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Effects of crude medicinal Thai flower extracts on cytotoxicity and FMS-like tyrosine kinase 3 protein expression in EoL-1 leukemic cell line

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ABSTRACT

Background: Leukemia is one cause of death worldwide. Nowadays, natural therapies such as the use of medicinal plants may reduce chemotherapy side effects of leukemia. Accordingly, the anticancer properties of medicinal plants have been recognized for centuries. This study focuses on Thai flowers from traditional drug recipes including phikun (*Mimusops elengi* Linn.), boonark (*Mesua ferrea* Linn.), ketawa (*Gardenia jasminoides* J.Ellis), and sarapi (*Mammea siamensis* (Miq.) T. Anders) for cytotoxicity and leukemia suppression. Fms-like tyrosine kinase 3 (FLT3) is a prognostic marker for acute myeloblastic leukemia (AML) and leukemic cell proliferation. Thus, FLT3 is a reliable marker for minimal residual disease (MRD) assessment in leukemia patients.

Objectives: To investigate the cytotoxicity of crude ethanolic extracts from four Thai flowers and to determine their effects on FLT3 protein expression in EoL-1 cells.

Materials and methods: Phikun, boonark, ketawa, and sarapi flowers were extracted by using 95% ethanol. An MTT assay was performed to evaluate cytotoxicity of each crude ethanolic flower extract. The effective crude ethanolic flower extract was further determined its inhibitory effect on FLT3 protein expression by Western blot analysis. Total cell number was determined by the trypan blue exclusion method.

Results: Crude boonark ethanolic extract demonstrated the strongest cytotoxic activity with the inhibitory concentration at 50% (IC_{50}) values of 62.5±3.9 µg/mL. Moreover, it could decrease the total cell number and FLT3 protein expression by a time- and dose-dependent manner.

Conclusion: The crude boonark ethanolic extract inhibited cell proliferation *via* the suppression of FLT3 expression. It could be suggested that crude boonark ethanolic extract is a promising approach for new anti-leukemic drug candidates.

Introduction

Leukemia is a group of blood diseases characterized by diversity of chromosomal and molecular changes. It was characterized by the hematopoietic progenitor cells losing their ability to differentiate normally and responding to normal regulators of proliferation. Some alterations of protein levels provided useful molecular biomarkers which have been evaluated in leukemia patients.[1,2] Previous studies showed that overexpression of Fms-like tyrosine kinase 3 (FLT3) are associated with leukemogenesis.[3-6]

FLT3 belongs to the type III receptor tyrosine kinase and has an important role in hematopoietic progenitor cell

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proliferation. It is a prognostic marker for acute myeloblastic leukemia (AML).[7,8] Ligand-FLT3 binding promotes receptor dimerization, 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.[7,9] 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.[10] 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.

Nowadays, chemotherapy is a generally effective treatment and is widely used for leukemia treatment. To avoid the severe side effects of drug chemotherapy, plants having anticancer activity are an alternative for cancer treatment. South-East Asian countries like Malaysia, Thailand, and Borneo (Indonesia) have a long history of using medicinal plant that offer considerable pharmaceutical potential. Lee and Houghton found that the South-East Asian region, owing to the vast bio-diversity of its flora, holds great promise for the discovery of novel biologically-active compounds.[11] According to the World Health Organization (WHO), most populations in the world still rely on traditional medicines for their psychological and physical health requirements. People living in rural areas from their personal experience know that these traditional remedies are valuable source of natural products to maintain human health.[12] Medicinal plants have long been used as a traditional source of healing in Thailand.[13] In the Thai folk medicine, five Thai flowers including mali (jasmine; *Jasminum sambac* (L.) Aiton), phikun (*Mimusops elengi* Linn.), boonark (*Mesua ferrea* Linn.), sarapi (*Mammea siamensis* (Miq.) T. Anders), and bualuang (sacred lotus or pink lotus-lily; *Nelumbo nucifera* Gaertn), have been used as a drug recipe.[14] This recipe has been used for heart tonic modulation. Phikun,[15] boonark,[16] and sarapi[17] were selected for the study to determine biological activities. Ketawa (*Gardenia jasminoides* J.Ellis)[18] was selected for the study because it also has been used in Thai folk medicine (skin disease treatment). The inhibitory effects of sarapi flower extract on FLT3, WT, and Bcr/Abl proteins expressions in leukemic cell lines have been previously reported.[17,19] In this study, the properties of crude ethanolic extracts from the Thai flowers of pikun, boonark, ketawa, and sarapi were investigated on FLT3 protein expressions in EoL-1 cell line.

