# CHAPTER IV

## Discussion

Leukemia is a hematological disease in the blood-forming tissues especially bone marrow. It characterized by the inhibition of differentiation with the resulting in accumulation of immature cells in the bone marrow and/or peripheral blood. Most leukemias are typically associated with a variety of genetic alterations, including gene rearrangements. However, exposure to chemicals, radiations, drugs and infections are possible causes of leukemias. These causes are suspected to overexpression or mutation of genes that involved in malignant transformation including inappropriate expression of oncogenes, loss of function of tumor suppressor genes, or a novel hybrid gene resulting from the fusion of two genes. Breakpoint Cluster Region/Abelson (Bcr/Abl) is a fusion protein encoded by *Bcr/Abl* fusion gene that occurs in 95% of patients with CML [60]. It is a product of t(9;22). The proto-oncogene *c-Abl* fusing with *Bcr* gene can result in the expression of Bcr/Abl fusion protein with increased tyrosine activity [61]. Bcr/Abl protein localizes mainly in cytoplasm [62], leading to the activation of a wide range of pathways involved in cell malignant transformation including JAK-STAT5, PI3K-AKT, and RAS [63-65]. *Wilms' tumor 1* gene is one of genes that involved in cell survival, differentiation, and proliferation [53]. Several studies showed that WT1 protein plays an important role during human development, being expressed in several tissues. WT1 protein is overexpressed in bone marrow and peripheral blood of leukemic cells in comparison to normal cells. The reduction of WT1 protein expression is associated with a decrease in cell proliferation of leukemic cells [66], suggesting that WT1 plays a key role in leukemogenesis. Fms-like tyrosine kinase 3 (FLT3), a receptor tyrosine kinase, is hematopoietic growth factor receptor. It involves in the cell proliferation, differentiation, and apoptosis of hematopoietic cells. Furthermore, FLT3 signaling plays an important role in regulating the survival and differentiation of lymphoid progenitors into B cell precursors in bone marrow. FLT3 is

expressed at high level in AML patients, resulting in aberrant signaling which is a crucial driving force in tumorigenesis [67]. It is used as a potential therapeutic target.

Medicinal plants have the ability to synthesize a wide variety of chemical compounds which are used to perform important biological functions. Several researches have been studied in their anti-cancer activity and applied for cancer treatment. In this study, *M. siamensis* or Saraphi is the plant of interest. It has been used for a longtime as traditional medicine. The flowers of this plant are mainly used for a heart tonic, reducing of fever and enhancement of appetite in Thai traditional medicine. Investigating the previous studies of plants in the genus Mammea, they were rich in secondary metabolites compounds, e.g. coumarins with cytotoxic, antioxidant, and antifungal properties [68, 69]. Recently, hexane fraction from seeds of *M. siamensis* possess cytotoxic effect and inhibitory effect on WT1 protein expression in K562 cell line [26]. However, nothing was known concerning the effects of *M. siamensis* flower extract on protein target markers related to leukemic cell proliferation.

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 the cytotoxic effects on Molt4, K562, and EoL-1 cell lines, whereas the MeOH fractional extract did not. The Hex fraction had the strongest cytotoxic effect on these three leukemic cell lines. The active compounds responding for the cytotoxic effect on leukemic cells are both in crude EtOH extract and Hex fraction. It will probably be nonpolar compounds. The result that was reported by Nguyen *et al.* demonstrated that the compound from the methanol extract of *M. siamensis* flower showed significant antiproliferative activities against human leukemic cell lines including HL-60, U937, THP-1, and Jurkat cell lines [27]. However, the results in these leukemic cells were different due to difference in leukemic cell-type and individual differences of *M. siamensis* flowers, location, and harvesting time [70].

The HPLC finger prints of crude EtOH extract and fractional extracts of Hex, EtOAc, and MeOH were examined by HPLC. The standard mammea E/BB was used as a standard marker [26]. The Hex fraction showed the highest amount of mammea E/BB. The mammea E/BB was 24.38% content in Hex fraction. Moreover, there was

the peak at the retention time of 37 min that closed to mammea E/BB peak (36 min) also showed the high percentage of content. This peak was assigned as an unknown compound. It's possible to synergist or combine their activities after treatment. The previous report showed that the flowers of *M. siamensis* have several unique coumarins, some of which have potential pharmacological and therapeutic properties [57, 71]. This result was similar to that of result in the previous study that demonstrated the main active compound in *M. siamensis* seeds hexane fraction is mammea E/BB [26].

