) and L-glutamine were obtained from GIBCO^{TM} Invitrogen (Grand Island, NY, USA). Penicillin was purchased from E.R. SQUIBB & SONS INC. (Princeton, NJ, USA) and Streptomycin was obtained from General Drugs House co., ltd. (Bangkok, Thailand). Ethyl alcohol, sodium nitrite ($NaNO_2$) and sodium hydroxide (NaOH) were purchased from MERCK (Germany).

Plant material

Houttuynia cordata Thunb. was collected from Lamphun province, in the northern Thailand. Its authenticity was identified and confirmed by Plants of Thailand research unit, Department of Botany, Faculty of Sciences, Chulalongkorn University, Bangkok.

Preparation of extracts

H.cordata Thunb. was cleaned and air-dried in room temperature, then incubator-dried at 45°C for 3 days. The dried of *H.cordata* Thunb. was extracted with 95% ethanol for 3 days at room temperature[1] and subsequently extracted with de-ionized distilled water for 5 hours at 70°C [11]. Then the extract was filtered and concentrated by using rotary evaporator at 45°C and lyophilized to dry.

Percent yield was calculated from formula:

$$\% \text{ yield} = \frac{\text{weight of lyophilized extract}}{\text{weight of dried H.}cordata} \times 100$$

The lyophilized *H.cordata* extracts were dissolved with RPMI-1640 to give final concentration of 5 mg/ml.

Total flavonoid content assay

Total flavonoid content was measured by aluminium chloride colorimetric assay as described by Kim et al.(2003) [12-13]. 1ml 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 culture

Human T cell leukemia (Jurkat, TIB-152) and human monocytic leukemia (U937, CRL-1593) cells were cultured in RPMI-1640 supplemented with 10% FBS, 1% L-glutamine and 1% Penicillin-Streptomycin (finally concentration of 100 units/ml penicillin and 100 μg/ml streptomycin). The cultures were maintained in a humidified incubator with 5% CO_2 at 37°C.

Cytotoxicity effect with XTT-based colorimetric assay

All leukemic cell lines were prepared at a concentration of 5.0 x 10^5 cells/ml and seeded 90 μl/well into 96-well plates. Serial concentrations of crude extracts were applied onto culture wells at a volume of 10 μl/well. Vincristine and RPMI-1640 were used as positive and negative controls. After 2 days incubation with a humidity atmosphere containing 5% CO_2 at 37°C, cells were determined for cytotoxicity with TOX2 by adding 20 μl/well and were incubated for 4 hours. The absorbance was measured by using an ELISA plate reader at 450 nm with a reference wavelength of 690 nm. Data were calculated as percent of cell viability by the following formula:

$$\% \text{ cell viability} = \frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control wells}} \times 100$$

The average cell viability obtained from triplicate determinations at each concentration was plotted as a dose response graph. The 50% inhibition concentration (IC_{50}) of the active substances was determined as the lowest concentration that reduced cell growth by 50% in treated compared to untreated culture.

Cytotoxicity effect with microscopic analysis

All leukemic cell lines were prepared in concentration of 5.0 x 10^5 cells/ml and seeded 90 μl/well into 96-well plates. Serial concentrations of crude extracts were applied onto culture wells at a volume of 10 μl/well. Vincristine and RPMI-1640 were used as positive and negative controls. After 2 days incubation with a humidity atmosphere containing 5% CO_2 at 37°C, cells were determined for cytotoxicity with inverted microscope compared to untreated cultures.

Enzyme-linked immunosorbent assay (ELISA) for cytokine assay

All leukemic cell lines were prepared in concentration of 5.0 x 10^5 cells/ml and seeded onto 6-well plate at a concentration of 5.0 x 10^5 cells/ml. U937 cells were differentiated to macrophage-like cells by treating with phorbol myristate acetate (PMA) 10 μg/ml for 24 hours. Macrophage-liked U937 and Jurkat were treated with *H.cordata* extracts for 6 hours then treated Jurkat was

stimulated with 12.5 ng/ml of PMA and 250 ng/ml of PHA for 24 hours to determine TNF-α levels. Also, treated U937 was stimulated with 1 μg/ml of LPS for 6 hours to determine TNF-α levels. The supernatants were collected and measured by commercially available ELISA kits (R&D Systems, Minneapolis, MN).

Statistical analysis

Numerical results are presented as mean±SEM. Student's *t*-test was used to compare two groups while multiple groups were compared by one-way analysis of variance (ANOVA). *p*-value < 0.05 was considered statistically significant.

