Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2012, 4(5):2590-2598

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Effects of *Houttuynia cordata* Thunb extract, Isoquercetin and Rutin on cell growth inhibition and apoptotic induction in K562 human leukemic cells

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Supported, in part, by the 90th anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot endowment fund) to C.W., and by the Thailand Research Fund (TRF) and Center for Research and Development in Molecular Hematology Sciences in the Chulalongkorn University Centenary Academic Development Project to S.C..

ABSTRACT

Houttuynia cordata Thunb. (H.cordata), which is local plant in Northern Thailand, has a wide range of biological activities including anti-cancer and anti-inflammatory activities. It consists of several components such as flavonoids, volatile oils and alkaloids. Major representatives of flavonol subgroup of flavonoids found in H.cordata are rutin, quercetin, quercitrin, isoquercitrin and hyperin. Despite the fact that flavonoids show various beneficial properties, there are few studies involving isoquercetin and rutin. In this study we aimed to investigate whether H.cordata extract, isoquercetin and rutin possess anti-leukemic effects through an apoptotic pathway in K562 human leukemic cells. Using the XTT assay we found that H.cordata extract, isoquercetin and rutin inhibited growth of K562 cells with IC_{50} values of 414.35 ± 5.78 µg/ml, 71.14 ± 4.49 µg/ml and 98.56 ± 6.35 µg/ml, respectively. In microscopic analysis, blebbing pattern of cell apoptosis was found in cells treated with isoquercetin (50 µg/ml) and H.cordata extract (250 and 300 µg/ml) at 48 h. Furthermore, H.cordata extract and isoquercetin induced a significant increase in K562 cells death through apoptosis at 12 and 24 h after analyzed by flow cytometry. In conclusion, our finding highlight the fact that H.cordata has anti-leukemic activity via apoptotic induction but certainly much more molecular mechanism research is needed.

INTRODUCTION

Leukemia is one of the most prevalent diseases and is a major cause of death worldwide. The mainstay of leukemia treatments including chemotherapeutic agents, bone marrow transplantation and radiation therapy are effective in an improvement of the survival rate and the quality of life of the patients. However, such treatments have been shown many side-effects. Therefore, an alternative treatment for leukemia must be studied and developed [1]. The research on screening and investigating of pharmaceutical anti-leukemic compounds in plants for reducing the treatment's side effects were important and needed [2]. Current plant-derived compounds for leukemia treatments used in clinical including vincristine, vinblastine, camptothecin and paclitaxel [3-6].

Houttuynia cordata Thumb.(*H.cordata*) or Plucao or Pak-Kao-Thong in Thai is a member of the Saururaceae family which is a local popular side-dish vegetable mainly found in the Northern region of Thailand. It is also used as medical herb according to its wide range of biological activities including anti-cancer, anti-allergy, anti-viral, anti-bacterial, anti-oxidant and anti-inflammatory activities. It composes of major active ingredients such as flavonoids, volatile oils and alkaloids [7, 8]. Flavonoids are polyphenolic and food-derived compounds which are prominent components in plant. Guo and Xu (2007) have reported a yield of flavonoids in *H.cordata* reached 0.586% [9]. The major identified flavonoids in *H.cordata* are rutin, quercetin, quercitrin, isoquercitrin and hyperin [8]. Previous

studies suggested that flavonoids play an important role in cancer prevention via reactive oxygen species production, inflammatory regulation, cell cycle arrest and growth inhibitory pathways, cell signal transduction pathways related to cellular proliferation and apoptosis [8-10]. Despite flavonoids show various beneficial properties there are few studies involving rutin and isoquercetin, the major representatives of the flavonol subgroup of flavonoids.

Apoptosis is a mode of programmed cell death that is necessary to maintain homeostasis during the development and multicellular organisms. Apoptosis is an energy dependent, controlled type of cell death, characterized by cell shrinkage, plasma membrane blebbing, phosphatidylserine (PS) externalization, nuclear condensation, chromatin aggregation, DNA fragmentation and formation of apoptotic bodies. Apoptosis can be triggered by two major mechanisms including the extrinsic pathway associated with stimulation of extrinsic signals to surface receptors including CD95/Fas, tumor necrosis factor (TNF) and death receptors (DRs) and the intrinsic pathway involving mitochondrial dysfunction and formation of a pore in mitochondrial membrane called the Permeability Transition pore (PT pore). The release of cytochrome C from the mitochondria is a particularly important event in the induction of apoptosis [11, 12]. Problems with the regulation of apoptosis have been implicated in a number of diseases including cancer. Recently, we demonstrated the anti-leukemic activity of *H.cordata* extract in Jurkat and U937 leukemic cell lines[13]. However, the molecular mechanisms underlying apoptotic induction by *H.cordata* extract are still unclear. In this study, we first investigated the effects of *H.cordata* extract, isoquercetin and rutin in terms of cytotoxicity and apoptotic induction on the K562 human leukemic cell line.