Materials and methods

Cell culture and condition

Eosinophilic leukemic (EoL-1) cells were used as human leukemic cell line model in this study. Cells were purchased from RIKEN BRC Cell Bank, Japan (RCB0641) and cultured in RPMI 1640 medium (GIBCO™, Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (Capricon, Ebsdorfergrund, Germany), 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin (GIBCO™, Thermo Fisher Scientific, MA, USA) at 37°C in a humidified incubator with 5% CO_2.

Plant material and preparation

The Thai flowers were used in this study were phikun, boonark, ketawa, and sarapi. The flowers were collected in the Chiang Mai Province of Thailand in 2014. The flowers were dried in a hot-air oven before being extracted by ethanol and were blended into a dehydrated powdered form. One hundred grams of each dried flower powder was macerated with 1,000 mL of 95% ethanol for 48 hr at room temperature. The liquid extracts were collected from three times of maceration, filtered, and then the solvent was removed under vacuum using a rotary evaporator at 45°C. Crude ethanolic flower extracts were obtained after complete removal of the solvent. The extracts were further dried in evaporator. The four flowers crude ethanolic extracts were kept in the refrigerator at -20°C until used and suspended in DMSO to prepare the stock solution (25,000 µg/mL).

MTT assay

Cytotoxicity of the crude ethanolic flower extracts, phikun, boonark, ketawa, and sarapi were determined by MTT assay. EoL-1 cells were seeded (5.0×10^4 cells/well) in 96-well plates and incubated overnight at 37°C with 5% CO_2. Cells were then treated with four extracts (0-100 µg/mL) for 48 hr. A 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 mg/mL) was added to each well and the plate was then incubated at 37°C for another 4 hr. Formazan crystal products were dissolved in 200 µL of DMSO, and the absorbance was measured at 578 nm by an AccuReader™ microplate reader (Metertech Inc, Taipei, Taiwan) with reference blank at 630 nm. The percentage of cell viability was calculated as following formula.

$$\% \text{ cells viability} = \frac{(\text{OD average of tested well} \times 100)}{\text{OD average of vehicle control}}$$

The average of cell viability obtained from triplicate experiments was plotted on a graph. The inhibitory concentration at 50% growth (IC_{50}) value was presented as the lowest concentration that decreases cell growth by 50%, whereas the IC_{20} value was determined as a non-cytotoxic dose and used for protein expression analysis.

Protein extraction and Western blotting

After treatment, leukemic cells were harvested, and total viable cell numbers were counted using 0.4% trypan blue. Thereafter, the total protein from the treated cells was harvested and extracted by using RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate ($C_{24}H_{39}NaO_4$), and 0.1% SDS). Protein concentration was measured by the 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™, 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™ Forte Western HRP substrate

(Millipore Corporation, Billerica, MA, USA). The protein band was quantified by a scan densitometer and Quantity One software, version 4.6.3 (Bio-Rad laboratories, Hercules, CA, USA). Density values of each FLT3 band were normalized to the GAPDH band.

Statistical analysis

Average of triplicate experiments and standard deviation (SD) were used for quantification. Levels of target protein expression were compared to vehicle control. The results were shown as mean±SD. Differences between means of each experiment were analyzed by One-way analysis of variance (One-way ANOVA). Statistic significances were considered at $p<0.05$, $p<0.01$, and $p<0.001$.

