CHAPTER I

Introduction

1.1 Historical background

Cancer is a world public problem. It causes 13 percent of all deaths which has more than 8 million deaths. In 2007, there were more than 18 million patients around the world and 9 million are new every year. The World Health Organization (WHO) predicts that in 2020 will be more than 11 million people who will die because of cancer around the world, 7 million people in developing countries. Cancer has been the first disease that caused the most death in Thailand for more than 10 years. In 2011 the dead were 61,082 with 35,437 male and 25,645 female that means 7 dead in every hour. WHO predicted that the new patients would be 118,600 each year, and would increase continuously [1]. Leukemia is normally found around the world including Thailand, every gender and age. Each race has different leukemia incidence. It varies to ages and races. Leukemia is known as severe cancer and distant. However leukemia is the first ten of the most cancers which was found. Nowadays the cancer patient has increased no matter what gender and age are. It will be about 260,000 especially on children [2]. The 53 percent of all cancer is leukemia. The 74 percent of leukemia is acute lymphoblastic leukemia (ALL) [3]. An incidence is increasing with high mortality. Chemotherapy is effective treatment for leukemia and it is accepted extensively. Most of chemotherapies destroy cancer cells by DNA creating obstruction while the normal cells are creating DNA also, therefore chemotherapy destroys both normal and cancer cells. That causes unpleasant side effects on patients. In the present, there is not cancer medicine which can destroy only cancer cells yet. Then, the concepts of using famous natural extract or daily-life consumed herbs are occurred. They might be an alternative for leukemia treatment instead of chemotherapy.

Thai herbs are accepted and famous for treatment and nourish body nowadays. Vegetables, plants, fruit, and herbs are well-known as the important antioxidant sources

[4]. There are many researches supported that the antioxidant from vegetables and fruits could reduce the risk of cancer occurrence. For example, the study on 83,234 cancer patients for 14 years showed that consumption of β carotene, vitamin E, and high vitamin C from vegetables and fruits could reduce the risk of cancer occurrence [5]. Moreover the research also found that many vegetables, plants, and herbs could prevent cancer. For instance, Phenolic compound in lead tree is an antioxidant. It can prevent oxidative stress. Every part of lead tree has phenol compounds; flavonoid, tannin, polyphenol, and saponin glycoside [6]. It also has methylglucuronin acid, glucuronin acid, and Galactose [6]. The research also found that curcuma extract (curcumin) could decrease liver cancer of guinea pigs [7]. It also found that flavonoid could impede cells increasing by inducing cells to G_2 phase [9]. Those reasons make present scientists are interested in herbs to study cancer prevention and treatment. In case of cancer treatment, many herbal forms are used such as usual having herbal plants for immunity increasing and cancer cell growth impedius [10]. The common researches are study on reactive ingredients from herbal extracts which have to be developed for chemotherapy replacement to decrease side effects on patients. It is known well that chemical substances from herbal plant extracts are medicine. The studies found that, apart from herbal extracts were antioxidant and also destroyed various cancer cells including leukemia cells. For example, curcuma extracts gave curcumin substance, guava, sweet basil, and kaffir lime leaves could be extracted also [10]. Oil from kaffir lime fruit and leaf could thwart KB and P388 cancer cells [8]. The kaffir lime extracts could stop leukemia cell growth, gene expression, and Wilms' tumor 1 (WT1) protein activity [12]. The *WT1* gene has found its high expression in leukemia cells and encoded WT1 protein that influences cancer cell mitosis control [13, 14]. Moreover, the tangeretin extract (flavonoid) in leech lime leave also impeded cells increasing. It induced cells to G_1 phase by thwarting cyclin-dependent kinases 2 (CDK2) and 4 (CDK4), then increasing CDK inhibitors effectiveness; p21 and p27 in human intestinal cancer cells [16]. Kiffir lime fractional extracts (different polarity solvents) were previously studied their effect on WT1 protein expression. The result showed that hexane extract was the best extract to decrease WT1 protein expression in leukemic cell lines. Moreover, partially purified hexane fractional extracts (fraction No. 1-13) after reverse phase chromatography were preliminary examined their cytotoxic effect in leukemic cell lines

(K562 and Molt4 cell lines). The result found that the partially purified fraction No. 9, 10, and 11 (F9, F10 and F11) from all 13 fractions had anti-leukemic activities by using MTT assay. Therefore, the partial purified fraction No. 9, 10, and 11 were further examined their effects on cell cycle progression in K562 and Molt4 leukemic cell models by flow cytometry and also studied cell cycle regulatory proteins related to cell cycle arrest Western blot analysis. This study would provide new knowledge of possible compound in the partially purified hexane fractional extracts from leech lime leaf on cell cycle progression. The new knowledge from this study should be data obtained for drug development in complementary and alternative medicine of leukemia treatment in the future or co-treatment with chemotherapeutic drugs to reduce their side effects in leukemia patients.

1.2 Objectives

1.2.1 To investigate anti-proliferative effects of partially purified fractions of kaffir lime (*Citrus hystrix* DC.) leaf in K562 and Molt4 leukemic cell lines.

1.2.2 To investigate effects of partially purified fractions from hexane fractional extract of kaffir lime (*Citrus hystrix* DC.) leaf on cell cycle progression in K562 and Molt4 leukemic cell lines.

1.2.3 To determine the inhibitory effects of partially purified fractions from hexane fractional extract of kaffir lime (*Citrus hystrix* DC.) leaf on cell cycle regulatory proteins in K562 and Molt4 leukemic cell lines.

1.3 Literature reviews

1.3.1 Leukemia

Leukemia is a type of cancer that starts in the bone marrow and results in the high numbers of abnormal white blood cells called leukemia cells. Leukemia cells can build up and crowd out normal blood cells. The low level of normal blood cells can make it harder for the body to get oxygen to the tissues, control bleeding, or fight infections. Also, leukemia cells can spread to other organs, such as the lymph nodes, spleen, and brain. Leukemia is the 11th most common cancer worldwide, with around 352,000 new cases diagnosed in 2012 (2% of the total) [17, 19]. The disease results from the neoplastic proliferation of hematopoietic or lymphoid cells. The cell in which the leukemic transformation occurs may be a precursor or pluripotential hematopoietic

stem cell that can differentiate into both myeloid and lymphoid cells [20]. Leukemia can spread to other organs such as lymph nodes, spleen, liver, central nervous system, and cause pathogenesis in these organs.