EXPERIMENTAL SECTION

Chemicals

Aluminium chloride ($AlCl_3$), *In Vitro* Toxicology assay kit, XTT Based (TOX2), Vincristine, Rutin and Isoquercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI Medium 1640, Fetal bovine serum, L-glutamine, Penicilin/streptomycin and Apoptosis Kit (VYBANT APOPTOSIS ASSAY KIT) were purchased from GIBCO^{TM} Invitrogen (Grand Island, NY, USA).Dimethyl sulphoxide (DMSO), Ethyl alcohol, Sodium nitrite ($NaNO_2$) and Sodium hydroxide (NaOH) were obtained from MERCK (Germany)

Plant materials

Houttuynia cordata Thunb. used in this study was collected from Lamphun province, in the Northern Thailand, and then was identified and confirmed its authenticity by the Plants of Thailand research unit, Department of Botany, Faculty of Sciences, Chulalongkorn University, Bangkok, Thailand.

Preparation of ethanolic extract and flavonoid standards

The stem and leaf of *H.cordata* was cleaned and air-dried at room temperature, then incubator-dried at 45 $^{\circ}$C for 3 days. The dried *H.cordata* was extracted with 95% ethanol for 3 days at room temperature. Then the extract was filtered and concentrated by using rotary evaporator at 55 $^{\circ}$C and lyophilized to dry.

Percent yield was calculated from the formula:

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

The lyophilized *H.cordata* extract and flavonoid standards (Rutin, Isoquercetin) were dissolved in DMSO and diluted in RPMI Medium 1640, where the concentration of DMSO did not exceed 0.1% in culture to give final concentration of 5 mg/ml for *H.cordata* extract and 1 mg/ml for isoquercetin and rutin.

Total flavonoid content assay

Total flavonoid content in *H.cordata* extract was determined using aluminium chloride colormetric assay as previously described by our group [13]. Rutin was used as standard at concentrations of (0, 7.8125, 15.625, 31.25, 62.5, 125, 250, 500, and 1,000 μg/ml). Total flavonoid content of *H.cordata* extract was expressed as mg Rutin equivalents (RE)/g of dried H.cordata material.

Cell culture

Human erythromyeloblastoid leukemia cell line (K562, CCL-243) were cultured in RPMI Medium 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The cultures were maintained in a humidified incubator with 5% CO_2 at 37 $^{\circ}$C. Cells in logarithmic growth phase were used for further experiments.

Cytotoxicity effect with XTT-based colorimetric assay

The cytotoxic effects of H.cordata extract, isoquercetin and rutin on K562 cells were determined by using XTT-based colorimetric assay as described by Jaturawat *et al*. [13]. Briefly, K562 cells were prepared at a concentration of 5.0 x 10^5 cells/ml and seeded 90 µl/well into 96 well plate. Serial concentration of *H.cordata* extracts and flavonoid standard as rutin and isoquercetin were applied onto culture wells at a volume of 10 µl/well. Vincristine and RPMI medium 1640 with 0.1% DMSO were used as positive control and negative control, respectively. After 48 hours incubation with 5% CO_2 at 37 $^{\circ}$C in a humidified incubator, cells were determined for cytotoxicity with TOX2 by adding 20 µl/well and 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 is represented as percent of cell viability by 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 (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

K562 cells were prepared at a concentration of 1.0 x 10^5 cells/ml and seeded 900 µl/well into 24 –well plates. Serial concentrations of *H.cordata* extract, Rutin and Isoquercetin standards were applied onto culture wells at a volume of 100 µl/well. Vincristine 10 ng/ml and RPMI medium 1640 with 0.1% DMSO were used as positive and negative controls, respectively. After 24 and 48 hours incubation in a humidified incubator with 5% CO_2 at 37 $^{\circ}$C, cells were assayed for cytotoxicity with an inverted light microscope compared to untreated cultures.