Results

Preparation of crude ethanolic extract from phikun, boonark, ketawa, and sarapi

One hundred grams of dried powder from each flower material was extracted by ethanol (without preservatives). The percentage yield recovery (% yield) of the four flowers crude ethanolic extracts (phikun, boonark, ketawa, and sarapi) were 20.66, 25.15, 27.16, and 29.50%, respectively.

Cytotoxicity of crude ethanolic extract from phikun, boonark, ketawa, and sarapi on EoL-1 cell line

The inhibitory concentration at 50% (IC_{50}) values of crude ethanolic flower extracts of phikun, boonark, ketawa, and sarapi on EoL-1 cells were >100, 62.5±3.9, >100, and >100 µg/mL, respectively (Figure 1).

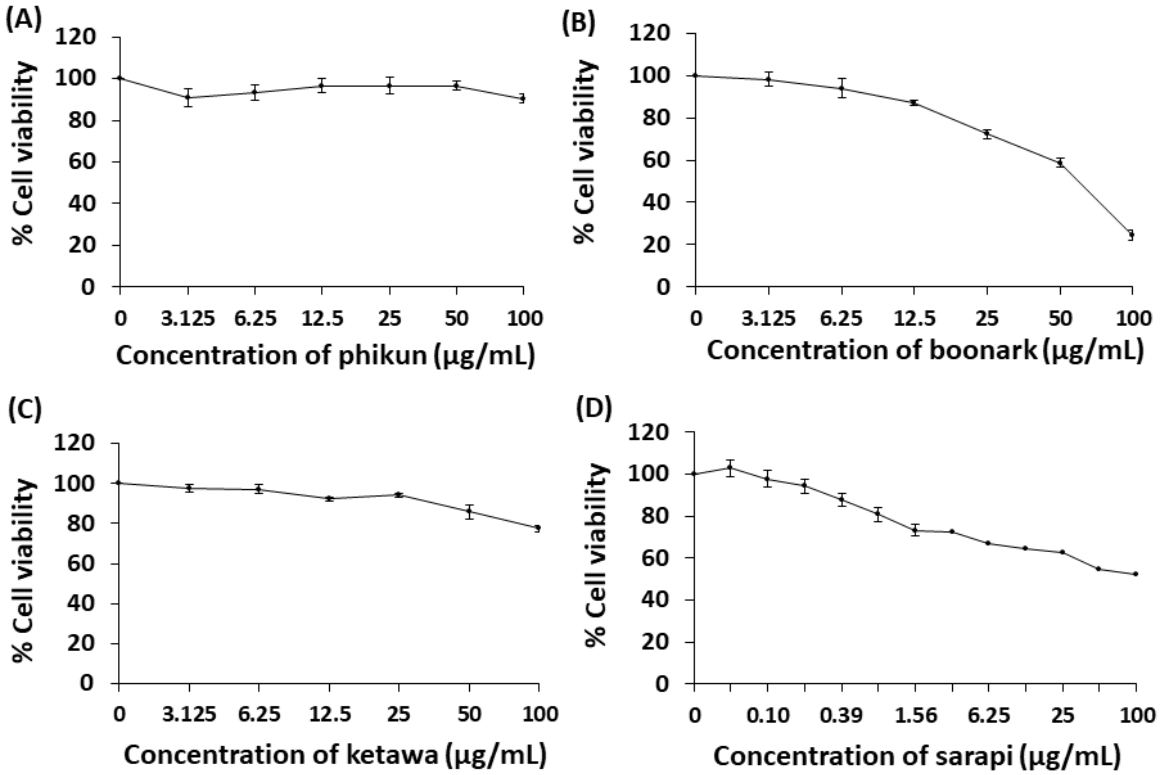

Figure 1. *Cytotoxicity of crude ethanolic extract from phikun, boonark, ketawa, and sarapi in EoL-1 cell line. EoL-1 cells (5×10^4 cells/mL) were cultured in the presence of various concentrations of crude ethanolic extract from (A) phikun, (B) boonark, (C) ketawa, and (D) sarapi (0-100 µg/mL) for 48 hr. The cell viability was determined by MTT assay. Each point presents the mean value±SD of three times independent experiments performed in triplicate.*