1.3.2 Risk factor of leukemia

The cause of leukemia in most patients is unknown; several factors are associated with increased risk of developing the disease. Many factors that influence risk of developing leukemia include:

1.3.2.1 Age

The risk of developing most types of leukemia increases steadily with age. For example, the trend for acute lymphoblastic leukemia (ALL) incidence shows the highest between the ages of 3-7 and rising again after the age of 40 [21]. The reason for this peak in early childhood ALL remains unclear.

1.3.2.2 Chemotherapy

There is a subset of acute myeloid leukemia (AML). Known as “therapy related myeloid leukemia” or “secondary AML” which can develop following treatment with chemotherapy. However, the exact mechanism remains uncertain. Prognosis for secondary AML is generally unfavorable compared to primary AML.

1.3.2.3 Ethnicity/gender

Leukemia occurs more commonly in those of white ancestry compared to those of Asian, Hispanic and black ancestry. However white and black origins have the same incidence rate of chronic myeloid leukemia (CML). Moreover, leukemia also occurs more frequently in males than females.

1.3.2.4 Inherited syndromes

Approximately 10% of children with Down syndrome are born with a “transient leukemia” that resolves spontaneously within months of birth [21]. Other inherited syndromes that increase risk of leukemia include Ataxia-telangiectasia, Bloom syndrome, Fanconi syndrome, Klinefelter syndrome and neurofibromatosis.

1.3.2.5 Ionizing radiation

Radiation has been suspected play a role in causing leukemia. Radiologists and other who were exposed to high level of irradiation for long periods such as the Japanese people in the atomic bombing of Hiroshima and Nagasaki could be developed.

1.3.2.6 Certain chemical exposures

The risk of AML is increased by exposure to certain chemicals. For example, longterm exposure to high levels of benzene or formaldehyde that is the risk factor for AML [21].

1.3.3 Classification of leukemia

Leukemia can be classified as acute or chronic types. Acute leukemia progresses more rapidly than chronic leukemia that requires immediate treatment. Moreover, leukemia is also classified by cell lineages as lymphocytic or myelocytic leukemia. Leukemias are broadly divided into: (i) acute leukemia, which if untreated, leads to death in weeks or months; and (ii) chronic leukemia, which if untreated, leads to death in months or years. They are further divided into lymphoid and myeloid leukemia. Thus, four types of leukemia are acute myeloblastic leukemia (AML), acute lymphocytic leukemia (ALL), chronic myelocytic leukemia (CML), and chronic lymphoblastic leukemia (CLL). Acute leukemia is characterized by a defect in maturation, leading to an imbalance between proliferation and maturation; since cells of the leukemic clone continue to proliferate without maturing to end cells and dying there is continued expansion of the leukemic clone and immature cells predominate. Chronic leukemia is characterized by an expanded pool of proliferating cells that retain their capacity to differentiate to end cells [13, 22].

1.3.3.1 Acute myeloblastic leukemia (AML)

Acute leukemia is a proliferation of immature myeloid cells in bone marrow derived cells (blasts) that may also involve in peripheral blood or solid organs. The percentage of bone marrow blast cells required of a diagnosis of acute leukemia has traditionally been set arbitrarily at 30% or more. However, more recently proposed classification systems have lowered the blast cell count to 30% for many leukemia types, and do not require any minimum blast cell percentage when certain morphologic

and cytogenetic features are present. AML is a malignant disease of the bone marrow in which hematopoietic precursors are arrested in an early stage of development. Most AML subtypes are distinguished from other related blood disorders by the presence of more than 20% blasts in the bone marrow. About 15,000 Americans will be diagnosed with AML in 2013 [23]. To date, AML accounts for nearly 80% of all adult acute leukemia cancer. In contrast, AML observed in childhood leukemia for 10-15% [23]. Moreover, AML also occurs more frequently in males than females and has high incidence in Europeans and Africans. The constant feature of AML is anemia and thrombocytopenia is nearly always present. More than 50% of patients have a platelet count less than 50,000/μL at the time of diagnosis [43]. In addition, more than 50%, of patients have the total white blood cell count less than 5,000 cells/μL, and the absolute neutrophils count less than 1,000 cells/μL [24].

1.3.3.2 Classification of acute myeloid leukemia

The French-American-British Cooperative Group (FAB) and the newer World Health Organization (WHO) classification have been used to classify AML into subtypes. The FAB divide AML into 9 subtypes (M0 to M7), distinct subtypes that differ morphologic features, immunophenotyping and cytochemical studies (Table 1.1).

1.3.3.2.1 Mo acute myeloblastic leukemia with minimally differentiated

The blasts in M0 AML usually resemble M1 myeloblasts or L2 lymphoblasts. In a minority of cases they resemble the monoblasts of M5 AML. Associated dysplastic features in erythroid and megakaryocyte lineages may provide indirect evidence that leukemia is myeloid not lymphoid accounts for approximately 5-10% of all AML. Forty percentages of patients showed leukocytosis and about 50% of patients showed leukocytopenia. Less than 3% of the blasts are positive for peroxidase or the Sudan black B reaction and Immunophenotyping is now widely used for identifying cases of M0 AML., the blasts are positive for immunophenotyping specific lymphoid markers CD13, CDI4, CD15, CD33, or CD34 and negative for B or T lineage marker (CD3, CD10, CD19, and CD5). Bone marrow smear was hypercellularity in all patients and contained many leukemic blast cells [25] with CD34+, human leukocyte

Table 1.1 French-American-British (FAB) classification of AML [24].