According to the study of Bcr/Abl, WT1, and FLT3 protein expressions in leukemic cell lines, non-cytotoxic doses ($IC_{20}$ values) of three *M. siamensis* flower extracts including crude EtOH extract, Hex fraction, and EtOAc fraction were used. After treatments with three *M. siamensis* flower extracts (crude EtOH extract, Hex fraction, and EtOAc fraction), the Hex fraction exhibited the strongest inhibitory effects on Bcr/Abl, WT1, and FLT3 protein expressions in a time- and dose-dependent manner in leukemic cells. It also showed its ability to suppress leukemic cell proliferation and did not alter the cell viability when compared to the vehicle control. As shown in the cytotoxicity test of *M. siamensis* flower extracts, the Hex fraction had also showed the strongest cytotoxicity on the three leukemic cell lines. The crude EtOH extract and EtOAc fraction could slightly inhibit WT1 protein expression in Molt4 and K562 cells by a time-dependent manner. Thus, the results from these two experiments revealed the active compounds dissolved in Hex fraction have ability to inhibit cell proliferation, destroy leukemic cells at high doses and downregulate the target Bcr/Abl, WT1, and FLT3 protein levels at non-cytotoxic doses. Furthermore, the result of mamme E/BB, pure compound from *M. siamensis* seeds significantly decreased both Bcr/Abl and WT1 expressions in line with the Hex fraction but did not affect to FLT3 expression. However, the mammea E/BB was suspected the main active compound contained in Hex fraction. The HPLC fingerprint of EtOAc fraction have four retention times different from Hex, possibly the active compounds in EtOAc fraction may be different from active compound which was found in Hex fraction, resulting in the lower inhibitory effect on Bcr/Abl, WT1, and FLT3 protein expressions than Hex fraction in all three leukemic cell lines. The active compound in EtOAc fraction may not only affect Bcr/Abl, WT1, and FLT3 protein, but also other proteins related cell proliferation in cell signaling pathway. The result in this study correlates to the previous study of the

hexane fraction from *M. siamensis* seed extracts on WT1 protein expression and cell proliferation in K562 cell line [26]. The main active compound which was found in this hexane fraction from seeds of *M. siamensis* was mammea E/BB (Figure 4.1). It could decrease the WT1 protein expression by 76.9±12.6%, which was similar to the effect of the HSS (hexane extract) (78.2±10.6%). Moreover, E/BB significantly decreased the total cell number at 72 h by 91.9±0.1% when compared to the vehicle control. The cell number was similarly decreased by HSS (90.6±0.1%). In this study, the Hex fraction form *M. siamensis* flowers exhibited the suppression of WT1 protein expression by 50.6±3.1% and decreased cell proliferation by 43.9±4.4%. It's possible that mammea E/BB from flower extract may be lower than that of seed extract.

The effective compounds in Hex fraction of *M. siamensis* flowers need further studies. These results suggesting that the active compound dissolved in hexane, a non-polar solvent, and may be essential oil. The essential oil in *M. siamensis* flower include many ingredients including mammea B/AC cyclo D, kayeassamin A, surangin C, theraphin B [24, 25, 72].

This study is the first study showing that *M. siamensis* flower extract has inhibitory effects on Bcr/Abl, WT1, and FLT3 proteins in leukemic cells. These results suggest that *M. siamensis* flower hexane fractional extract have various abilities to inhibit various target proteins related to leukemic cell proliferation and potentially used to be developed into new anti-cancer drug candidates since these compounds had no inhibitory effect against normal cell lines.



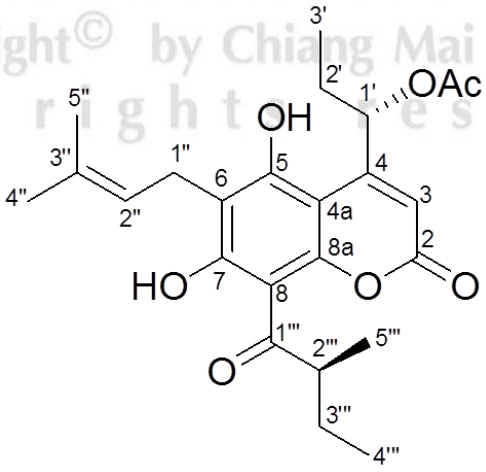


**Figure 4.1 Chemical structure of mammea E/BB.**