Crude ethanolic extract from phikun, boonark, ketawa, and sarapi suppressed FLT3 expression in EoL-1 cell line

When examining the effect of crude ethanolic extracts from phikun, boonark, ketawa, and sarapi on FLT3 protein expression, their IC_{20} values (100, 18.6, 85.4, and 0.9 µg/mL, respectively) were used. *n*-Hexane extract of sarapi (Hex-sarapi) with concentration of 0.07 µg/mL was used as a positive control.[17] EoL-1 cells were examined with those of Hex-sarapi and crude ethanolic extracts on FLT3 protein expression. After treatment of EoL-1 cells with four crude ethanolic extracts for 48 hr, the crude ethanolic extract from boonark at 18.6 µg/mL showed the most effective suppression against FLT3 expression by 58.4±3.7% ($p<0.01$) when compared to the vehicle control (Figure 2A), Hex-saraphi, phikun, ketawa, and sarapi could suppress FLT3 protein expression by 6.4±1.8, 15.4±2.1, 2.8±0.7, and 35.3±4.9%, respectively. The total cell numbers were significantly decreased after treatment with positive control (Hex-saraphi) and crude ethanolic flower extract from phikun, boonark, ketawa, and sarapi with the values of 29.0±3.5, 42.9±4.0, 64.3±1.5, 10.0±6.4, and 47.7±2.6%, respectively, when compared to the vehicle control (Figure 2B). The percentages of dead cells were in the range of 0-0.1% of that determined by trypan blue exclusion method.

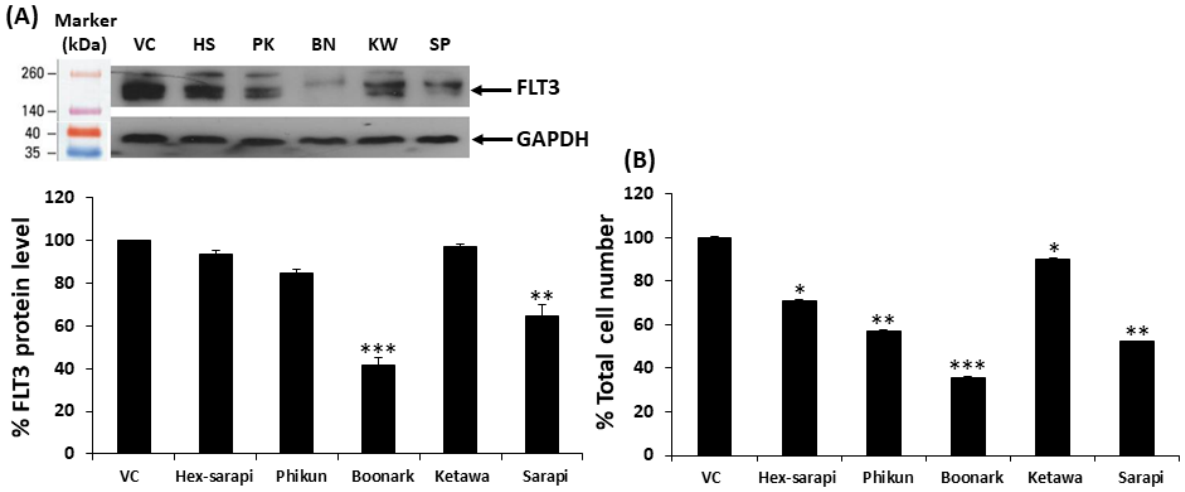

Figure 2. *Effect of crude ethanolic extracts from phikun (PK), boonark (BN), ketawa (KW), and sarapi (SP) on FLT3 protein expressions in EoL-1 cells. (A) Level of FLT3 protein expression after treatments were assessed by Western blotting; GAPDH was used as the loading control. Hex-sarapi (HS) was represented as positive control. (B) Total cell numbers of EoL-1 cells after treatment with Hex-sarapi and four flower crude ethanolic extracts were determined by trypan blue exclusion method. Data points were mean values±SD of three independent experiments. Asterisks (*), double asterisks (**) and triple asterisks (***) denote values that were significantly different from the vehicle control (VC) at p<0.05, p<0.01, and p<0.001, respectively.*

