

ผลของสารสกัดจากดอกสารภี (*Mammea siamensis*) ต่อการแสดงออกของ  
โปรตีนฟิมส์ไลค์ ไทโรซีนไคเนส 3 ในเซลล์มะเร็งเม็ดเลือดขาวเพาะเลี้ยงชนิด EoL-1

Effects of Saraphi (*Mammea siamensis*) flower extracts on cell proliferation and  
Fms-like tyrosine kinase 3 expression in leukemic EoL-1 cell line

รุ่งกาญจน์ สังขรักษ์<sup>1</sup>    สิงห์คำ ทิมา<sup>1</sup>    เมธี รุ่งโรจน์สกุล<sup>2,3</sup>  
Rungkam Sangkaruk<sup>1</sup>    Singkome Tima<sup>1</sup>    Methee Rungrojsakul<sup>2,3</sup>  
สาวตรี เขียมพานิชยกุล<sup>1</sup>    ทรงยศ อнуชปรีดา<sup>1</sup>  
Sawitree Chiampanichayakul<sup>1</sup>    Songyot Anuchapreeda<sup>1</sup>

<sup>1</sup>แขนงวิชาจุลทรรศน์ศาสตร์คลินิก ภาควิชาเทคนิคการแพทย์ คณะเทคนิคการแพทย์ มหาวิทยาลัยเชียงใหม่ จังหวัดเชียงใหม่

<sup>1</sup>Division of Clinical Microscopy, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand

<sup>2</sup>ภาควิชาวิทยาศาสตร์เภสัชกรรม คณะเภสัชศาสตร์ มหาวิทยาลัยเชียงใหม่ จังหวัดเชียงใหม่

<sup>2</sup>Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Chiang Mai Province, Thailand

<sup>3</sup>วิทยาลัยการแพทย์ทางเลือก มหาวิทยาลัยราชภัฏจันทรเกษม กรุงเทพมหานคร

<sup>3</sup>Alternative Medical College, Chandrakasem Rajabhat University, Bangkok, Thailand

\* ผู้รับผิดชอบบทความ (Email: songyot.anuch@cmu.ac.th)

\* Corresponding author (Email: songyot.anuch@cmu.ac.th)

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### บทคัดย่อ

**บทนำ:** สารภี (*Mammea siamensis*) เป็นพืชสมุนไพรที่ใช้ในทางการแพทย์แผนไทยมาเป็นระยะเวลานาน ดอกมีสรรพคุณช่วยบำรุงหัวใจ แก้ไข้ และทำให้เจริญอาหาร FLT3 (Fms-like tyrosine kinase 3) เป็นโปรตีนบ่งชี้มะเร็งเม็ดเลือดขาวแบบเฉียบพลัน ชนิด acute myeloblastic leukemia (AML) มีบทบาทสำคัญในการควบคุมการแบ่งตัวของเซลล์มะเร็งเม็ดเลือดขาว

**วัตถุประสงค์:** เพื่อศึกษาผลของสารสกัดหยาบจากเอทานอล (EtOH) และสารสกัดหยาบแยกส่วนในเฮกเซน (Hex) เอทิลอะซิเตต (EtOAc) และเมทานอล (MeOH) จากดอกสารภี ต่อการทำลายเซลล์มะเร็งเม็ดเลือดขาวเพาะเลี้ยงชนิด EoL-1 และผลของสารสกัดทั้งหมดต่อการแสดงออกของโปรตีน FLT3 ในเซลล์มะเร็งเม็ดเลือดขาวชนิดนี้ด้วย

**วัสดุและวิธีการ:** สกัดสารจากดอกสารภีโดยใช้เอทานอล และสกัดแยกส่วนต่อด้วยเฮกเซน เอทิลอะซิเตต และเมทานอล ตามลำดับ ทดสอบฤทธิ์ของสารสกัดในการทำลายเซลล์โดยวิธี MTT สารสกัดที่ให้ผลดีในการทำลายเซลล์มะเร็งเม็ดเลือดขาวจะถูกนำมาศึกษากลไกการยับยั้งการแสดงออกของโปรตีน FLT3 ด้วยวิธีเวสเทิร์น บลอต (Western blot) และนับจำนวนเซลล์หลังจากการทดสอบด้วยวิธีทริฟแพนบลู ดาย เอ็กชคลูชัน (trypan blue exclusion)