Flow cytometry analysis of apoptosis

K562 cells were prepared at a concentration of 1.0 x 10^6 cells/well and cultured in 6-well plates and treated with fresh medium containing *H.cordata* extract at final concentration of 250 µg/ml, Rutin and Isoquercetin at final concentration of 50 µg/ml. Vincristine 1 ng/ml and RPMI medium 1640 with 0.1% DMSO were used as positive and negative controls, respectively. Cultured cells were incubated for 12 and 24 hours in a humidified incubator with 5% CO_2 at 37°C. Approximately 1x10^6 cells were analyzed for annexin-V binding using Annexin V- Alexa488 and propidium iodide (PI). Data were analyzed by FACSCalibur flow cytometer (Becton Dickinson, USA).

Statistical analysis

Numerical results were evaluated statically expressed as mean ± SEM. and evaluated using one-way analysis of variance (ANOVA). *p*-value < 0.05 was considered statistically significant.

RESULTS

Yield extracts and total flavonoid of *H.cordata*

In order to determine percentage yield, the dried of *H.cordata* was extracted with 95% ethanol and percentage yield was 10.28%. Total flavonoid content of *H.cordata* extract is 258.12±8.78 mg RE/g dried *H.cordata*.

Effects of *H.cordata* extract, Isoquercetin and Rutin on cell viability

The cytotoxicity of the *H.cordata* extract, isoquercetin and rutin were assessed as the cell viability. K562 cells were treated with *H.cordata* extract and 2 flavonols at various concentrations. After incubation, cell viability was determined using the XTT assay as shown the results in Figure 1. The concentration required for inhibiting growth by 50% (IC_{50}) for 48 h on K562 cells was 414.35 ± 5.78 µg/ml of *H.cordata* extract, 71.14 ± 4.49 µg/ml of isoquercetin and 98.56 ± 6.35 µg/ml of rutin, respectively. The data of cytotoxicity effect experiments have shown that *H.cordata* extract and the 2 flavonols can inhibit leukemic cell growth. Rutin compared with isoquercetin at the same concentration was less cytotoxic to K562 cells.