Effect of different time points of crude boonark ethanolic extract on FLT3 protein level in EoL-1 cells

To examine the effect of time period of the most effective crude ethanolic flower extract on FLT3 protein expression in EoL-1 cells, the cells were treated with crude boonark ethanolic extract with the concentration of 18.6 µg/mL (IC_{20} value) for 24, 48, and 72 hr. FLT3 protein levels after treatment with crude boonark ethanolic extract were decreased by 4.0±1.9, 49.9±4.5 (p<0.01), and 88.1±3.0% (p<0.001), respectively, when compared to the vehicle control (Figure 3A). The total cell numbers after treated with crude boonark ethanolic extract from were decreased by 8.3±1.3 (p<0.05), 45.9±6.3 (p<0.01), and 53.9±3.3% (p<0.001), respectively when compared to the vehicle control (Figure 3B).

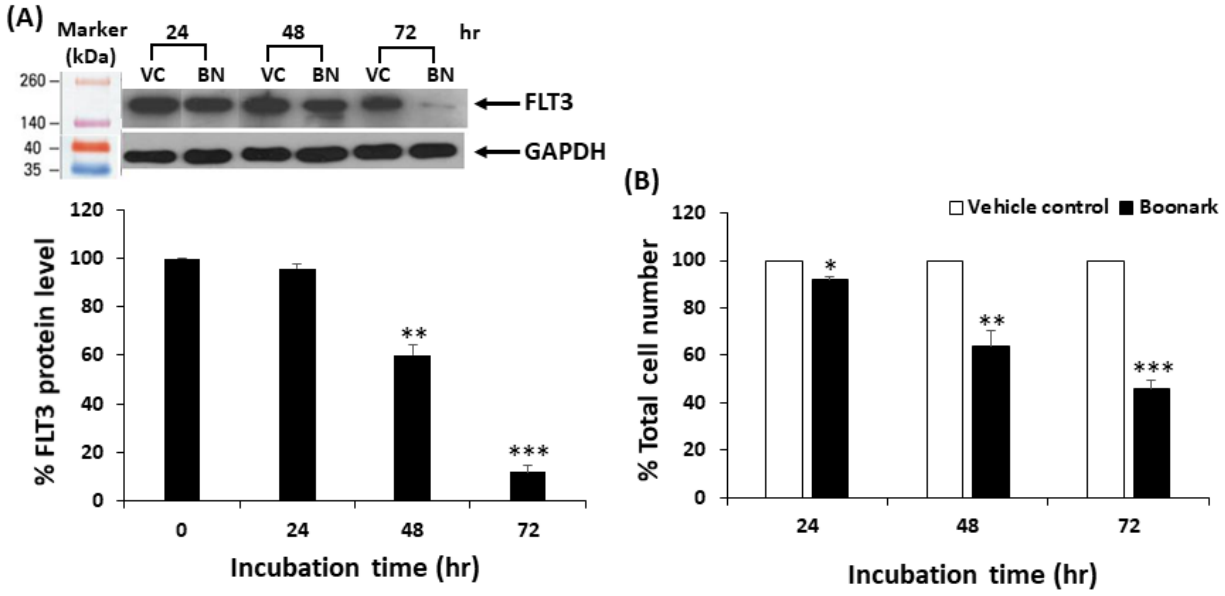

Figure 3. *Effect of incubation time period of crude boonark ethanolic extract on FLT3 protein expressions in EoL-1 cells. (A) Level of FLT3 protein expression after treatments were assessed by Western blotting; GAPDH was used as the loading control. (B) Total cell numbers of EoL-1 cells after treatment with crude boonark ethanolic extract (BN) were determined by trypan blue exclusion method. Data points were mean values±SD of three independent experiments. Asterisks (*), double asterisks (**) and triple asterisks (***) denote values that were significantly different from the vehicle control (VC) at p<0.05, p<0.01, and p<0.001, respectively*