RESULTS

Yield of *H.cordata* extracts

In order to determine percentage yield, the dried of *H.cordata* Thunb. was extracted with deionized distilled water and 95% ethanol. Percentage yield of *H.cordata* water extract and ethanolic extract are 14.1% and 14.28%, respectively (Table 1).

Table 1. Percent yield and total flavonoid content in *H.cordata* extract (*n*=3)

H.cordata extract	%yield	Total flavonoid (mg QE/g)
Water extract	14.1	130.79±4.89
Ethanolic extract	14.28	224.90±2.80

Determination of total flavonoid of *H.cordata*

Total flavonoids content of H.*cordata* water extract is 130.79±4.89 mg QE/g dried *H.cordata* and ethanolic extract is 224.90±2.80 mg QE/g dried H.*cordata* (Table 1). Total flavonoid content of water extract has significantly lower than ethanolic extract ($p < 0.05$) which means ethanol can extract the flavonoid component from *H.cordata* better than water.

Cytotoxicity effect of *H.cordata* extracts cell viability

After incubation of leukemic cell lines (Jurkat and U937) with *H.cordata* extracts in various concentrations. The cytotoxicity of *H.cordata* extract and three flavonoids were determined and showed in Table 2. IC_{50} values of water extract was higher than that of ethanolic extract. The cytotoxicity in Jurkat cells was higher than U937 cells as shown in Figure 1 and Figure 2. The data of cytotoxicity effect experiments have shown that *H.cordata* extract can induce the apoptosis of both Jurkat and U937 cell lines suggesting that its alkaloids and flavonoids play a critical role in program cell death induction of leukemic cells. Several previous studied had reported that the alkaloids and flavonoids have several activities such as blocking the tyrosine phosphorylation [14], arresting the cells in late G1 phase [15-16], inducing of caspase-3 activity and inducing the release of cytochrome C to activation of caspase-9 [17], inhibiting DNA topoisomerase I [18] and inducing the expression of CD95 to induced apoptosis [19].

Table 2. IC_{50} values (μg/ml) of crude extracts of *H.cordata* on Jurkat and U937 cells (*n*=3)

	IC_{50} (μg/ml) (Mean±SEM)	
	Jurkat	U937
Ethanolic extract	403.30±5.35	386.20±5.28
Water extract	445.10±6.49	416.40±6.78

Figure 1. Effect of *H.cordata* extracts on Jurkat. Treated Jurkat with various concentrations of *H.cordata* extracts. (*n*=3) (A) *H.cordata* water extract and (B) *H.cordata* ethanolic extract

A. *H.cordata* water extract B. *H.cordata* ethanolic extract

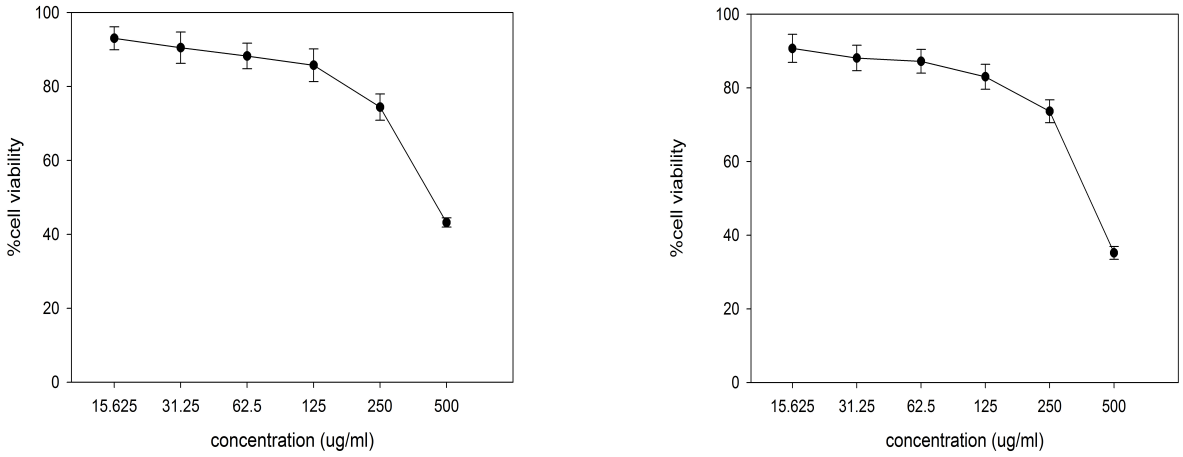


Figure 2. Effect of *H.cordata* extracts and flavonoids on U937. Treated U937 with various concentrations of *H.cordata* extracts. (*n*=3) (A) *H.cordata* water extract and (B) *H.cordata* ethanolic extract