**ผลการศึกษา:** สารสกัดแยกส่วนด้วย Hex สามารถทำลายเซลล์มะเร็งเม็ดเลือดขาวเพาะเลี้ยงชนิด EoL-1 ได้ดีที่สุด โดยมีค่า IC<sub>50</sub> เท่ากับ 3.8±0.8 µg/mL และลดการแสดงออกของโปรตีน FLT3 และจำนวนเซลล์ โดยเป็นไปตามระยะเวลาและความเข้มข้นที่เพิ่มมากขึ้น เมื่อใช้ความเข้มข้นที่ไม่เป็นพิษต่อเซลล์ที่ค่า IC<sub>20</sub>

**สรุปผลการศึกษา:** สารสกัดแยกส่วนด้วย Hex ของดอกสารภี สามารถทำลายเซลล์มะเร็งเม็ดเลือดขาวเพาะเลี้ยงชนิด EoL-1 และยับยั้งการแบ่งตัวของเซลล์โดยการยับยั้งการแสดงออกของโปรตีน FLT3 ดังนั้น สารสกัดจากดอกสารภีจึงเป็นสมุนไพรที่น่าสนใจและอาจเป็นทางเลือกใหม่สำหรับพัฒนาเป็นยาด้านมะเร็งเม็ดเลือดขาวต่อไป

วารสารเทคนิคการแพทย์เชียงใหม่ 2559; 49(2): 286-293. Doi: 10.14456/jams.2016.19

**คำรหัส:** *Mammea siamensis* ดอกสารภี ฟิมส์ไลค์ ไทโรซีนไคเนส 3 มะเร็งเม็ดเลือดขาว EoL-1

## Abstract

**Introduction:** Saraphi (*Mammea siamensis*) is a medicinal herb used in Thai traditional medicine. Its flowers were traditionally used for heart tonics, fever, and enhancement of appetite in Thailand. FLT3 (Fms-like tyrosine kinase 3) is a prognostic marker for acute myeloblastic leukemia (AML) and it can control leukemic cell proliferation.

**Objective:** To investigate the cytotoxic effects of crude ethanolic extract (EtOH) and fractional extracts including hexane (Hex), ethyl acetate (EtOAc), and methanol (MeOH) fractions from *M. siamensis* flowers and to determine the effects on FLT3 expression in EoL-1 cells.

**Materials and methods:** Flowers of *M. siamensis* were firstly extracted using ethanol. The EtOH extract was further fractionated by Hex, EtOAc, and MeOH, respectively. MTT assay was performed to evaluate cytotoxic effects of each extract. The effective extracts were used to determine their inhibitory effects on FLT3 protein expression by Western blot analysis. Total cell number was determined by trypan blue dye exclusion assay.

**Results:** Hex fraction demonstrated the strongest cytotoxic activity with  $IC_{50}$  of  $3.8 \pm 0.8$   $\mu\text{g/mL}$ . Moreover, FLT3 protein expression and total cell numbers of Hex fraction treated EoL-1 cells were decreased in a time- and dose-dependent manner at the concentration of  $IC_{20}$  value.

**Conclusion:** The Hex fraction from *M. siamensis* flowers inhibited cell proliferation via the suppression of FLT3 expression. It could be suggested that Hex extraction of *M. siamensis* flower is a promising approach for new anti-leukemic drug candidates.