Acute myelocytic leukemia	**FAB classification**
M0	Myeloblastic without maturation or undifferentiated leukemia Blast > 90% of nonerythroid bone marrow cells and conventional cytochemistry negative
M1	Myeloblastic with minimal maturation Blast > 90% of nonerythroid bone marrow cells and promyelocytes < 10%
M2	Myeloblastic with maturation Myeloblastic 30-89% of nonerythroid Granulocytic component > 10% and Monocytic component < 20%bone marrow cells
M3	Promyelocytic Promyelocytes ≥ 30% Abnormal promyelocytes with heavy granulation and some with multiple Auer rods (faggots). Typical reniform or bilobed nucleus
M4 Subtype: M4eo M4baso	Immature monocytes comprising 20-80% of of nonerythroid bone marrow cells Blast > 30%, monocyte component > 20% M4 with eosinophil > 5% M4 with basophil maturation
M5a	Monoblastic Monoblast > 80%, granulocytic component < 20%
M5b	Promonocytic Mixture of monoblast and more mature monocytic cells
M6	Erythroleukemia Erythrod precursor ≥ 50% and myeloblast > 30%
M7	Megakaryoblastic Megakaryoblast > 30%

antigen DR (HLA-DR), and terminal deoxymucleotidyl transferase (TdT). CD7 is more often expressed than in other FAB categories of AML [26]. In most case, the blast cells were small to medium-sized round cells with an eccentric nucleus and mature myeloid cells were not seen. The nucleus is often had flattened shape and was sometimes lobulated or cleaved and contained fine chromatin with several distinct nucleoli. The cytoplasm was lightly basophilic without granules. Auer rods are not found.

1.3.3.2.2 MI Acute myeoblastic leukemia without maturation

AML-M1 is found in all aged groups with the highest incidence seen in adult and in infants. Estimated 50% of patients show the increase of white blood cells count at the time of diagnosis. The peripheral blood smear is usually shows a poorly differentiated myeloblast with finely reticulated chromatin and prominent nucleoli. M1 blasts are usually medium to large in size, a round or oval nucleus, one or more nucleoli, and cytoplasm that sometimes contain Auer rods. For the diagnosis of acute myeloid leukemia of M1 AML, blasts must be ≥ 30% of bone marrow cells and ≥ 90% of bone marrow non-erythroid cells. Promonocytes to monocytes should be ≤ 10% of non-erythroid cells. At least 3% of the blast cells are positive for myeloperoxidase or sudan black B stains indicating granulocytes differentiation but negative for PAS, alpha-naphthyl acetate esterase and naphthol AS-D esterase staining. Moreover, about 50% of the patients will have acquired clonal chromosome aberrations in the leukemic cells. The myeloid markers, M1 AML blasts are usually positive for CD13, CD33, and HLA-DR, and may express CD34 [27] .The most common cytogenetic abnormalities are t(9;22) (q34; q11).

1.3.3.2.3 M2 Acute myeloblastic leukemia with maturation

AML-M2 is the most common subtype. The symptoms for M2 AML are similar to those of the M1 type. Myeloblast may be the predominant cell type found in the blood smear. Pseudopelger-Huet and hypogranular neutrophils being most common cells are found in M2. The bone marrow smear shows the blasts account for 30-83% of non-elytroid cells. The monocytic components are less than 20%, differentiating M2 from M4. Basophils in some patient (M2baso) were increased. Eosinophil's and their precursor may be abundant, and in some cases accounts for up to 15% or myelogram [28].

The neutrophils may show many abnormal nuclear segmentations and Auer rods. The granulocytic nature of AML-M2 is usually demonstrated clearly by the presence ofmaturing cells in the granulocytic series. Myeloblasts of M2 AML always express HLA-DR, CD13, CD15, and CD33. Expression of CD34 and CD117 are infrequently found.

1.3.3.2.4 M3 Acute promyelocytic leukemia (APL)

AML-M3 is found in all aged groups with highest incidence seen in younger adult. Promyelocyte are the predominant cell type found in the blood smear. Myeloblasts are also increased in the peripheral blood. AML-M3 is divided into two major morphologic subtypes: hypergranular and hypogranular. The hypergranular variant is characterized by the presence of atypical promyelocytes heavily loaded with azurophilic granules. Auer rod is common and may appear in bundles in some of the promyelocytes (faggots cells). In the hypogranular variant, promyelooytes contain fewer and finer azurophilic granules. The nucleus is often bilobed or markedly indented and a nucleolus can be seen in each lobe displaying a monocyte-like morphology.The leukemia cells in M3 AML are strongly MPO and SBB positive. A small proportion of cases may also demonstrate NSE reactivity. Moreover, CD13, CD33, CD11, and CD15 antigens are often expressed but HLA-DR and CD14 are negative. Association of the lymphoid markers is CD2 and CD19 with myeloid markers and the negativity of HLA-DR and CD34.

1.3.3.2.4 M4 Acute myelomonocytic leukemia (AMML)

AML-M4 can distinguish from MI, M2, and M3 by an increased proportion of leukemia monocytic cells in the bone marrow or blood or both. Gingival hyperplasia combination with gingival bleeding is present in AML-M4 patients. The white blood cell count is usually shows the increase of monocytic cells (monoblast, promoncyte, monocytes) anemia, and thrombocytopenia are present in almost al lcases. The marrow smear differs from AML-M1, M2, and M3 in those monocytic cells exceed 20% of the non-erythroid nucleated cells. The sum of the myelocytic cells including myeloblasts, promyelocytes, and later granulocytes is > 20% and < 80% of nonerythroid cells. Cytochetmistry stains were shown the positive reactions of sudan black B or peroxidase and both specific and non-specific esterase. A few cases of M4 AML are characterized by increased marrow eosinophils and classified as M4e [48]. Immuno-

logical studies revealed the expression of CD13, CD33, CDI1b, and CD14. The most common cytogenetic abnormalities are: inv (16) (p13: q22) and del (16) (q22).

1.3.3.2.5 M5 Acute monoblastic leukemia (AMoL)

AML-M5 is accounting for 10% of all AML cases. The criterion for a diagnosis of M5 is that 80% or more of all non-elytroid cells in the bone marrow are monocytic cells. AML-M5 is divided into two subtypes characterized by the presence of all developmental stages of monocytes; monoblast, promonocyte, monocyte.

