CHAPTER V

Conclusion

This research study can be concluded as followings:

A) Four fractional extracts (*n*-hexane, ethyl acetate, *n*-buthanol, and water fractional extracts) can be extracted from the seeds of *M. siamensis*. Cytotoxicity test against K562 cell line demonstrates that the *n*-hexane fractional extract is the most effective with the IC_{50} values of 8.7±1.0 and 5.1±1.1 µg/mL after incubation with the cells for 48 and 72 h, respectively.

B) Mammea E/BB, surangin A and surangin C are the active compounds which can be extracted from the *n*-hexane fractional extract of *M. siamensis* seeds. Purification of these compounds can be performed using silica gel column chromatography with the gradient elution from 100% *n*-hexane to 50% ethyl acteate/*n*-hexane. Subfraction 3.3 was identified as mammea E/BB. The presence of mammea E/BB can be confirmed by NMR analysis using the ^1H NMR spectra of a Bruker AV 400 spectrometer. The chemical shifts can be recorded in ppm (δ) in $CDCl_3$ using residual solvent peak (δ 7.26) as internal reference.

C) Cytotoxicity comparative test among the three active compounds of *M. siamensis* seeds (mammea E/BB, suragin A, and suragin C) against K562 cell line demonstrated that mammea E/BB has less cytotoxic effect than surangin A and surangin C with the IC_{50} values of 21.2±3.0 and 10.4±1.0 µg/mL at incubation time of 48 and 72 h, respectively. The IC_{50} values of surangin A and surangin C against the cells at incubation time of 48 h were 6.5±1.0 and 3.3±0.2 µg/mL, respectively, wheras that of 72 h were 3.0±0.0 and 2.4±0.0 µg/mL, respectively.

D) Comparison of the effects on WT1 mRNA and WT1 protein of these three active compounds of *M. siamensis* seeds (mammea E/BB, surangin A, and surangin C) it is found that mammea E/BB significantly shows the highest potential on inhibition of

WT1 protein (76.9±12.6%) than surangin A and surangin C (26.4±9.4, 42.2±14.2%). Moreover, the *n*-hexane fractional extract and mammea E/BB have similar activities to inhibit both WT1 mRNA and WT1 protein levels by a dose- and time- dependent manner. A cell number after *n*-hexane fractional extract and mammea E/BB treatment were also decreased by a time- and dose-dependent manner without cell death due to decreasing the cell proliferation. Thus the main active compound in the *n*-hexane fractional extract is mammea E/BB.

E) Mammea E/BB-mediated down-regulation of WT1 was not the result of protein or mRNA degradation processes.

F) Both chromatin immunoprecipitation (ChIP) and luciferase reporter assays indicate that mammea E/BB interferes with WT1 binding to its DNA consensus site at the proximal promoter of the *WT1* gene, nullifying WT1's positive auto-regulatory role. Therefore, mammea E/BB functions to inhibit WT1 expression at the transcriptional regulatory level.

G) Cell cycle arrest at the S phase was observed after mammea E/BB treatment due to the decreasing of *WT1* gene expression. This result related to the inhibition of cell proliferation. Cell cycle checkpoint protein at the S phase, cyclin A was suppressed by dose-dependent manner. Moreover, the proteins cyclin B and cdc2 were also decreased by dose-dependent manner at 72 h.

H) The inhibition of mammea E/BB on cell signaling pathway by the Intracellular Signaling Array Kit revealed that mammea E/BB induced cell apoptosis signal transduction after cell cycle arrest during 72 h at the S phase in K562 cells. Moreover the result showed in line with Molt4 cells. The phosphorylated target proteins related to cell apoptosis including Bad, cleaved PARP, and cleaved caspase-7 proteins were observed. Furthermore, increasing of p53 levels strongly supported its effect on cell cycle arrest. The suppression of p-ERK1/2 (extracellular signal-regulated kinase 1/2) levels related to the decreasing of cell proliferation after mammea E/BB treatment because p-ERK1/2 is the protein generally promotes cell proliferation and cell survival. The p-ERK1/2 always phosphorylates c-Fos/ AP-1. This phenomenon was correlated to

the decreasing of c-Fos/AP-1– DNA binding after mammea E/BB treatment in K562 cells by ChIP assay.

In summary, this research study demonstrates that mammea E/BB-mediated down-regulation of WT1 was not the result of protein or mRNA degradation processes. Rather, both chromatin immunoprecipitation (ChIP) and luciferase reporter assays indicate that mammea E/BB interferes with WT1 binding to its DNA consensus site at the proximal promoter of the *WT1* gene, nullifying WT1's positive auto-regulatory role. Therefore, mammea E/BB functions to inhibit WT1 expression at the transcriptional regulatory level. This novel mechanistic knowledge of how mammea E/BB affects WT1 transcriptional function in leukemic cells may be useful in the future development of therapeutic treatment of leukemia patients.