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**Keywords:** *Mammea siamensis*, Saraphi flower, FLT3, leukemia, EoL-1

## Introduction

Leukemia is a blood disease characterized by diversity of chromosomal and molecular changes. It was characterized by the hematopoietic progenitor cells losing their ability to differentiate normally and to respond to normal regulators of proliferation. Some alterations of protein levels provided useful molecular biomarkers which have been evaluated in leukemia patients.<sup>1,2</sup>

FLT3 (Fms-like tyrosine kinase 3) belongs to the class III receptor tyrosine kinase and has an important role in hematopoietic progenitor cell proliferation. It is a prognostic marker for acute myeloblastic leukemia (AML).<sup>3,4</sup> Ligand-FLT3 binding promotes receptor dimerization and subsequent signaling and phosphorylation of multiple cytoplasmic proteins as well as the activation of several

downstream signaling pathways, such as Ras/Raf, MAPK, and PI3 kinase cascades.<sup>3,5</sup> Previous study showed that upregulation of FLT3 and its ligand by leukemic cells creates an autocrine signaling loop which stimulates proliferation of EoL-1 cell line.<sup>6</sup> Furthermore, high levels of wild-type FLT3 have been reported for blast cells in 20-25% of AML patients without FLT3 mutations. This may be considered to represent an attractive therapeutic target in AML.

*Mammea siamensis* (Miq.) T. Anders, belonging to the Guttiferae family, is a Thai medicinal plant, named "Saraphi". Its flowers have been traditionally used as a heart tonic in Thailand. Several coumarins and xanthone have been isolated from Saraphi flower and some of which have potential pharmacological and therapeutic effects.<sup>7,8</sup> Recent study revealed that isolated compounds from flower

extract of *M. siamensis* showed significant anti-proliferative activities against leukemia and stomach cancer cell lines.<sup>9</sup> However, there are no data concerning the effects of *M. siamensis* flower extracts on FLT3 protein expression in leukemic cells. The objectives of this study were to investigate cytotoxic and inhibitory effects of fractional crude extracts (ethanol, hexane, ethyl acetate, and methanol) from *M. siamensis* flowers on FLT3 expressed EoL-1 leukemic cell line.

## Materials and Methods

### Cell culture and condition

EoL-1 (Cat. No. RBRC-RCB0641; acute myeloblastic cell line) was purchased from RIKEN BRC CELL BANK and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Invitrogen,<sup>TM</sup> Carlsbad, CA, USA), 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin at 37 °C in a humidified incubator with 5% CO<sub>2</sub>.

### Plant material and preparation

*M. siamensis* flowers were collected between February-April, 2014 in Chiang Mai Province, Thailand. A voucher specimen (J.F. Maxwell, No. 92-70) was deposited by the CMU herbarium, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand. The flowers were dried in hot-air oven before extraction by ethanol (EtOH). Crude EtOH extract was further fractionated by quick column chromatography packed with silica gel and partitioned with hexane, ethyl acetate, and methanol successively yielding the Hex, EtOAc, and MeOH fractions, respectively. Concentrated solution was completely dried by a rotary evaporator. Four fractions were kept at -20 °C until used and suspended in DMSO to prepare the stock solution (25 mg/mL).

### MTT assay

Cytotoxicity of crude EtOH extract and three fractional extracts (Hex, EtOAc, and MeOH) from *M. siamensis* flowers were determined by MTT assay. EoL-1 cells were seeded ( $1.0 \times 10^4$  cells/well) in 96-well plates, and incubated

for overnight at 37 °C with 5% CO<sub>2</sub>. Cells were then treated with 4 extracts (0-100 µg/mL) for 48 hrs. Complete medium with DMSO was used as a vehicle control (VC). After incubation, 15 µL of MTT dye (Sigma-Aldrich, St Louis, MO, USA) solution (5 µg/mL) was added to each well and plate was incubated at 37 °C for another 4 hrs. Formazan crystal products were dissolved by 200 µL of DMSO, and the absorbance was measured at 578 nm by an AccuReader<sup>TM</sup> microplate reader (Metertech Inc, Taipei, Taiwan) with reference blank at 630 nm. The percentage of cell viability was calculated as following formula.

$$\% \text{ Cell viability} = \frac{\text{Absorbance of test}}{\text{Absorbance of vehicle control}} \times 100$$

The average of cell viability obtained from triplicate experiments was plotted as graph. The inhibitory concentration at 50% growth (IC<sub>50</sub>) value was presented as the lowest concentration that decreases cell growth 50%, whereas IC<sub>20</sub> value was determined as a non-toxic dose and used for protein expression analysis.