M5a (maturation index < 4%) demonstrates minimal morphological evidence of monocytic differentiation. Monoblasts account for 80% or more of the leukemic cells. They have a variable amount of gray-blue or deep blue cytoplasm, often round or oval nuclei and a single or a few very prominent nuclei.

M5b (maturation index > 4%) displays partial monocytic differentiation with a mixture of monoblasts, promyelocytes and more mature monocytic cells. Auer rods are detected in a small proportion of the M5b subtype.

Non-specific esterase stains and alpha-naphthyl esterase are positive and PAS is negative myeloperoxidase and sudan black B are weak diffuse activity in the monoblast. Immunological studies demonstrate positivity with CD11b and CD14 There is a strong association between AML M5/M4 and deletion and translocations involving band 11q23.

1.3.3.2.6 M6 Acute erythroblastic leukemia

The AML-M6 accounts for about 5% of all AML cases, most AML-M6 cases are preceded by a refractory anemia. The criteria for diagnosis of AML-M6 are bone marrow containing erythroblast ≥ 50% and non-erythroid blasts ≥ 30%. Erythroid dysplasia may manifest as binuclearity, nucleocytoplasmic asynchrony and vacuolation. Other features such as fragmentation, Howell-Jolly bodies, ring sideroblast, megaloblastic changes are common. Blood smear often shows abnormal red blood cell morphology and marked aniso-poikilocytosis. Myeloperoxidase and Sudan black B stains may be positive in the myeloblasts. The iron stain may show ringed sideroblasts end PAS may be positive in the erythroid precursors in a block or diffuse pattern. The erythroid precursors express glycophorin A, transferrin receptor (CD71), hemoglobin and spectrin, and express CD13, CD33, HLA-DR. Cytogenetic studies may demonstrate structural abnormalities in chromosome 3, 5, and 7 or trisomy 8.

1.3.3.2.7 M7 Acute megakaryoblastic leukemia (AMkl)

M7 AML is predominantly megakaryoblasts. They are polymorphic and vary in size, amount of cytoplasm, chromatin density, and the number of nuclei. Bone marrow fibrosis, as a consequence of unsuccessful marrow aspiration, is one of the characteristic features of M7 AML observed in over 70% of cases. Megakaryoblasts often appear in clusters trapped within the fibrotic tissue. M7 AML comprises about 5% of all AML cases but is probably the most common type of AML associated with Down's syndrome. Bone marrow biopsy shows increased fibroblasts and/or increased reticulin and presence of greater than 30% blast cells. Bone marrow fibrosis one of the characteristic features of AML-M7 observed in over 70% of cases. Megakaryoblasts often appear in clusters trapped within the fibrotic tissue. Cytochemical positivity for α-naphthyl acetate esterase reaction and negative reaction with α-naphthyl butyrate esterase is unique to megakaryoblast. Immunological techniques of AMkl are CD41, CD42 and CD61 positivity. Trisomy 21 and t(1;22) have been reported in *de novo* AML-M7. Moreover, trisomy 8 and structural abnormalities of chromosomes 5 and 7 have been found in therapy-related leukemia.

1.3.3.3 Acute lymphoblastic leukemia (ALL)

Acute lymphoblastic leukemia most commonly affects children between 3 and 10 years of age. It accounts for about 85% of childhood leukemia and is the most common type of cancer children. It also affects adults, mainly those between 30 and 50 years of age, accounting for 20% of all adult acute leukemia [22]. ALL is divided into a number of different subtypes based upon the clinical, morphological, laboratory und in some cases, cytogenetic features. The current version of the FAB classification, which recognizes three distinct subtypes solely on morphological studies, and the present WHO classification are shown table 1.2.

1.3.3.4 Chronic myelocytic leukemia (CML)

Chronic myelocytic leukemia (CML) is a clonal myeloproliferative expansion of transformed, primitive hematopoietic progenitor cells [31]. It was the first hematolo-gical malignancy involved with a specific chromosome abnormality, known as the Philadelphia chromosome [32]. The Bcr/Abl fusion results in constitutive activa-

tion of tyrosine kinase, which leads to uncontrolled proliferation of myeloid cells [31]. In CML, leukemia cells tend to build up in the body over time. Most cases of CML occur in adults accounting for 15% of all leukemia, and most cases occur in patients at around 60 years of age [33].

Table 1.2 FAB classification of acute lymphocyte leukemia [22].

ALL type	FAB classification of ALL
L1	Small cells with scant cytoplasm; nucleoli indistinct and not visible
L2	Large, heterogeneous cells with moderately abundant cytoplasm; clefting and indentation of nucleus; large and prominent nucleoli
L3	Large cells with moderately abundant cytoplasm; regular, oval-to-round nucleus; prominent nucleoli; prominent cytoplasmic basophilia and cytoplasmic vacuoles

1.3.3.4.1 Phases of Chronic myelocytic leukemia

CML is divided into 3 phases based the number of immature white blood cells are seen in the blood or bone marrow. There are chronic phase, accelerated phase, and blast crisis phase.

1.3.3.4.1.1 Chronic phase

Patients in the chronic phase may be asymptomatic. Common symptoms include fatigue, night sweats, and splenomegaly with abdominal discomfort. Some cases, patients may present with a hyperviscosity syndrome, stroke, pianism, and stupor or visual changes caused by hemorrhage [34].

CML-chronic phase may appear for several years and most patients are diagnosed in this phase. CML-chronic phase is characterized by accumulation of myeloid precursor and mature cells in bone marrow, peripheral blood, and extramedullary sites. A peripheral blood examination shows the white blood cell count is usually more than 50×10^9 cells/L, with the range of 20×10^9 cells/L to 800×10^9 cells/L. About 50% of patients who diagnosed as CML are present anemia and the

platelet count is elevated. During this phase, leukemic cells can also retain the ability to differentiate normally, a full spectrum of myeloid cells from blast to neutrophils are found in the peripheral blood, with blasts less than 5% of all white blood cell differential, basophilia and eosinophilia are common. The bone marrow examination shows hypercellularity with granulocytic and megakaryocytic hyperplasia, basophilia and blasts cells less than 5% [35].