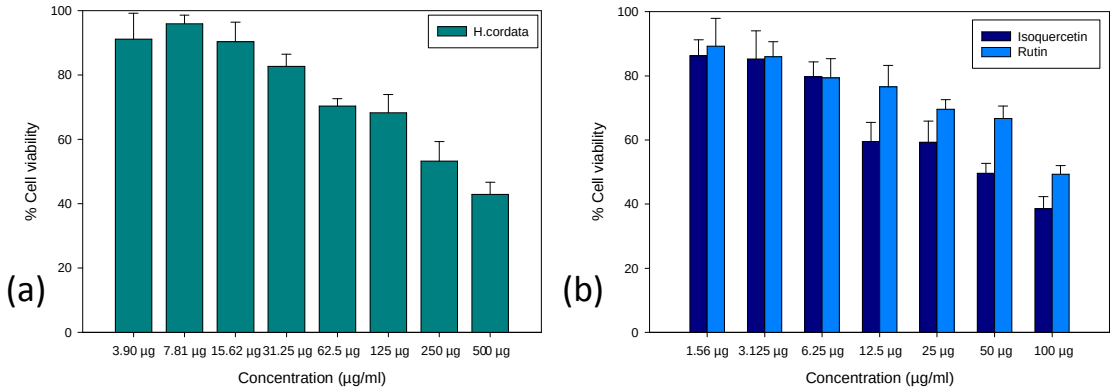
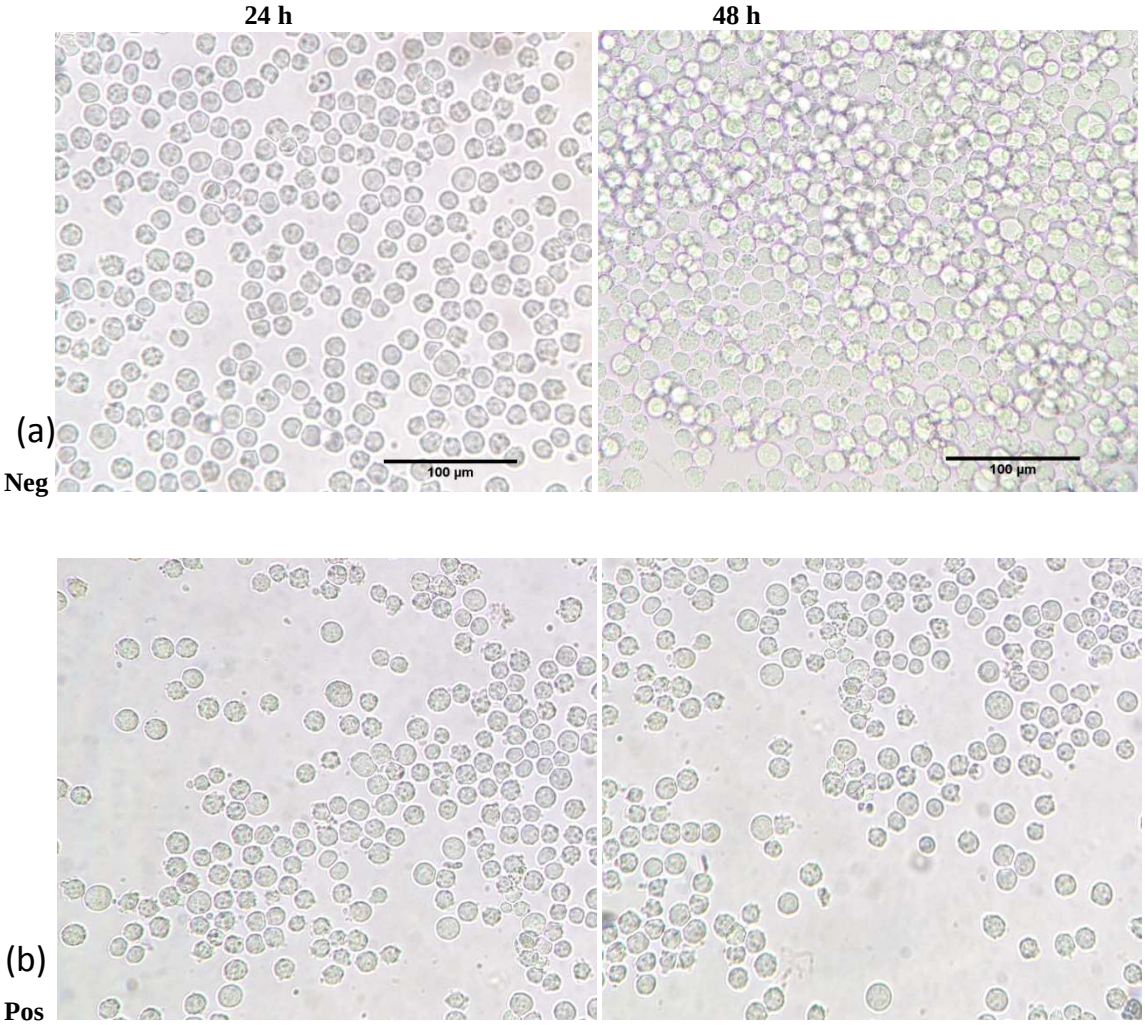