### Protein extraction and Western blotting

After treatment, leukemic cells were harvested and viable cell number was counted using 0.4% trypan blue. Thereafter, total protein from treated cells was extracted using RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate (C<sub>24</sub>H<sub>39</sub>NaO<sub>4</sub>), and 0.1% SDS). Protein concentration was measured by Folin-Lowry method. FLT3 protein was separated by 7.5% SDS polyacrylamide gel and detected using rabbit polyclonal anti-FLT3 antibody (1:1,000 in blocking buffer, Invitrogen<sup>TM</sup>, Carlsbad, CA, USA). GAPDH (Santa Cruz Biotechnology, CA, USA) was used as a loading control with a dilution of 1:1,000. HRP-conjugated goat anti-rabbit IgG (1:20,000) was used as a secondary antibody. Protein of interest was detected by Luminata<sup>TM</sup> Forte Western HRP substrate (Millipore Corporation, Billerica, MA, USA). Protein band was quantified by a scan densitometer and Quantity One software, version 4.6.3 (Bio-Rad laboratories, Hercules, CA, USA). Density value of each FLT3 band was normalized to GAPDH band.

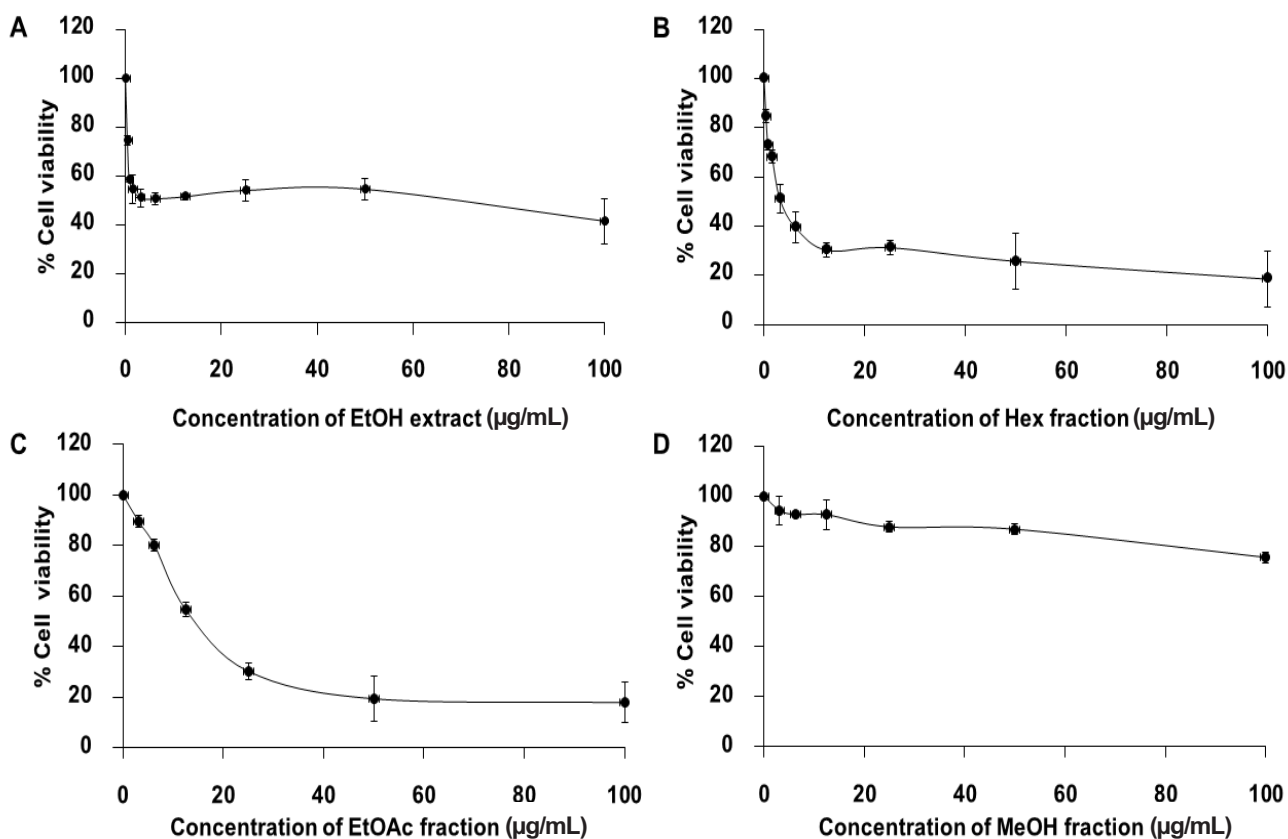
## Statistical analysis

Average of triplicate experiments and standard error of mean (SEM) were used for quantification. Level of target protein expression was compared to vehicle control. The results were shown as mean $\pm$ SEM. Differences between-means of each experiment were analyzed by One-way analysis of variance (One-way ANOVA). Statistic significances were considered at  $p < 0.05$ .