Effect of different concentrations of crude boonark ethanolic extract in EoL-1 cells

To examine the effect of concentrations of crude boonark ethanolic extract on FLT3 protein expression in EoL-1 cells, cells were treated with medium containing DMSO (vehicle control) and various non-cytotoxic doses of crude boonark ethanolic extract (12, 15, and 18 µg/mL) for 48 hr. The protein levels of FLT3 were decreased by 14.8±1.6 (p<0.05), 48.4±0.5 (p<0.01), and 61.4±1.1% (p<0.001) in response to concentrations of 12, 15, and 18 µg/mL, respectively when compared to the vehicle control (Figure 4A). The total cell numbers were decreased by 30.5±5.0 (p<0.01), 59.2±3.8 (p<0.001), and 66.6±4.9% (p<0.001) in response to concentrations of 12, 15, and 18 µg/mL of crude boonark ethanolic extract when compared to the vehicle control (Figure 4B).

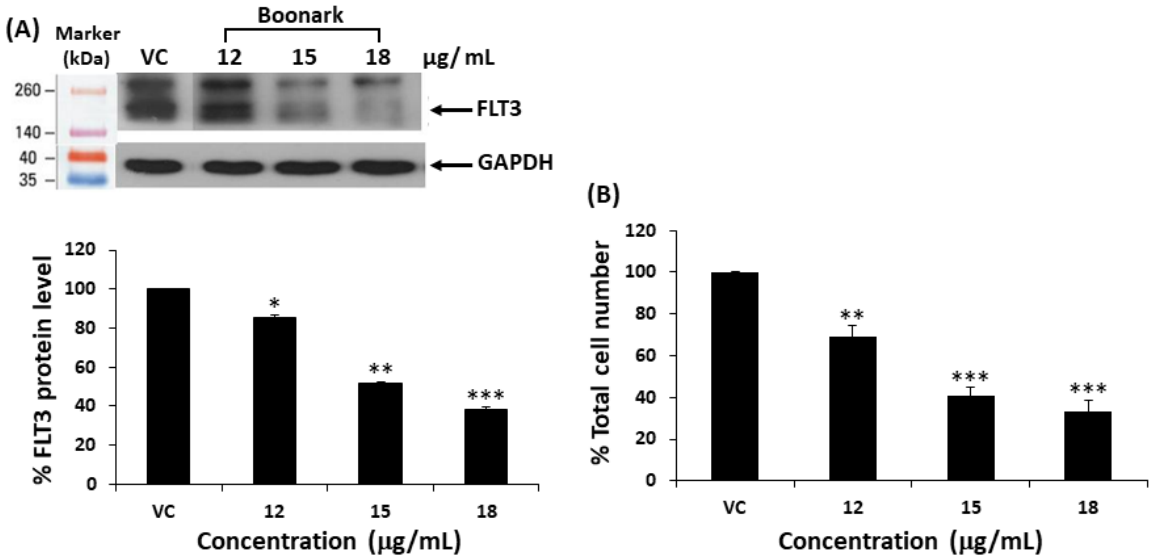

Figure 4. *Effect of dose of crude boonark ethanolic extract on FLT3 protein expressions in EoL-1 cells. (A) Level of FLT3 protein expression after boonark (BN) treatments were assessed by Western blotting; GAPDH was used as the loading control. (B) Total cell numbers of EoL-1 cells after treatment with crude boonark ethanolic extract were determined by trypan blue exclusion method. Data points were mean values±SD of three independent experiments. Asterisks (*), double asterisks (**), and triple asterisks (***) denote values that were significantly different from the vehicle control (VC) at p<0.05, p<0.01 and p<0.001, respectively.*