1.3.3.4.1.2 Accelerated phase

Approximately 2-5 years of untreated CML, the disease will progress from the chronic to the accelerated phase. Patients in the accelerated phase may have progressive splenomegaly, bone pain and other complaints such as fatigue, fever, poor appetite, and weight loss. They do not respond to treatment as well as during the chronic phase. Criteria for diagnosing transition into the accelerated phase are variable. The World Health Organization (WHO) criteria indicate a diagnosis if one or more of the following is present [36].

- Blast 10% to 19% of bone marrow cells or peripheral blood with cells
- Peripheral blood basophils at least 20%
- Persistent thrombocytopenia ($<100\times10^9$ cells/L) unrelated to therapy, or persistent thrombocytosis ($>1000\times10^9$ cells/L) unresponsive to therapy.
- Increasing spleen size and increasing WBC count unresponsive to therapy.
- Cytogenetic evidence of clonal evolution (the appearance of an additional genetic abnormality that was not present in the initial specimen at the time of diagnosis of chronic phase CML.
- Megakaryocytic proliferation in sizable sheets and clusters, associated with marked reticulin or collagen fibrosis, and/or severe granulocytic dysplasia, should be considered as suggestive of CML-AP. These findings have not yet been analyzed in large clinical studies, however, so it is not clear if they are independent criteria for accelerated phase, they often occur simultaneously with one or more of the other features listed.

1.3.3.4.1.3 Blastic Phase (Blast crisis)

The terminal phase of CML is blast crisis phase, patients often feel oven worse. This phase is characterized by the rapid expansion of a population of myeloid or lymphoid differentiation-arrested blast cells. Cytochemistry stain of CML shows the markedly abolished of leukocyte alkaline phosphatase (LAP) score. However, elevated LAP may be seen in CML during remission, pregnancy, blast crisis, and bacterial infection. The blast crisis of CML is characterized by the presence of blast cells about 20% or more of peripheral blood white cells or bone marrow cells. The blast cells show very variable morphological and cytochemical characteristics. The most obviously seen is appearance of myeloid precursors containing PAS material.

1.3.3.4.2 Chronic lymphocytic leukemia (CLL)

Chronic lymphocytic leukemia (CLL) or, chronic lymphatic leukemia, is the most common type of leukemia in the Western people and affects mainly elderly individuals [37]. CLL is usually a slow growing leukemia and some patients with CLL for survive for many years with minimal or no treatment. However, in other case, the disease is more aggressive and requires more intensive treatment. Most cases of CLL occur in older adult patients at around 71 years of age or older. It is rarely seen in people under the age of 40 and is very rare in children. About 14,620 Americans will be diagnosed with CML in 2015 and about 4,650 Americans were died from CML in 2015 [48]. In most case of CLL the cells are B-cell lineage, although the less frequently occurring T cell is well recognized. In most case of CLL, the bcl-2 protein, protein that produced from the *bcl-2* proto-oncogene and functions as an inhibitor to apoptotic cell death in overexpressed [38]. Approximately 80% of cases are present chromosomal abnormalities. Most common are deletions of chromosomes 13ql4 and 11q22-23 as well as trisomy 12 [39].

1.3.3.5 Leukemia treatments

In general, there are five major approaches to the treatment of leukemia including chemotherapy, interferon therapy, radiotherapy, stem cell transplantation (SCT), and surgery.

1.3.3.5.1 Chemotherapy

Chemotherapy is the use of drugs to kill leukemia cells. The classical chemotherapy functions by destroying the cells that divided rapidly one of the main properties of most cancer cells.

1.3.3.5.2 Interferon therapy

Interferons are a class of proteins that are released by virus-infected cells. They help normal cells to make antiviral proteins. Interferons also help the body to reduce leukemia cell proliferation (growth and reproduction) and promote immune system.

1.3.3.5.1 Radiotherapy

Radiotherapy is the use of high-energy radiation to shrink tumors and kill cancer cell [39]. There are three types of radiation used for cancer treatment including X-rays, gamma rays, and charged particles.

1.4 Cell cycle

The cell cycle or cell-division cycle is the series of events that takes place in cell leading to its duplication (replication) and division. The cell-division cycle is a vital process by which a single-celled fertilized egg develops into a mature organism, as well as the process by which hair, skin, blood cells, and some internal organs are renewed. In eukaryotic cells, the cell cycle can be divided into four major phases; G1 is the first gap phase in which the cell prepares for DNA replication; S phase is the period of DNA synthesis during which a second copy of the entire genome is generated. Duplicated chromosomes consist of two identical strands of DNA. The two pieces are not completely separated at this point. They are held together at a pinched in region called the centromere. Each identical stand is called a sister chromatid. On either side of the centromere region, protein discs called kinetochores provide the place of attachment for kinetochore fibers which are part of mitotic spindle. G2 is a second gap phase in which the cell prepares for division, and M phase or mitosis is the period during which the two copies of DNA segregate and the cell divides into two genetically identical daughter cells [40] (Figure 1.1). Postmitotic cells in multicellular organisms can “exit” the cell cycle and remain for days, weeks, or in some cases (e.g., nerve cells and cells of the eye lens) even the lifetime of the organism without proliferating further. A cell may also

exit the cell cycle to undergo processes of differentiation or programmed cell death [41]. Many complex signals interact to determine a cell's fate, specifying whether it should be quiescent, divide, differentiate, or undergo apoptosis. Most postmitotic cells in vertebrates exit the cell cycle in G1, entering a phase called G0. A G0 cell is often called "quiescent". Many G0 cells are busy carrying out their functions in the organism. G0 cells can be followed by reentry into the cell cycle with proper stimulation. They can be stimulated to reenter the cell cycle at G1 and proceeded on to new rounds of alternating S phases and mitosis. Cancer cells cannot enter G0 and are destined to repeat the cell cycle indefinitely [42].