## Results

### Cytotoxicity of *M. siamensis* flower extracts

In the present study, it was demonstrated that crude EtOH extract and Hex fraction exhibited a strong inhibitory effect on EoL-1 cells, with  $IC_{50}$  of  $5.5 \pm 0.7$  and  $3.8 \pm 0.8$   $\mu\text{g/mL}$ , respectively, while  $IC_{50}$  values of EtOAc and MeOH fractions were  $14.9 \pm 1.0$  and  $>100$   $\mu\text{g/mL}$ , respectively (Figure 1). The finding indicated that Hex fraction may have a potent anti-proliferative properties against FLT3-expressing leukemic cells.

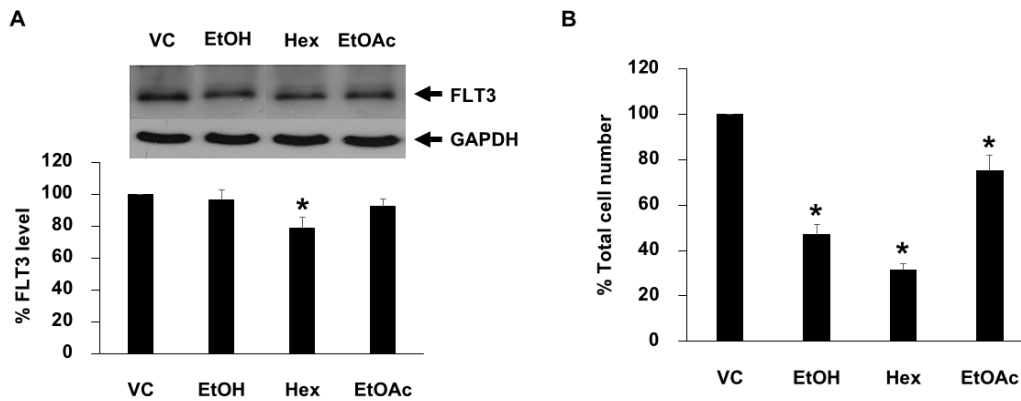


**Figure 1** Cytotoxic effect of *M. siamensis* flower (A) crude EtOH extract and fractional extracts of (B) Hex, (C) EtOAc, and (D) MeOH against EoL-1 cell line. Average of cell viability was obtained from three independent experiments.

### *M. siamensis* flower extracts suppressed FLT3 expression in EoL-1 cell line

After treatment of EoL-1 cells with crude EtOH extract and fractional extracts of Hex and EtOAc for 48 hrs, Hex fraction showed the most effective in inhibition of FLT3 expression by  $21.1 \pm 6.3\%$  compared to vehicle control but not crude EtOH extract and EtOAc fraction (Figure 2A).

