CHAPTER IV
DISCUSSION

Leukemia is a disease resulting from the neoplastic proliferation of hematopoietic cells. It results from mutation of a single stem cell that forms a clone of the leukemic cell. Genetic events contributing to malignant transformation include inappropriate expression of oncogenes, a mutation or dysregulation in proto-oncogenes, or a novel hybrid gene resulting from the fusion of two genes, or loss of function of tumor suppressor genes [68]. A major property of cancer cells is their ability to escape the anti-proliferative signals and undergo cellular expansion. The human Wilms' tumor (*WT1*), gene encoding a zinc finger transcription factor, is important in cell survival, differentiation and proliferation [13]. In recent years, the study of WT1's involvement in malignant cells has unexpectedly revealed a potential role for WT1 as an oncogene, especially in leukemia. WT1 is highly expressed in the bone marrow or peripheral blood of a variety of leukemias in comparison to normal bone marrow and normal progenitor cells [18, 19]. The expression of WT1 varies between and within different forms of human leukemia. In CML, WT1 levels are usually low in the chronic phase but frequently increase in the accelerated and blast crisis phase. In the acute leukemias, increased levels of WT1 can be found in both AML and ALL. The reduction of WT1 mRNA expression is associated with decreased cell proliferation in leukemic cells and leukemic cell lines (K562 and HL-60) [17], suggesting that WT1 plays a role in leukemogenesis. Nowadays, chemotherapy is the most common

approach for leukemia treatment. Its main functions are to interfere with cell division or DNA synthesis. However, there are some newer agents do not directly interfere with the DNA, instead directly targeting a molecular abnormality in certain types of cancer; these are known as targeted therapies.

Medicinal plants are a major source of nutrients and antioxidants. They have been studied fir their anti-cancer activity and applied in many types of cancer treatment, including leukemia. Many studies have reported that dietary and medicinal plant extracts, such as turmeric, cabbage, guava, and basil, have cytotoxic activity on cancer cells. Kaffir lime has been used for a long time as a flavoring agent and medicine in Southeast Asia. The essential oil from kaffir lime peel and leaf is the source of active chemical components such as β-pinene, limonene, sabinene, and citronellal. The oil and extracts from kaffir lime possess many biological activities, such as antioxidant [26], anti-microbial [26, 27], anti-inflammatory [28], and anti-cancer [25, 29] activities. From previous studies, kaffir lime leaf and peel ethanol fractional extracts have cytotoxic effects on K562, Molt4, U937 and HL60 leukemic cell lines [195]. Kaffir lime leaf extract showed the strongest activity on all leukemic cell lines and had an inhibitory effect on *WT1* gene expression, but nothing was known concerning the effects of kaffir lime leaf fractional extracts.

Crude kaffir lime leaf fractions that were extracted by ethanol, hexane, ethyl acetate, and n-butanol; these exhibited similar cytotoxic effects on K562, Molt4, U937, and HL60 leukemic cell lines, whereas the methanol fraction did not. Kaffir lime leaf crude extracts extracted by ethyl acetate had the strongest cytotoxic effect on all of the leukemic cell

lines used in this study, followed by the hexane fraction, which had an IC_{50} value less than 50 µg/mL. Ethanol and n-butanol fractions of kaffir lime leaf extracts showed lower cytotoxic effects (IC_{50} > 50 µg/mL), and the methanol fraction had no cytotoxic (IC_{50} > 100 µg/mL) effects, compared to the ethyl acetate and hexane fractions. According to the Standard National Cancer Institute criteria, crude extracts possessing an IC_{50} values less than 20 µg/mL are considered active against the tested cancer cells [197]. The extracts with IC_{50} values less than 20 µg/mL against the cancer cell lines were the ethyl acetate fractions, with IC_{50} values of 21.8, 19.8 and 19.0 µg/mL on Molt4, U937 and HL60 cell lines, respectively. Ampasavate *et. al.* (2010) found that ethanolic extract had strong cytotoxicity, with IC_{50} values less than 20 µg/mL on Molt4, U937, and HL60 cell lines (11.9, 9, and 17.1 µg/mL, respectively) [195]; whereas ethanolic extracts had lower cytotoxicity as compared to those for the cell lines used in this study. The IC_{50} values of the ethanolic fraction in the same cell type were different due to individual differences of kaffir lime plants, location, and harvesting time. The study of Manosroi *et. al.* (2006) found that the essential oil extracted from kaffir lime leaf showed anti-proliferative effect on KB and P388 cell lines with the IC_{50} values of 1,147.9 µg/mL and 397.7 µg/mL, respectively [25], which were much higher than that of the ethyl acetate fraction. It is possible that differences in extraction methods and cell lines contributed to the observed differences in IC_{50} values.