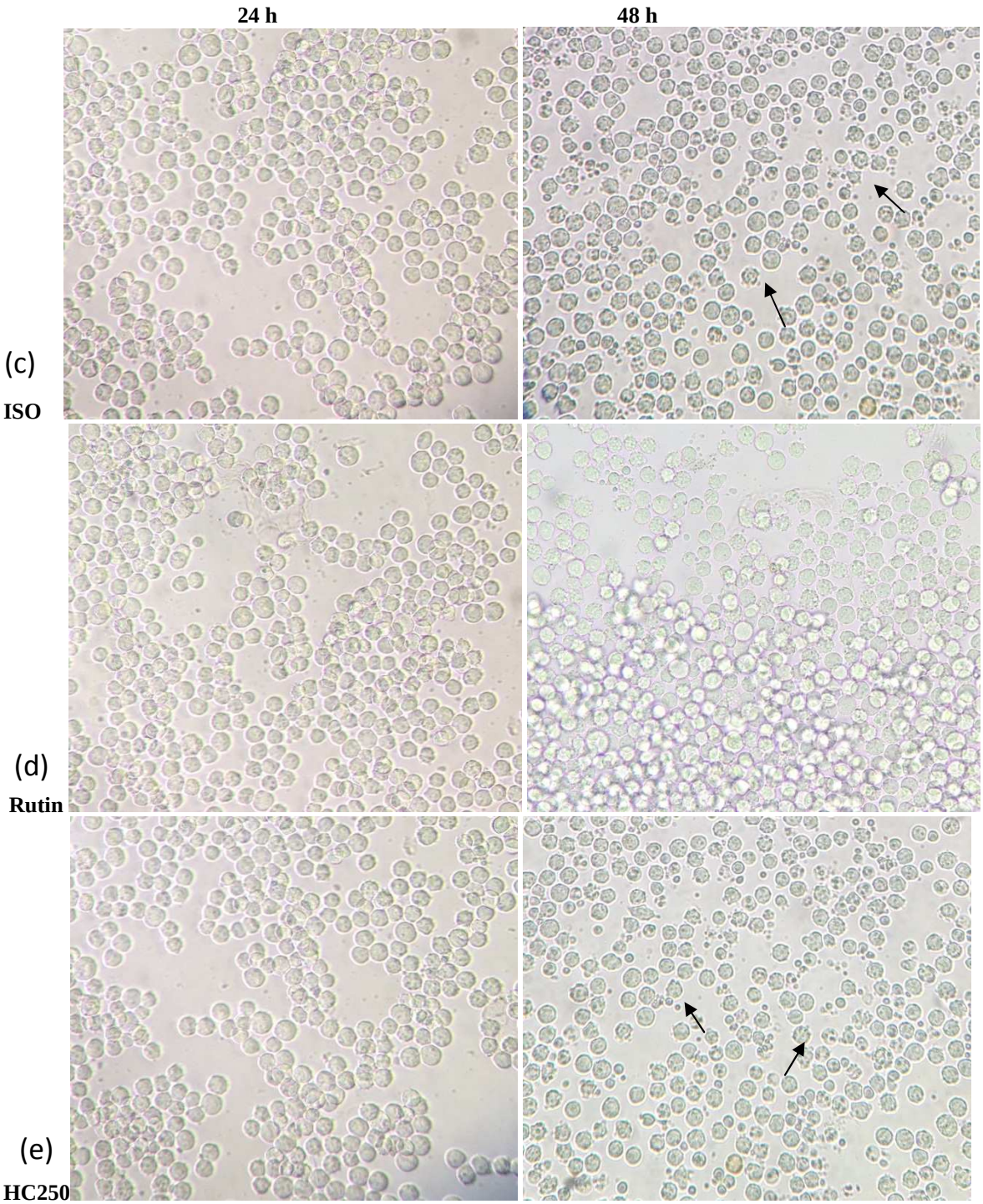