Total cell numbers after treatment with crude EtOH extract and fractional extracts of Hex and EtOAc were significantly decreased by  $52.8 \pm 4.1$ ,  $68.3 \pm 2.3$ , and  $24.7 \pm 6.5\%$ , respectively when compared to vehicle control (Figure 2B). Percentages of dead cells were in the range of 0-3%.

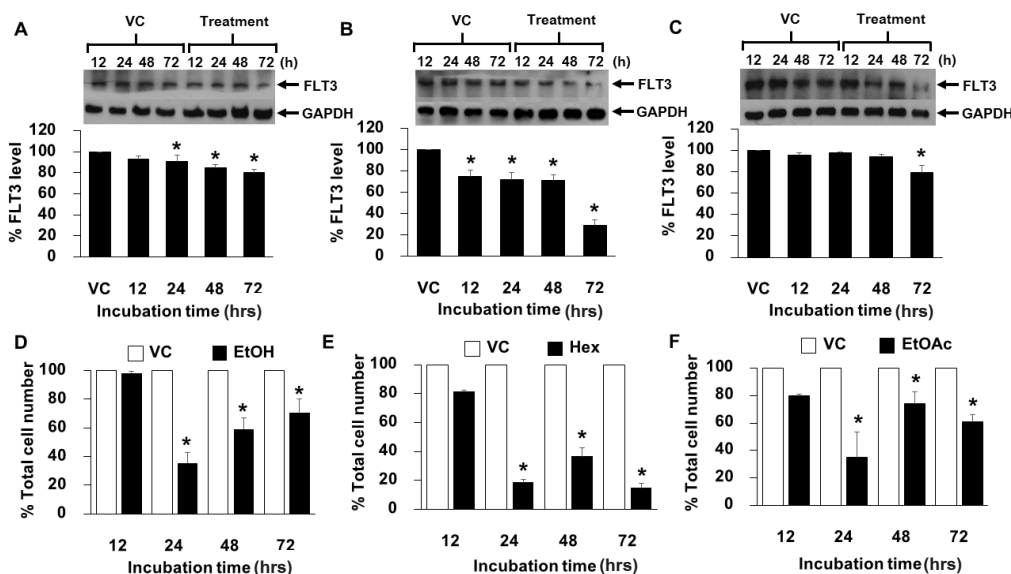


**Figure 2** Effects of crude EtOH extract and fractional extracts of Hex and EtOAc on FLT3 protein expression in EoL-1 cells. (A) Level of FLT3 protein expression after treatment was evaluated by Western blotting; GAPDH was used as a loading control. Density of each FLT3 band was normalized to GAPDH. (B) Total cell numbers of EoL-1 cells after treated with EtOH extract, Hex fraction, and EtOAc fractions were determined by trypan blue exclusion method. Data were mean values $\pm$ SEM of three independent experiments. Asterisk (\*) denotes a significant difference from the control group ( $p < 0.05$ ).

### Suppression of FLT3 expression in *M. siamensis* flower extracts treated EoL-1 cell line was time dependent

In this experiment, EoL-1 cells were treated with crude EtOH extract, Hex, and EtOAc fractions for 12, 24, 48, and 72 hrs and FLT3 was determined by Western blotting. Crude EtOH, Hex, and EtOAc fractions reduced FLT3 expression in time-dependent manner compared to vehicle control (Figure 3A). However, crude EtOH and Hex fraction showed more activity to suppress FLT3 than

EtOAc fraction. Total cell number after 48 hrs-treatment was also decreased without induction of cell death (Figure 3B). Percentage of dead cells was in the range of 0-3%. From these results, the active compound of *M. siamensis* flower extract was most likely less polar compound found in low to non-polar compartment, especially in Hex fraction. Thus, Hex fraction was selected as a candidate *M. siamensis* flower extract for further examination.