A. *H.cordata* water extract B. *H.cordata* ethanolic extract

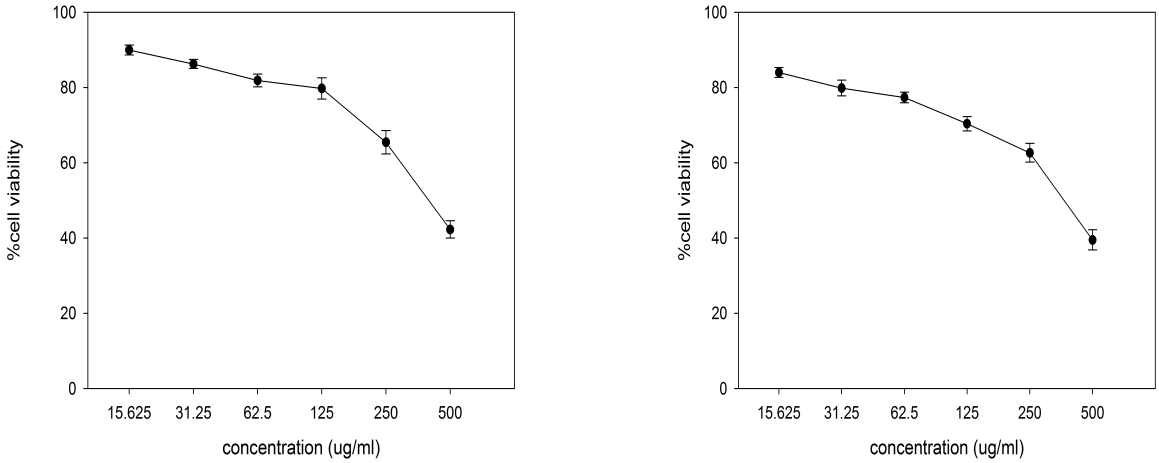
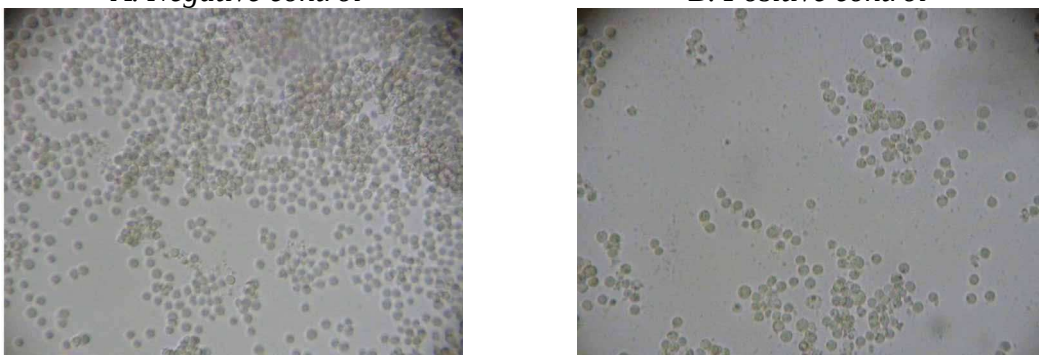
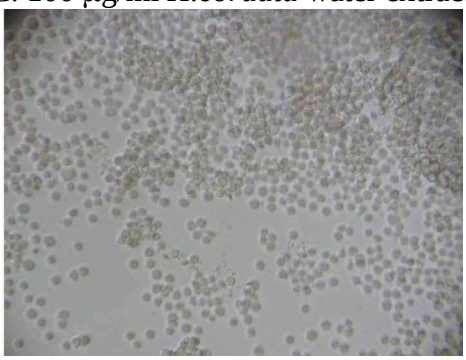


Figure 3. Cytotoxicity effect of *H.cordata* extracts on Jurkat cells by Microscopic analysis. Treated Jurkat cells with 100 µg/ml and 500 µg/ml. A. Negative control, B. Positive control, C. 100 µg/ml *H.cordata* water extract, D. 500 µg/ml *H.cordata* water extract, E. 100 µg/ml *H.cordata* ethanolic extract and F. 500 µg/ml *H.cordata* ethanolic extract.