Figure 1. Effects of ethanolic *H.cordata* extract, isoquercetin and rutin on K562 cells; (a) *H.cordata* extract, (b) isoquercetin and rutin

Cytotoxicity effects of *H.cordata* extract, Isoquercetin and Rutin with microscopic analysis

After incubation of K562 cells with *H.cordata* extract (250 and 300 µg/ml), isoquercetin and rutin (50 µg/ml) for 24 and 48 h, microscopic analysis showed the blebbing pattern of cell apoptosis in isoquercetin and *H.cordata* extract (250 and 300 µg/ml) at 48 h as shown in Figure 2 suggesting that cytotoxicity of isoquercetin and *H.cordata* extract might involve the apoptosis process. Moreover, K562 cell contents treated with *H.cordata* extract, isoquercetin and rutin at 48 h were significantly lower than negative control, which means *H.cordata* extract and the flavonols can inhibit cell growth with similar results with the XTT assay as described above.




24 h
48 h
(c)
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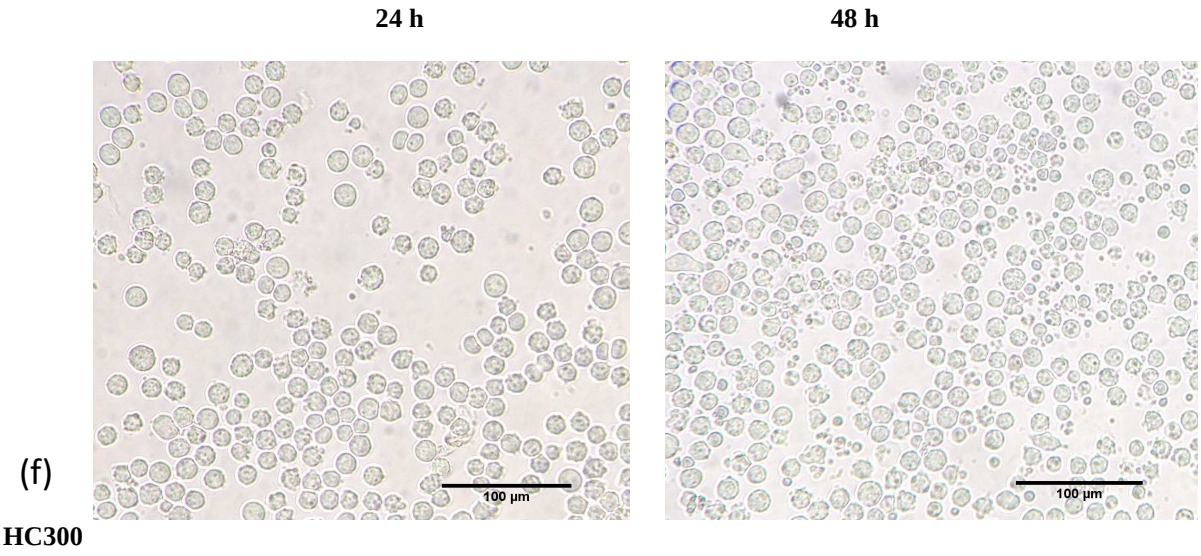
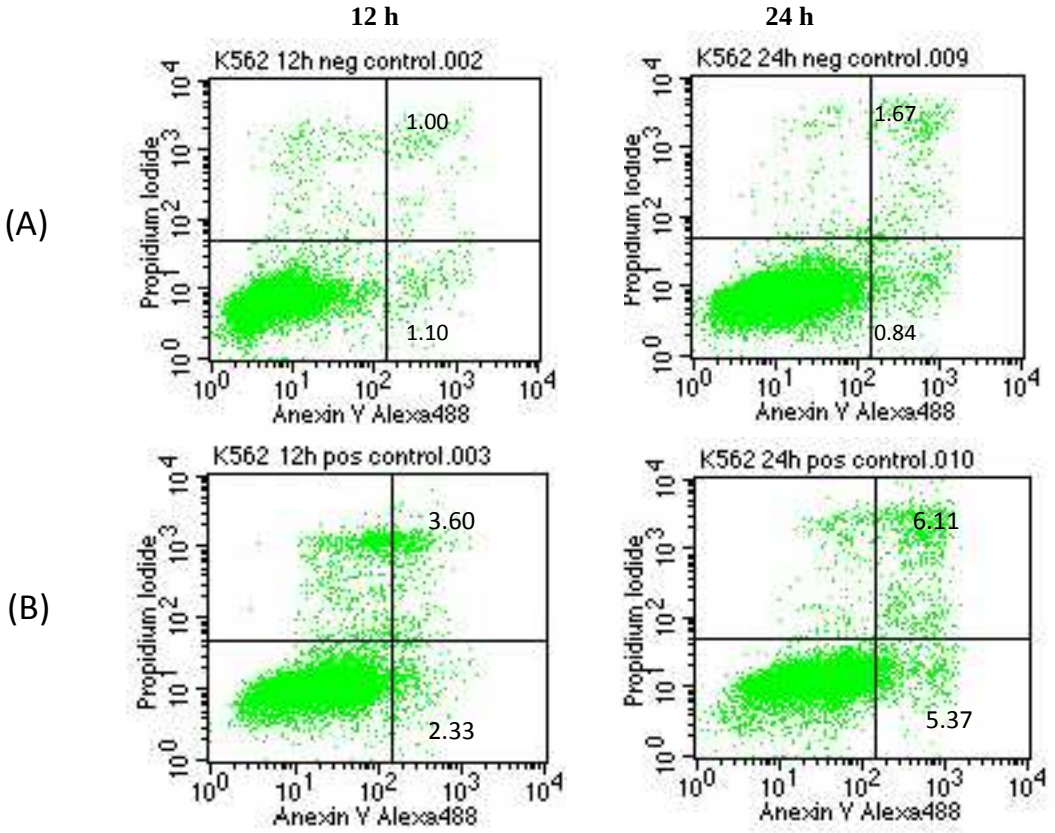


Figure 2.Cytotoxic effects of *H.cordata* extract, isoquercetin and rutin on K562 cells by microscopic analysis; (a) Negative control, (b) Positive control, (c) Isoquercetin, (d) Rutin, (e) *H.cordata* extract 250 µg/ml, (f) *H.cordata* extract 300 µg/ml after 24 and 48 hours treated cells.

Effect of *H.cordata* extract, rutin and isoquercetin with flow cytometry analysis

In order to confirm the finding of apoptosis in microscopic analysis, we determined the effects of phosphatidylserine on the cell surface after incubation of K562 cells with *H.cordata* extract (250 µg/ml), rutin and isoquercetin (50 µg/ml) for 12 and 24 h. Cells were stained with Annexin V- Alexa488 (AnxV) and propidium iodide (PI) and apoptotic cells were determine by flow cytometry, the frequency of early apoptosis (Anx V-/PI+) and late apoptosis (Anx V+/PI+) cells is indicated by numbers in the corresponding quadrants. *H.cordata* extract and isoquercetin induced a significant increase in K562 cells death through apoptosis at 12 and 24 h as shown in Figure 3 and Figure 4.