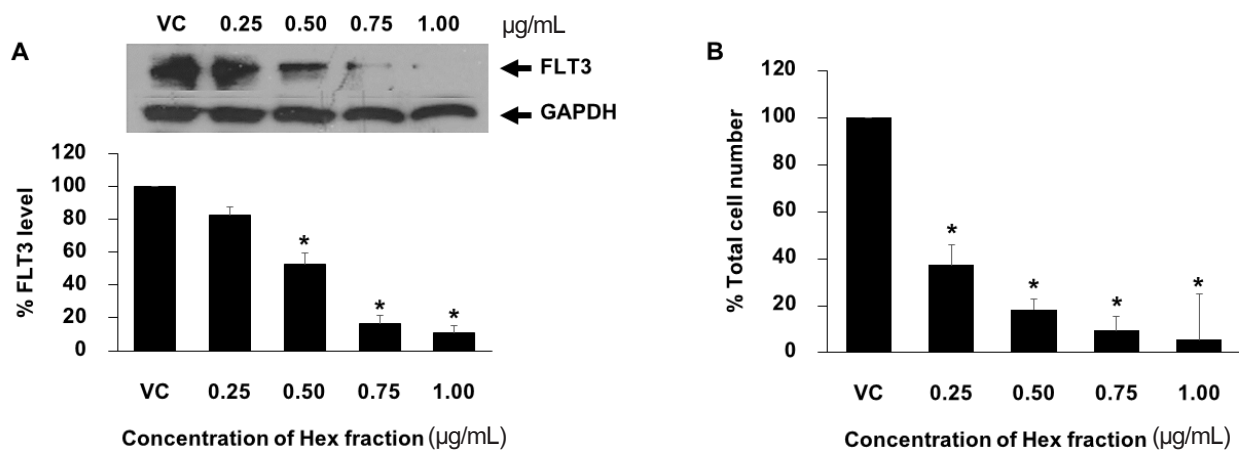


**Figure 3** Effect of incubation time period of crude EtOH extract and fractional extracts of Hex and EtOAc treatments on FLT3 expression in EoL-1 cells. Levels of FLT3 expression after treated with (A) crude EtOH extract, (B) Hex fraction, and (C) EtOAc fraction was evaluated by Western blotting; GAPDH was used as a loading control. Density of each FLT3 band was normalized to GAPDH. (D-F) Total cell numbers after 12, 24, 48, and 72 hrs were counted using trypan blue exclusion method. Data were mean values $\pm$ SEM of three independent experiments. Asterisk (\*) denotes a significant difference from the control group ( $p < 0.05$ ).

### Suppression of FLT3 expression in *M. siamensis* flower extracts treated EoL-1 cell line was dose dependent

Various non-cytotoxic doses of Hex fraction (0.25, 0.50, 0.75, and 1.0 µg/mL) of *M. siamensis* flowers were examined for effects on FLT3 protein expression. Protein levels of FLT3 were decreased 11.2±8.6, 67.5±4.7, 83.4±5.9, and 88.8±19.6% in response to concentrations

of 0.25, 0.50, 0.75, and 1.00 µg/mL of Hex fraction, respectively compared to vehicle control (Figure 4A). Total cell numbers after Hex fraction treatments were significantly decreased 62.6±0.4, 81.7±0.3, 90.5±0.8, and 94.6±0.9% at 0.25, 0.50, 0.75, and 1.00 µg/mL of Hex fraction, respectively compared to vehicle control. Percentage of dead cells was in the range of 0-1% (Figure 4B).



**Figure 4** Effect of dose of Hex fraction on FLT3 expression in EoL-1 cells. Cells were treated with various concentrations (0.25, 0.50, 0.75, and 1.00 µg/mL) for 48 hrs. (A) FLT3 protein expression after treatment was evaluated by Western blotting; GAPDH was used as a loading control. Density of each FLT3 band was normalized to GAPDH. (B) Total cell numbers after 48 hrs were counted using the trypan blue exclusion method. Data were mean values±SEM of three independent experiments. Asterisk (\*) denotes a significant difference from the control group ( $p<0.05$ ).