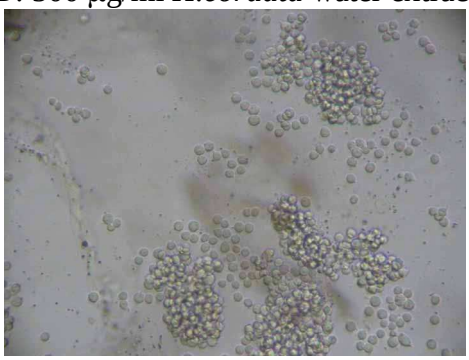
A. Negative control *B. Positive control*



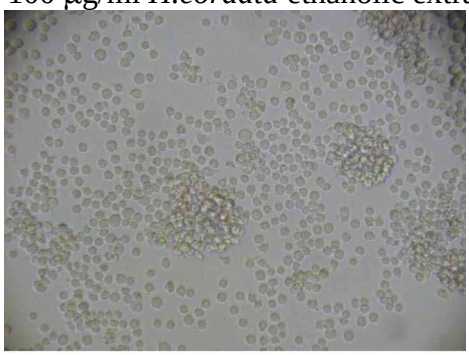
C. 100 µg/ml *H.cordata* water extract



D. 500 µg/ml *H.cordata* water extract



E. 100 µg/ml *H.cordata* ethanolic extract



F. 500 µg/ml *H.cordata* ethanolic extract

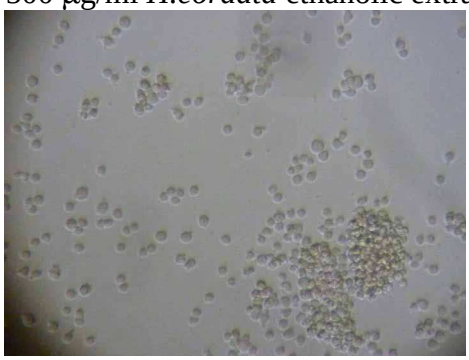
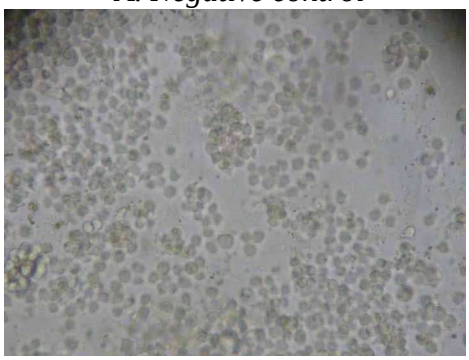
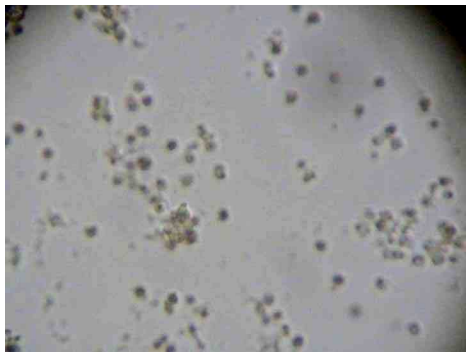


Figure 4. Cytotoxicity effect of *H.cordata* extracts on U937 by Microscopic analysis. Treated U937 cells with 100 µg/ml and 500 µg/ml. A. Negative control, B. Positive control, C. 100 µg/ml *H.cordata* water extract, D. 500 µg/ml *H.cordata* water extract, E. 100 µg/ml *H.cordata* ethanolic extract and F. 500 µg/ml *H.cordata* ethanolic extract.

