CHAPTER IV

Discussion

Leukemia is a disease characterized by accumulation of abnormal blood cells with neoplastic proliferation and abnormal differentiation. This phenomenon shows abnormal cells in both bone marrow and peripheral blood circulation. The numbers of patients with leukemia increase currently, chemotherapy is widely used for leukemia treatment. However, it causes many side effects in patients, such as hair loss, anorexia, and even kills normal cells. The incidence of death in leukemia patients is still nigh [21].

Natural compounds have been used for a long time, they are a major sources of nutrients and chemical compounds and plays an important role by treatment of various diseases. Natural compounds have been studied for their ability, especially anti-cancer activity for cancer treatment including leukemia. Many studies have reported that natural compound extracts from herbs and medicinal plants, such as turmeric curcuminoids, galic acid, and α-mangostin, had cytotoxic effects on cancer cells [81]. Kaffir lime has been used for a long time as a traditional medicine in Southeast Asia. The essential oil from kaffir lime peels and leaves is the main source of chemical components such as β-pinene, limonene, sabinene, and citronellal [74, 79]. Previous studies showed that kaffir lime leaf and fruit extracts had anti-oxidant [74], free radical scavenging [84], anti-microbial [80], and anti-inflammatory activities [84]. Kaffir lime essential oil has previously been shown to have an anti-proliferative activity on human mouth epidermal carcinoma (KB) and murine leukemia (P388) cell lines [8]. Furthermore, purified fractional extracts from hexane of kaffir lime leaf showed cytotoxic effect against HL60 (promyelocytic leukemia), K562 (chronic myelocytic leukemia), Molt4 (lymphoblastic leukemia), and U937 (monocytic leukemia) cell lines [12]. Kaffir lime leaf extract also showed its ability to induce cell cycle arrest at the G2/M phase and inhibit cell cycle checkpoint such as cyclin B1, cdc2, cdc25c, and $p21^{WAF1/CIP1}$ [22, 93]. To study the cytotoxic effects of purified fractions from hexane

fractional extract from kaffir lime leaf including fraction No. 9, 10, and 11 in K562 and Molt4 cell lines, cells were treated and assessed by MTT assay. The inhibitory concentrations at 20% and 50% of cell growth (IC_{20} and IC_{50}) values were determined. IC_{20} value (non-cytotoxic dose) was used for studying cell cycle arrest and proteins that control a cell cycle checkpoint. The IC_{50} values were used to compare cytotoxic activities. Chueahongthong *et al,* was previously reported that ethyl acetate and hexane fractions of kaffir lime leaf extracts had the strongest cytotoxic effect on K562, Molt4, U937, and HL60 cells [12]. In this study, the cytotoxic effects of purified fractions No. 9 had the strongest cytotoxic effect on K562 and the cytotoxic effects of purified fractions No.11 had the strongest cytotoxic effect on Molt4 cell lines, respectively. This result indicates that the IC_{50} value of purified fraction No. 9 nearly with No. 11. Tamhakeaw previously showed that purified fraction No. 9 from hexane fractional extract from kaffir lime leaf had the inhibitory effect on WT1 protein expression [89]. It is possible that differences in active compounds and cell lines contributed to the observed differences in IC_{50} values. The purified fractions No. 9 from hexane fractional extract had cytotoxic effects on K562 and Molt4 cells proliferations. Tunjung *et al,* was previously reported that anti-proliferative effects of hexane fractional extract of kaffir lime leaf had cytotoxic effects on cervical cancer and neuroblastoma cell lines including HeLa, UKF-NB3, IMR-5, and SK-N-AS cell lines with the IC_{50} values of 40.8, 28.4, 14.1, and 25.2 μg/mL, respectively [94]. Thus, the purified fraction No. 9 had a main active compound more than purified fractions No.10 and 11.

The most effective purified fractional No. 9 was further studied its effects on cell cycle progression and the protein checkpoints of cell cycle were determined by Flow cytometry and Western blot analysis, respectively. The non-cytotoxic doses (IC_{20} values) were used. The cell cycle control is an important process in a cell proliferation and cell growth. The regulatory proteins that control cell cycle are divided into 2 groups which are cyclin and cyclin-dependent kinase (cdks) [42]. Some natural compounds have been reported their effect on cancer cell inhibition, apoptosis, and/or cell cycle arrest [42]. The purified fraction No. 9 was shown to undergo accumulation of cell population in G2/M phase in K562 cells when compared to vehicle control at 24 h. Moreover, the proportion of cell apoptosis was assessed after sub G1 peak determination. The percentage of sub G1 and G2/M phase populations increased after

purified fraction No. 9 treatment in K562 cells by a dose dependent manner. The effect of purified fraction No. 9 in Molt4 cell line showed only sub-G1 peak and did not show cell cycle arrest. The results demonstrated that the purified fraction No. 9 inhibited K562 cell proliferation by arresting the cell cycle at G2/M phase and further induced cell apoptosis while low doses of purified fraction No. 9 could induce Molt4 cell cycle arrest and cell apoptosis in short time period.

To determine effects of purified fraction No. 9 on regulatory proteins of cell cycle in K562 and Molt4 cell lines by Western blotting using non-cytotoxic doses (IC_{20} values), the result showed that purified fraction No. 9 could decrease cyclin A, cyclin B, cyclin E, and cdc2 protein expressions in both cell lines at 48 h. In addition, it could up-regulate the p53 protein expression in K562 cells. Moreover, the results also showed that purified fraction No. 9 decreased the cyclin B and cdc2 protein expressions and up-regulated the p53 levels in Molt4 cells at 48 h. This result indicated that purified fraction No. 9 induced G2/M phase arrest in both leukemia cell lines. The purified fraction No. 9 responds in a dose-dependent manner. The cell cycle control plays an important role in the control mechanisms that ensure the proper execution of cell cycle events. This study reveals that purified fraction No. 9 inhibits the cell cycle progression at G2/M phase by G2/M phase regulatory protein inhibition. The regulatory proteins involved in G2/M phase are p53, cyclin B, cyclin A, and cdc2 proteins. The G2/M checkpoint, blocks entry into mitosis when DNA is damaged [42]. The increasing of p53 protein level was detected after purified fraction No. 9 treatment and then G2/M phase arrest was induced in both cell lines.

The p53 is tumor suppressor protein. It plays an important role in preventing cancer development, by arresting or killing potential tumor cells. Mutations within the *p53* gene, leading to the loss of p53 activity, are found in about half of all human cancers [47]. Moreover, p53 can regulate G1 arrest and also contributes to G2/M phase arrest in cell cycle *via* induction of p21 protein [47]. The p21 protein is an inhibitor of cyclin/cyclin-dependent kinase complexes and interacts with other regulators of signal transduction [47]. In G2/M arrest, p53 acts by inhibiting cdc2 or by inhibiting binding of cdc2 with cyclin B [49]. Many previous studies found that natural compounds had inhibitory effects on cancer cell proliferation by blocking of cell proliferation at G2/M checkpoint of cell cycle progression. Moragoda *et al.* (2001) investigated curcumin

from turmeric, had strong effect of anti-cancer activity, have been shown to inhibit proliferation by decreasing cdc2 kinase activity with a concomitant increase in p53 and p21 and arresting cells at G2/M phase [48].

This study has provided the new knowledge concerning the anticancer effect of kaffir lime leaf extracts can inhibit leukemic cell proliferation by cell cycle arrest and led to the induction of cell apoptosis.