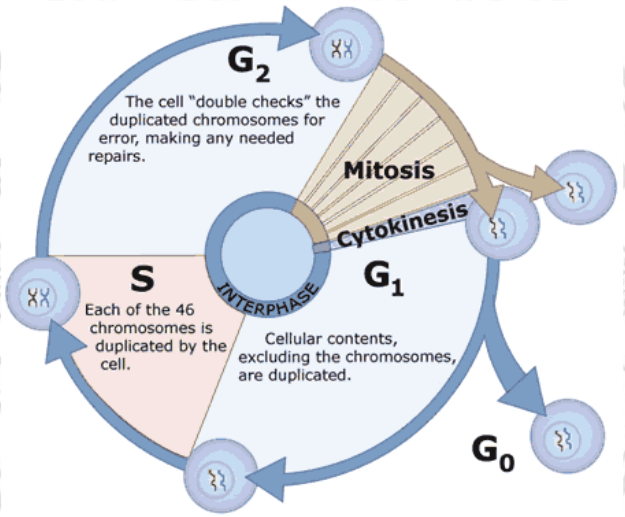

Figure 1.1 Cell cycle progression [53].

1.4.1 Cell cycle control

In multicellular organisms, precise control of the cell cycle during development and growth is critical for determining the size and shape of each tissue. Cell replication is controlled by a complex network of signaling pathways that integrate extracellular signals about the identity and numbers of neighboring cells and intracellular cues about cell size and developmental program. The master controllers of cell cycle event are composed of two core components, cyclin-dependent kinases (cdks) and cyclins. Without their cyclin partner, cdks are inactive. Each cdk catalytic subunit can associate with different cyclins, and the associated cyclin determines which proteins are phosphorylated by the cdk-cyclin complex. A variety of cyclin:cdk complexes are formed during distinct phases of cell cycle (Figure 1.2), each dedicated to the phosphorylation of a distinct set of target proteins. In general, the levels of cdks are

relatively constant throughout the cell cycle, while the levels of the cyclins are restricted by transcriptional regulation of cyclin-encoding genes and by ubiquitin-mediated degradation. This indicates that the activity of cyclin:cdk complexes is determined in part by the levels of available cyclins [42]. The principle cdks active in most mammalian cells have been named cdk1, 2, 4, and 6. Mammalian cells express multiple cyclins include cyclin A, B, D, and E. An assemblage of cyclin: cdk complexes orchestrates the advance of the cell though the phase of its growth cycle. In brief, as cells emerge from quiescence in response to mitogenic stimuli, the synthesis of cyclin D is induced. The continued presence of mitogens ensures that the levels of these cyclins remain high throughout the remainder of the cell cycle. Once synthesized, the cyclin D associates with cdk4 and cdk6. In mid/late G1, several hours before the onset of S phase, cyclin E is induced and form complexes with cdk2. The activity of this complex appears to be essential for entrance into S phase, when cyclin A appears in concert with the onset of DNA synthesis. Cyclin A associates initially with cdk2 and later with cdk1 (cdc2). This association continues until the late G2 phase cyclin B appear, forming complexes with cdk1 and triggering the complex series of events associated with mitosis [43].

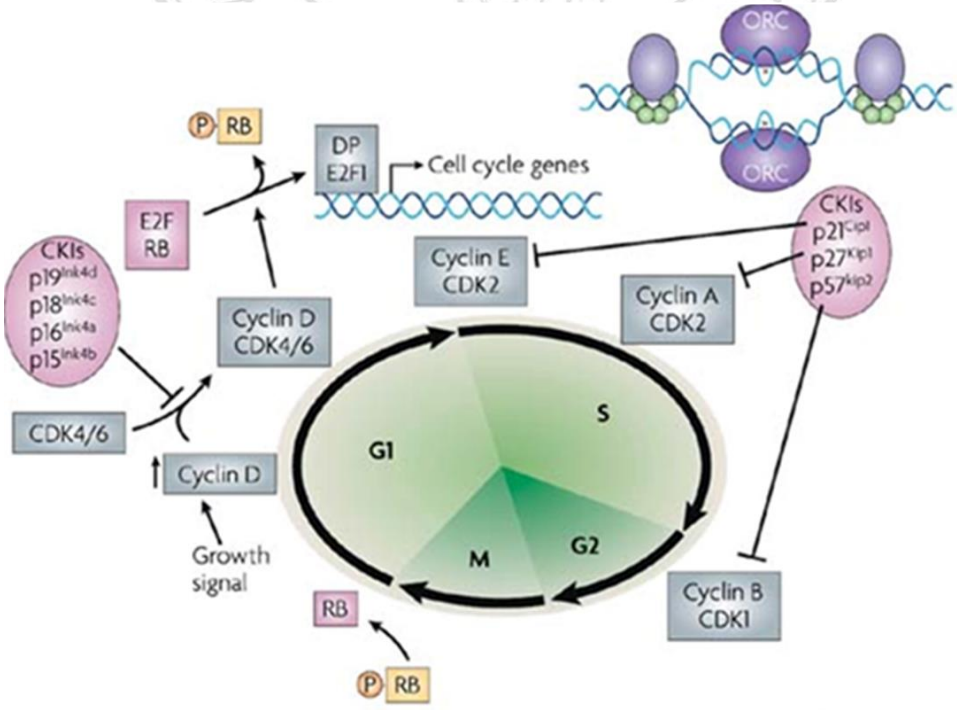

Figure 1.2 Activity of mammalian cdk-cyclin complexes in each phase of cell cycle [43].

1.4.2 Checkpoints in cell cycle regulation

Checkpoint is quality control of the cell cycle. The occurrence of such mistakes in cell cycle events, a cell's progression through the cycle is monitored at four key checkpoints [41]. Control mechanisms that operate at these checkpoints ensure that chromosomes are intact and that each stage of the cell cycle is completed before the following stage is initiated [42].