In this study, the percentages of yield of each crude kaffir lime leaf fractional extract varied according to the solubility of the solvents. The highest percentage of kaffir lime leaf fraction was due to ethanol, as it can dissolve many compounds present in kaffir lime

leaf. However, crude kaffir lime leaf ethyl acetate and hexane fractions had high cytotoxicity compared to the other fractions. Although the IC_{50} values for the hexane fraction were slightly higher than these of ethyl acetate fraction in all leukemic cell lines, the trends of cytotoxic effect on each cell type were similar. The active compounds responding for the cytotoxic effect on leukemic cells may be extracted and dissolved in the ethyl acetate and hexane fractions.

The study of effects of kaffir lime leaf fractional extracts on *WT1* gene expression in K562, Molt4, U937, and HL60 using non-cytotoxic doses of crude extracts at IC_{20}, suggested that all crude extracts could decrease the WT1 mRNA levels. Nevertheless, only the hexane fraction had strong inhibitory effect on *WT1* gene expression, and the concentrations of the hexane fraction used in four leukemic cell lines were lower than for the other crude extracts used in this study. Referring to the study of cytotoxicity of crude kaffir lime leaf fractional extracts, the hexane fraction had great cytotoxic effect on the four leukemic cell lines as well. Thus, the results from two experiments demonstrate that the active compounds dissolved in the hexane fraction may have the ability to destroy leukemic cells at high doses and can downregulate WT1 mRNA level at non-cytotoxic doses. Possibly the active compounds in the ethyl acetate fraction may be different from those found in hexane, or the amount of active ingredients are not equal, resulting in the low inhibitory effect on *WT1* gene expression of the ethyl acetate fraction in all leukemic cells. Moreover, the active compound in the ethyl acetate fraction may not only affect *WT1* gene and WT1 protein expression, but also other cell proliferation pathways, causing the ethyl acetate fraction to have greater cytotoxic effect than the hexane fraction.

Because the hexane fraction had the largest inhibitory effect on *WT1* gene expression, it was used to study the effect of concentrations and time points of crude kaffir lime leaf extract on *WT1* gene expression in the K562 cell line, used as a representative of all leukemic cell lines. The WT1 mRNA level decrease followed the increase of non-cytotoxic concentrations of the hexane fraction (5-20 µg/mL) in the K562 cell line. The experiment also showed that treatment of the K562 cell line with the hexane fraction at a concentration of IC_{20} for 1, 2, and 3 days could inhibit *WT1* gene expression in a time dependent manner.

The WT1 protein was detected in K562 and Molt4 leukemic cell lines but could not be detected U937 and HL60 cell lines as the level of WT1 in these cells is too low to detect by the methods used in this study. However, different mechanisms of *WT1* gene expression at the transcriptional level of each type of leukemic cell will be subsequently investigated. The crude kaffir lime leaf hexane fraction extract had the strongest inhibitory effect on WT1 protein expression in both K562 and Molt4 cell lines, and decreased WT1 protein levels in the K562 cell line, used as a representative of all leukemic cell line. All of the results supported the hypothesis of a role for WT1 in leukemogenesis.

The effective components in the crude kaffir lime leaf hexane fraction extract need further study. The current results suggest that the active compound dissolved in hexane, a non-polar solvent, and may thus be an essential oil. The essential oils are major compounds in kaffir lime leaves and include many active ingredients, such as citronellal, limonene and α/ β pinene [172, 176]. Studies have shown that essential oil and extract from kaffir lime leaf has anti-cancer activity [25, 195]. The substances extracted in the

hexane fraction should be analyzed by HPLC and further tested for cytotoxicity and inhibitory effect on *WT1* gene and WT1 protein expression.

This study is the first report showing that kaffir lime leaf extract has inhibitory effect on leukemic cells. The results suggest that the crude kaffir lime leaf hexane fraction extract can potentially be used as a chemotherapeutic agent in human leukemias and may lead to clinical trials. The active substances in the hexane fraction and their mechanisms should be studied in regard to natural anti-leukemic drug research.