### Discussion

This is the first report revealed the effect of *M. siamensis* flower extracts on the molecular inhibitory mechanism of FLT3 protein in leukemic cells. FLT3, a receptor tyrosine kinase, involves in cell proliferation, differentiation, and apoptosis of hematopoietic cells. In this study, *M. siamensis* or Saraphi was interested. It has been used for a long time as a Thai traditional medicine. Saraphi is in the genus *Mammea* and enriches of secondary metabolites compounds, e.g. coumarins and xanthone.<sup>10,11</sup> Recently, hexane fraction of seeds of *M. siamensis* was shown to have cytotoxic and inhibitory effects on WT1 protein expression in K562 cell line.<sup>12</sup>

The cytotoxic effect of crude EtOH extract and fractional extracts (Hex, EtOAc, and MeOH) from *M. siamensis* flowers were assessed by MTT assay. Crude EtOH extract and fractional extracts of Hex and EtOAc showed cytotoxic effects on EoL-1 cells but not MeOH fractional extract. Hex fraction provided the strongest cytotoxic effect followed by crude EtOH extract and EtOAc fraction. The results obtained from Tung *et al.* demonstrated that compound from methanol extract of *M. siamensis* flower exhibited significant antiproliferative activity against human leukemic cell lines including HL-60, U937, THP-1, and Jurkat cell lines.<sup>9</sup> Furthermore, after treatment with three *M. siamensis* flower extracts (crude EtOH extract,



Hex fraction, and EtOAc fraction), Hex fraction had the strongest inhibitory effect on FLT3 protein expression in leukemic cells both in time- and dose-dependent manner. Result also showed its ability to suppress leukemic cell proliferation without cell viability alteration. However, crude EtOH extract and EtOAc fraction could slightly inhibit FLT3 protein expression in a time-dependent manner. Taken together, it revealed that active compounds dissolved in Hex fraction have ability to inhibit cell proliferation, destroy leukemic cells at high doses and downregulate the target FLT3 protein level at non-cytotoxic doses. Previous study showed that main active compound found in Hex fraction from seeds of *M. siamensis* was mammea E/BB.<sup>12</sup>

The effective compounds in Hex fraction of *M. siamensis* flowers need further on studies. These results suggested that the active compound dissolved in hexane, a non-polar solvent, may be essential oil. The essential oil in *M. siamensis* flower, includes mammea B/AC cyclo D, kayeassamin A, surangin C, theraphin B.<sup>13,14</sup> Flower extract of *M. siamensis* was reported to inhibit *Staphylococcus aureus* and *Bacillus subtilis*. Moreover, methanolic extract of flowers could inhibit nitric oxide (NO) production in lipopolysaccharide-activated RAW264.7 cells with IC<sub>50</sub> value of 28.9 µg/mL, whereas ethyl acetate extract exhibited cytotoxic effects by MTT assay. The ethyl acetate soluble fraction had 3 coumarins; mammeasin A, kayeassamin G, and mammea A/AD.<sup>15</sup>

Results from this study suggested that Hex fractional extract of *M. siamensis* flower has potentially used to develop into new anti-cancer drug candidates.

## Conclusion

The flower extracts of *M. siamensis* strongly possessed anti-proliferation of leukemic cells although MeOH fraction had no cytotoxic and inhibitory effects on EoL-1 leukemic cell line. Furthermore, Hex fraction decreased FLT3 protein level in both time- and dose-dependent manner. This is the first report of inhibitory effects of *M. siamensis* flower extracts on FLT3 protein expression in leukemic cells. Therefore, an active compound from the fraction will be further investigated in the future. This result indicates that flower extracts from *M. siamensis* used in traditional Thai medicine may be useful as an alternative therapeutic agent in human acute myeloblastic leukemic cells. The study has provided a basis for the future study of *M. siamensis* to confirm its effect on leukemia treatment.

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