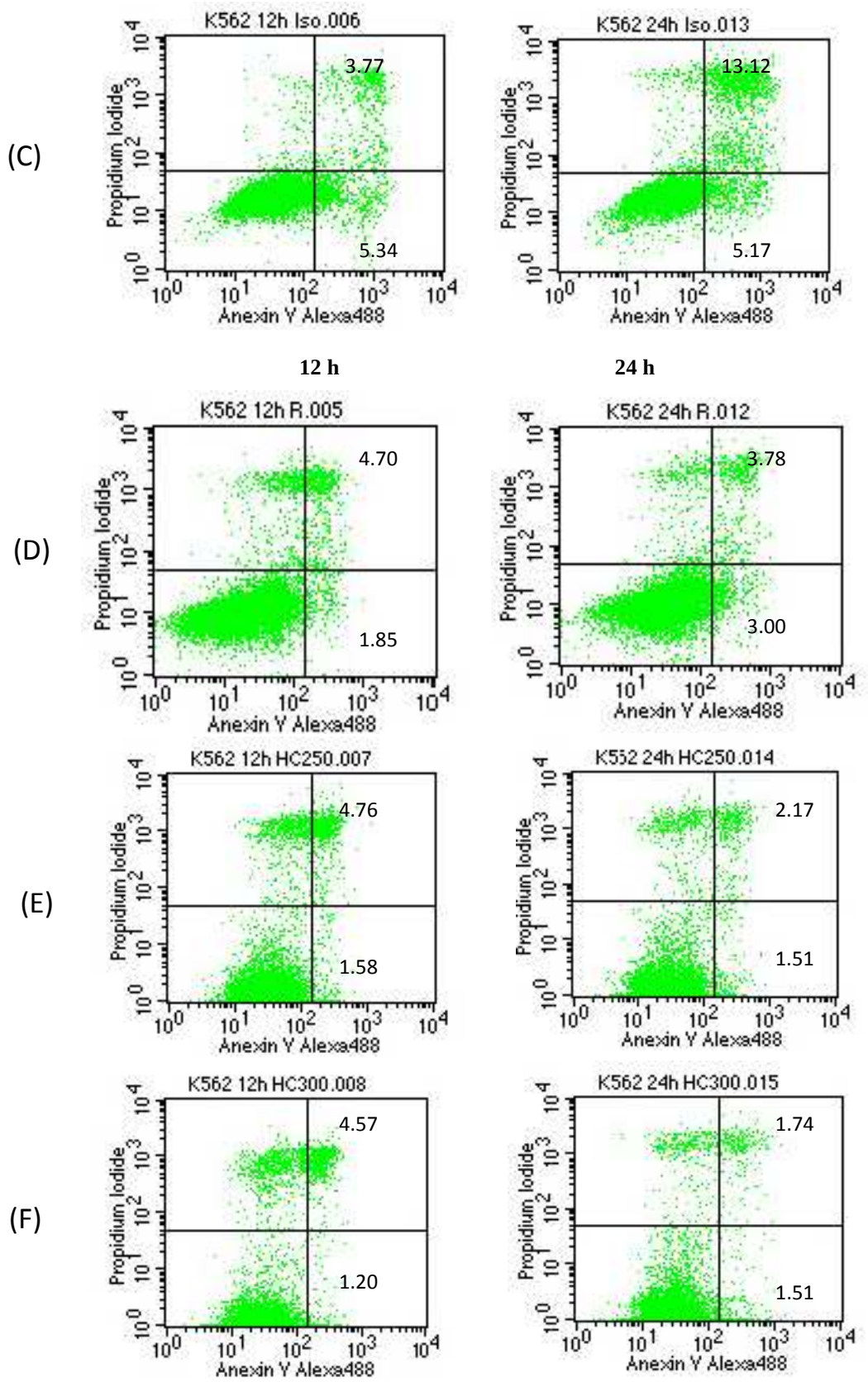


Figure 3. Apoptosis of K562 cells under effect of *H.cordata* extract, isoquercetin and rutin by flow cytometry; (A) Negative control, (B) Positive control, (C) Isoquercetin, (D) Rutin, (E) *H.cordata* extract 250 µg/ml, (F) *H.cordata* extract 300 µg/ml after 12 and 24 hours treated cells.