Checkpoint signaling may also result in activation of pathways leading to programmed cell death if cellular damage cannot be properly repaired. Defects in cell cycle checkpoints can result in gene mutations, chromosome damage, and aneuploidy, all of which can contribute to tumorigenesis [44]. The presence of unreplicated DNA prevents entry into mitosis. Cell that fail to replicate all their chromosomes do not enter mitosis. Operation of this checkpoint control involves the recognition of unreplicated DNA and inhibition of mitosispromoting factor (MPF) activation. MPF is a heterodimer composed of a mitotic cyclin and a cdk. The protein kinase activity of MPF stimulates the onset of mitosis by phosphorylating multiple specific protein substrates [45].

Improper assembly of the mitotic spindle leads to arrest in anaphase. The mitotic spindle checkpoint monitors the microtubule structure and chromosome attachments of the mitotic spindle and delays chromosome segregation during anaphase until defects in the mitotic spindle apparatus are corrected. When the mitotic spindle has not assembled properly, a checkpoint control prevents activation of anaphase-promoting complex (APC) polyubiquitination system that normally leads to degradation of the anaphase inhibitor, required for onset of anaphase, and later to the degradation of mitotic cyclins, required for the exit from mitosis [46]. Cells whose DNA is damaged by irradiation with UV light or X-rays or by chemical modification become arrested in G1 and G2 phase until the damage is repaired. Arrest in G1 phase prevents copying of damaged bases, which would fix mutations in the genome. Replication of damaged DNA also causes chromosomal rearrangements at high frequency. Arrest in G2 phase allows DNA double-stranded breaks to be repaired before mitosis. If a double-stranded break is not repaired, the broken distal portion of damaged chromosome is not properly segregated because it is not physically linked to a centromere, which is pulled toward a spindle pole during anaphase. Genes whose inactivation contributes to the development of a tumor are called tumor-suppressor genes. The most commonly mutated tumor-suppressor genes associated with human cancers is p53. The p53 protein functions in

the checkpoint control that arrests human cells with damaged DNA in G1 phase, and it contributes to arrest in G2 phase. Cells with functional p53 arrest in G1 or G2 phase when exposed to ɣ-irradiation, whereas cells lacking functional p53 do not arrest in G1phase [47]. Moderate DNA damage activates p53, a transcription factor that stimulates expression of p21CIP. This cyclin-kinase inhibitor then binds to and inhibits all cdk cyclin complexs, causing arrest in G1 and G2 phase. In response to extensive DNA damage, p53 activates gene that induce apoptosis [48].

1.4.3 Phase of cell cycle

1.4.3.1 G1 phase

G1 phase (Gap1) is cell preparation before DNA creating; protein or significant biomolecule synthesis for cell mitosis. After cell mitosis, the DNA creating does not occur; this is the most important point of cell cycle because it is the beginning of cell mitosis preparation. Cells must not have any abnormality inside and be ready for mitosis. Cells influence protein synthesis that controls important process such as more cyclin D production. The cyclin D protein combines with cyclin-dependent kinase (CDK4 and 6) to be cyclin D/CDD4-CDK6. It is be activated by CDK-activating enzyme (CDK = cyclin H/CDK7) by adding phosphate to cyclin D/CDK4/CDK6, then cyclin D/CDK4/CDK6 works by adding phosphate to retinoblastoma (Rb) [49]. Actually a few hypophosphorylation combines with E2F protein, then E2F cannot activate protein synthesis. When Rb protein is added by a lot of hyperphosphorylation [49, 50], Rb protein is isolated from E2F then E2F could activate any proteins in cell cycle. The cyclin E can be synthesized more and combine with CDK2 to be cyclin E/CDK2 which can increase positive feedback of phosphate adding to Rb protein. Meanwhile cyclin A is produced and combines with CDK2 enzyme to be cyclin A/CDK2 and impedes E2F function; cell cycle finished restriction point (R) at the end of G1 phase. After passing restriction point, heading to S phase without obstructs or back although there is not any activations from mitogen or cell mitosis activated substances. Some cells do not replicate; such as neuron, so they are in silent period which is called G0 phase; protein synthesis does not happen. In cell cycle controlling in G1 phase, apart from protein creating which is used to activate cell cycle, cells also control cell cycle by protein creating which can stop cycle cell in G1 phase. They are proteins in inhibitor of CDK4/alternative reading frame family; INK4a/ARF family,

which is p15, p16, p18, and p19, they can stop cyclin D/CDK4/CDK6 function. Other proteins are called cip/kip family which can stop cyclin D/CDK4/CDK6, cyclin E/CDK4/CDK6 and cyclin A/CDK2 functions which are p21, p27, and p57 [51-54]. Those proteins influence normal cell cycle. If those proteins works improperly, uncontrolled cell mitosis is happened which is one cause of cancer.

1.4.3.2 S phase (synthesis)

When cells send signal for mitosis, DNA synthesis increasing is started and other biomolecules are prepared. In this phase, the amount of DNA is twice with is controlled by cyclin A/CDK2. When DNA synthesis is correct, it will become another phase of cell cycle. However, if there are any dysfunctions in this phase; DNA synthesis or DNA is destroyed, it activates p53 protein to be created. The p53 activates p21 protein synthesis, in this stage the cell cycle is temporary stopped for giving repairing time to DNA. However, if there are too many DNA dysfunctions or DNA are seriously damaged, p53 influences cells to apoptosis. The p53 increases when DNA damage is occurred, p21 synthesis in cancer cells will be happened. Normally found that the mutated p53 cannot control cell cycle anymore, then abnormal cells are happened and uncontrolled; cancer cells. Apart from p53, Rb protein is another one which controls cell cycle, cell differentiation, and apoptosis. Rb is phosphoprotein with 928 amino acids, it obstructs E2F function; transcription factor, which is significant protein in S phase to activate and control cell cycle. Therefore, Rb controls cell cycle and is tumor suppressor. When the abnormality happens, E2F and non-phosphorylate Rb cannot activate. They cannot match gene promotor and begin transcription. In S phase, Rb is added phosphate, E2F isolates from Rb and matches gene promotor then activate gene transcription. In any case of Rb mutation, cells will replicate unstoppably then cancer happens. In the study on CML, that the blast cells in CML normally influence mutation of p53 tumor suppressor. Moreover, not only p53 and Rb which are tumor suppressor [55, 56], another protein is Wilms' tumor 1; WT1, which is controlled on its creation by *WT1* gene. Type of protein is zinc finger motif. WT1 protein is a significant protein on cancer control. When there is abnormal cells because of abnormal gene, WT1 has high expression on abnormal cells' expression control. *WT1* is reported that it related to children Wilms' tumor disease; childhood kidney tumor. The WT1 expression is more significant than p53 and Rb's. WT1 expresses on some areas such as gonads, uterus,

kidney, and mesothelium. Apart from tumor suppressor, WT1 has found that was oncogene, related to leukemia, and also tumor marker of leukemia. WT1 has 1,000-10,000 times of expression in bone marrow and normal blood stream when compared with white blood cells in leukemia patients [57].