Discussion

This study is the first report of Thai recipe flower extracts on FLT3 protein expression in leukemic cells. FLT3 is a receptor tyrosine kinase involved in hematopoietic cell proliferation, differentiation, and apoptosis.[20] Moreover, FLT3 has been defined as a type III of receptor tyrosine kinases, and it plays an important role in leukemogenesis.[3-6] In this study, crude ethanolic extracts from three out of five flowers in Thai traditional flower recipes (mali (jasmine), phikun, boonark, sarapi, bualuang)[14] were tested. They have been used for a long time as a Thai traditional medicine. The cytotoxic effect of four crude ethanolic extracts from phikun, boonark, ketawa, and sarapi in EoL-1 cells were examined by MTT assay. Crude boonark ethanolic extracts showed the best cytotoxicity in EoL-1 cells, whereas crude ethanolic extracts from phikun, ketawa, and sarapi did not show cytotoxicity (IC_{50} >100 µg/mL) in EoL-1 cells, however cytotoxicity of sarapi trended to decrease by a dose-dependent manner (Figure 1D). Sangkaruk et al. previously reported that crude sarapi ethanolic extract had cytotoxic effects on EoL-1 cells with IC_{50} values of 5.5 µg/mL.[17] The results were reported in different IC_{50} values when compared to the results in this study. It is possible that the sarapi flowers were collected in different location, plant, and harvesting time.[21,22] Moreover, a cell line in different time of cell culture might show in different IC_{50} values.

According to the effects of four crude flower extracts on FLT3 protein expressions by Western blotting in EoL-1 cells at non-cytotoxic doses (IC_{20} values), boonark flower extract (18.6 µg/mL) was the most effective extract to decrease the FLT3 protein level by 58.4±3.7% without affecting cell death suggesting that boonark flower extract suppressed EoL-1 cell proliferation. FLT3 protein was previously reported to be inhibited by Hex-sarapi (1.0 µg/mL) flower extract by 21.1±6.3%17 while Hex-sarapi (0.07 µg/mL) in this study could inhibit FLT3 protein by 6.4±1.8%. Boonark flower extract showed better inhibitory effect than Hex-sarapi flower extract (0.07 µg/mL) for 2.8-fold when compared to that result from Sangkaruk et al.[17] Next, the most effective crude ethanolic extracts were used for further experiments. As shown in the cytotoxicity test of four flowers crude ethanolic extracts, the crude boonark ethanolic extract had also showed the strongest cytotoxicity on EoL-1 cells. It decreased FLT3 protein levels and inhibited EoL-1 cell proliferation by a time- and dose-dependent manner when compared to the vehicle control and did not alter cell viability. Thus, the results from these experiments exhibited that crude boonark ethanolic extract could inhibit cell proliferation and downregulate the target FLT3 protein levels at non-cytotoxic doses. Boonark (*M. ferrea* L.) belongs to the family Clusiaceae (Guttiferea). The plant has shown various pharmacological activities including anti-neoplastic, immunomodulatory, anti-oxidant, anti-inflammatory activities, etc.[23] Flowers and stamens of boonark have

reported the various constituents such as glycosides, coumarin, flavonoids, xanthones, triglycerides, and resins.[12] The crude extract of *M. ferrea* L. showed a strong cytotoxic activity toward T lymphocyte leukemia cells and weak antimicrobial activities against *Staphylococcus aureus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*.[24]

This is the first report that revealed the effect of crude boonark ethanolic extract have the inhibitory mechanisms *via* FLT3 protein expression in EoL-1 cells. However, the target molecule of crude boonark ethanolic extract to inhibit *FLT3* gene expression is unclear because FLT3 involved in three main signal transduction networks, including phosphatidylinositol-3-kinase (PI3K), MAP kinase, and Jak-STAT pathways. Activation of these pathways is critical for leukemic cell proliferation and survival.[20] Furthermore, the active compounds of boonark extract are of interest for further study.

Conclusion

The crude boonark ethanolic extract possessed anti-proliferation of EoL-1 cells. Furthermore, crude boonark ethanolic extract decreased FLT3 protein level in both time- and dose-dependent manner. This is the first report of the inhibitory effects of crude boonark ethanolic extract 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 crude boonark ethanolic extract 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 crude boonark ethanolic extract to confirm its effect on leukemia treatment.

Conflict of interest

The authors declare that they have no conflict of interest.

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