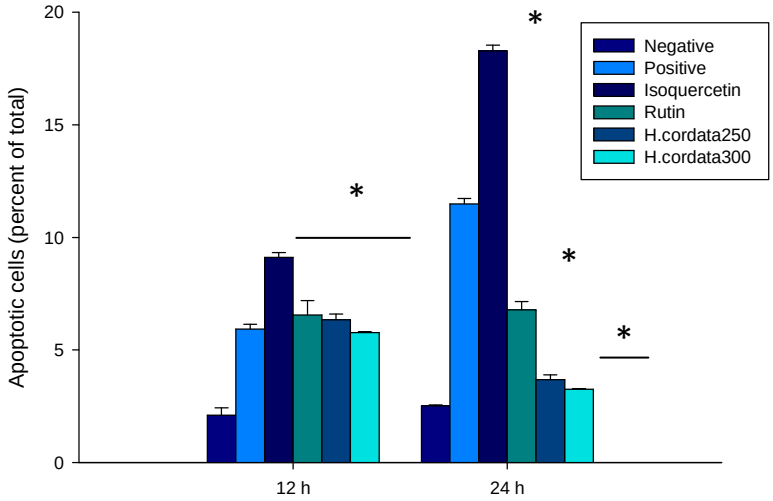


Figure 4. Apoptosis of K562 cells under the effect of *H.cordata* extract, isoquercetin and rutin by flow cytometry. Cell death is shown as percentage of total apoptotic cells (early apoptosis; Anx V-/PI+ and late apoptosis; Anx V+/PI+). Negative cells were treated cells with 0.1% DMSO and positive control for apoptosis were 10 ng/ml vincristine treated cells. * p< 0.05; significance compared with negative control.

DISCUSSION AND CONCLUSION

Previously, we have demonstrated that total flavonoids from ethanolic extract of *H.cordata* were higher than water extract and can reduce the TNF-α secretion in Jurkat and U937 cells [13]. In present study, we investigated the molecular mechanism of *H.cordata* extract, isoquercetin and rutin on apoptotic induction in K562 cells. We found that *H.cordata* extract, isoquercetin and rutin could substantially induce apoptosis in K562 cells by microscopic analysis. Late apoptotic cell exhibited cytoplasmic blebbing and irregularity in shape apoptotic bodies as shown in Figure 2. Apoptotic death cells were characterized by staining with annexin V and PI and were analyzed in early apoptosis (Annx V+/PI-) and late apoptosis (Annx V+/PI+), our results once again confirmed the effect of *H.cordata extract*, isoquercetin and rutin on apoptotic induction in K562 leukemic cell as shown in Figure 3. Several studies, Chang *et al* (2001) demonstrated that *H.cordata* extract could inhibit 5 leukemic cells; U937, K562, Raji, P3HR1 and L1210 cells (IC_{50} between 478 µg/ml to 662 µg/ml but is well tolerated by healthy human cell IC_{50} > 1,000 µg/ml) but the molecular mechanisms of *H.cordata* extract induced cell death remained unclear[14]. Tang *et al* (2009) have reported that 50% ethanol extract of *H.cordata* increased ROS production and loss of mitochondrial membrane potential (MMP) in human colon adenocarcinoma HT-29 cells (IC_{50} was 435 µg/ml). *H.cordata* extract caused the release of cytochrome C, Apaf-1, pro-caspase-9 and AIF from mitochondria in treated HT-29 cells. They suggest that 450 µg/ml of *H.cordata* extract may induce apoptosis in HT-29 cells through a mitochondria-dependent pathway [15]. In addition, recently report investigated the anticancer effect of *H.cordata* extract and the associated molecular mechanism in human primary colorectal cancer and found that *H.cordata* extract induction of apoptosis through mitochondria-dependent apoptotic signaling pathway. The decrease in the level of MMP was associated with a decrease in the ratio of Bax/Bcl-2 and led to activation of caspased-9 and caspased-3 [16]. The possible molecular signaling pathway of cell death in response to *H.cordata*, isoquercetin and rutin flavonoids may be via intrinsic pathway. Nevertheless, intrinsic and extrinsic pathways or autophagy-associated cell death pathway still need further study to identify in deep.

In conclusion, our results demonstrated that the ethanolic extract of *H.cordata*, isoquercetin and rutin flavonoids have anti-leukemic activity and could inhibit leukemic cell growth by induction of apoptosis and *H.cordata* may be useful in anti-leukemic therapy in the future.

Acknowledgements
This study was supported, in part, by the Thailand Research Fund (TRF) and Center for Research and Development in Molecular Hematology Sciences in the Chulalongkorn University Centenary Academic Development Project to SC., and by the 90th anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot endowment fund) to CW.. The authors would like express gratitude to Faculty of Allied Health Sciences and Faculty of Medicine, Chulalongkorn University for scientific equipments. Finally, we gratefully acknowledge to Prof.Dr.Pornthep

Tiensiwakul and Assist.Prof.Jittawat Rungjerdpha for meaningful guidance, Dr. Supranee Buranapraditkun for the technical support, and Dr. James M. Brimson for critical reading our manuscript.

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