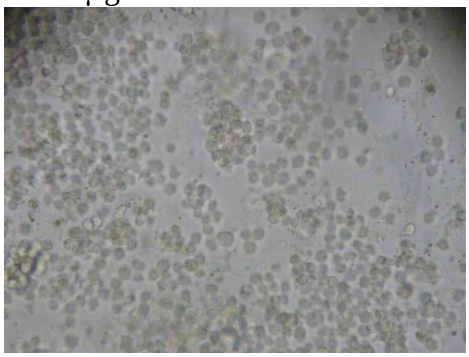
A. Negative control



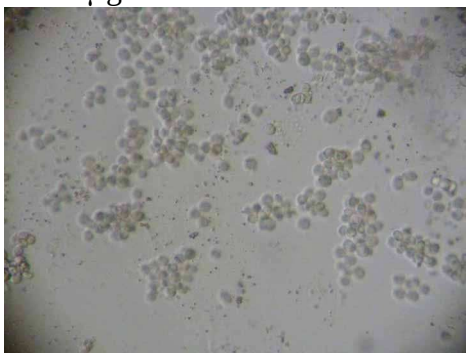
B. Positive control



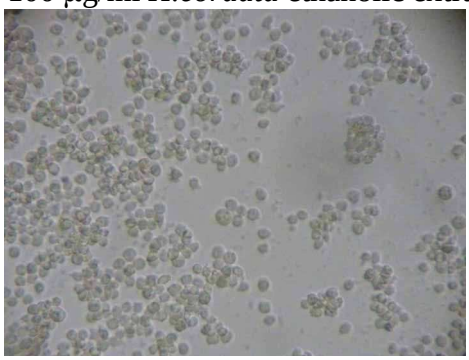
C. 100 µg/ml *H.cordata* water extract

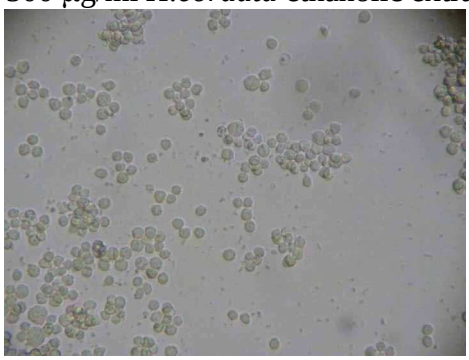


D. 500 µg/ml *H.cordata* water extract



E. 100 μg/ml *H.cordata* ethanolic extract F. 500 μg/ml *H.cordata* ethanolic extract





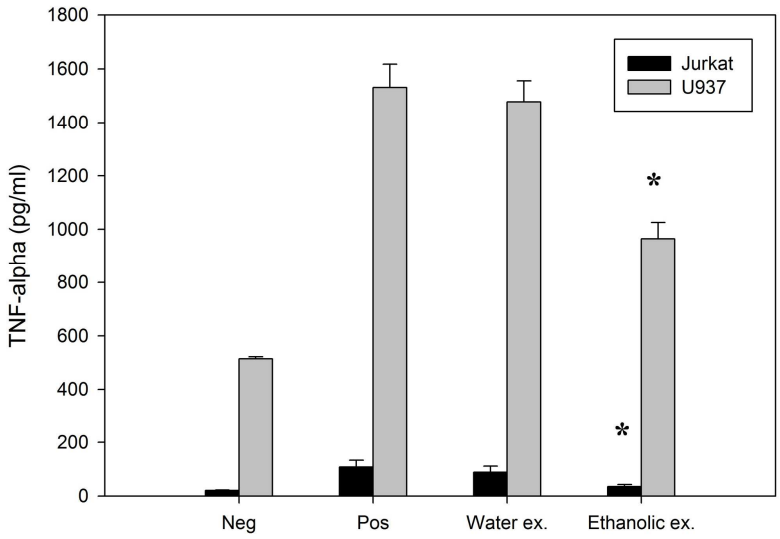
Cytotoxicity effect of *H.cordata* extracts by microscopic analysis

After incubation of leukemic cell lines (Jurkat and U937) with *H.cordata* extracts in 100 and 500 μg/ml. Jurkat cell contents in 500 μg/ml are lower than 100 μg/ml in Figure 3 and 4, which means *H.cordata* extract can induce the apoptosis of both Jurkat and U937 cell lines with similar results with the XTT assay.

Effect of *H.cordata* extracts on TNF-α production

The cytokine productions of Jurkat and U937 cells were determined by ELISA and illustrated in Figure 5. As shown in Table 3, *H.cordata* ethanolic extract can reduce the stimulated TNF-α production in Jurkat and U937 cells. The data showns *H.cordata* ethanolic extract can substantially reduce the level of TNF-α suggesting that *H.cordata* ethanolic extract is a potent anti-leukemic agent. The TNF-α expression of Jurkat cells was regulated by NF-κB and AP-1 from the MAPK pathway [20-21] and by NF-κB activation in U937 cells [22]. That means *H.cordata* ethanolic extract can reduce the TNF-α secretion by blocking the tyrosine kinase in the MAPK pathway and blocking the phosphorylation of IκB to prevent the NF-κB dissociation from IκB.

Figure 5. Effect of *H.cordata* extracts on TNF-α production by ELISA (*n*=3).



** statistically significant at p <0.05*

Table 3. Effect of *H.cordata* extracts on TNF-α production by ELISA (*n*=3)

	TNF-α (pg/ml)(Mean±SEM)	
	Jurkat	**U937**
Negative control	20.76±2.02	513.73±8.25
Positive control	107.83±25.39	1531.22±86.86
Water extract 350 µg/ml	88.51±22.66	1477.71±77.80
Ethanolic extract 350 µg/ml	35.26±7.72*	964.93±60.41*

** statistically significant at p <0.05*

CONCLUSION

From our study, *H.cordata* has anti-leukemic activity and may be one of alternative medicinal plant for leukemic treatment.

Acknowledgements
This work was supported by Thailand Research Fund (TRF) and Center for Research and Development in Molecular Hematology Sciences in the Chulalongkorn University Centenary Academic Development Project. We gratefully acknowledge Dr.Ponlapat Rojnukarin for his guidance and Dr. James M. Brimson for critical reading our manuscript.

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