1.4.4.3 G2 and M phases

The significant proteins for cell mitosis are produced, including cell cycle protein on next phase; cyclin A/CDK4/CDK6 is activated. This phase is the longest period of cell cycle. Chromatin is heterochromatin. M phase is cell mitosis with short period. Chromosome condenses in this phase and there is not any RNA synthesis (Chromatin condenses therefore RNA polymerase cannot function). The time periods of G1, S, G2, and M phases on cell line are 5, 7, 1, and 1 h, respectively.

1.5 Phytochemicals from plants

Phytochemicals are defined as bioactive non-nutrient plant compounds in fruits, vegetables, grains, and other plant foods that have been linked to reduce the risks of major chronic diseases and cancers. Phytochemicals in common fruits and vegetables can have complementary and overlapping mechanisms of action, including of gene expression in cell proliferation, cell differentiation, oncogenes and tumor suppressor genes; induction of cell cycle arrest and apoptosis; modulation of enzyme activities in detoxification, oxidation and reduction; stimulation of the immune system; regulation of hormone metabolism; as well as antibacterial and antiviral effects [58]. Polyphenols particularly are among the diverse phytochemicals that show health benefits. The polyphenolic phytochemicals are virtually ubiquitous in plant materials. They are very important to plants and have multiple functions. The most important role of plant phenolics may be in plant defense against pathogens and herbivore predators [59]. Phenolic compounds comprise a large group of biologically active ingredients (about 8,000 identified compounds), varying from simple phenol molecules to polymeric structures with molecular mass above 30,000 Da [60]. The group of simple phenols contains “phenolic acids” or phenol with carboxyl group. Flavonoids are the most studied phenolic compounds. More than 4,000 flavonoids have been identified in different plant species [61]. Phenolic compounds possess various biological activities such as antioxidant, antimutagenic, anticarcinogenic, metal chelators, and signaling

agent. A diet rich in fruit and vegetables has been linked to human health, such as reducing the risk of developing coronary heart disease, cancer, hypertension, diabetes, and inflammatory processes [62]. In addition, these phytochemicals have anticancer property. Experimental studies show that antioxidant vitamins, plant polyphenols and flavonoids selectively induce apoptosis in cancer cells and prevent angiogenesis and metastasis. They may have potential as adjuvants in cancer therapy [63]. The plants have been used on disease since a long time ago. Although the synthetic chemical drugs industrial has grown and using herb has decreased, now people realize about toxicity of chemical drugs and side effects, herbal plants are more used. Many plants extracts cure disease and give less side effects than synthetic chemical drugs [64]. For example, diterpne alcohol which is extracted from plaonoi leaf can be used for stomach and intestine lesions [61]. Moreover, there are many significant medicines which are extracted from herbal plants. They do not come from chemical synthesis. For example, gingolide B which is from gingko leave, it enhances and protects capillary wall, balances blood circulation, and anti-inflammation and swelling [65]. Vegetables, plants, fruits, and herbs are significant source on antioxidant substances. Free radicals are main cause of old and other diseases; heart disease, coronary artery disease, degeneration of neurons, inflammation, and cancer. The example antioxidants are S-allylcysteine (SAC) and S-allylmer captocysteine (SAMC). Those are main chemical substances which are found in garlic. They are antioxidant, and protect inner wall of blood vessel from damage; the cause of vascular disease. They work by match the antioxidant or increase function of antioxidant substances in cells [65]. The antioxidant is assumed coming from various compounds such nutrients; vitamin E [21] or vitamin D [65, 66], and phytochemical; carotenoid [67] and phenol [68]. The antioxidant differentially effects on vary cancers risking rate decreasing. For instance, it impedes carcinogenic, anti-cancer substance, stop growing and autoinoculation of cancer, reduce cancer mutation, and enhance immune system. Some studies stated that getting antioxidant from vegetable and fruit reduced cancer risk occurrences [68]. Herbal plants were researched on cancer treatment. Some research data reported that various herbal plant extracts could destroy leukemia and reduced WT1 expression in both gene and protein. For example, the curcuma extracts (curcuminoids) had cytotoxic effects on K562, U937, HL60, and Molt4 leukemic cell lines and decreased *WT1* gene and protein expressions by a dose-

and time-dependent manner [69, 70]. Moreover, kaffir lime leaf extracts also affected on leukemia cells impedius, and on *WT1* gene and WT1 protein expressions.

1.6 Kaffir lime (*Citrus hystrix* DC.)

Kaffir lime is in the Citrus genus. Its scientific name is *Citrus hystrix* DC, which is the in Rutaceae family [71]. One leaf looks like two leaves connected to each other. The leaves are dark green on top but light green other side. The skin is smooth, vernicose, and thick, fragrant from oily gland around the leaf. It looks like two leaves are connected to be another leaf. The flowers are cluster, white petal, yellow pollen, fall easily, and fragrant. The fruit is dark green like lime with rough skin. The calyx looks like cork. The young fruit is dark green and turns to bright yellow when it is ripe as in figure 1.3. The size of the leaves can vary quite a bit, from 2.5 to 4 cm. wide and 4 to 7 cm. long [72]. Under the leaves, there are many oil glands, making the leaves very fragrant [73]. Flowers are clustered into 3 to 5 with white petals. The pollen is yellow and aromatic [73]. The kaffir lime trees are shrubs to medium-size trees up to about 5 m (16 ft) in height. They are single-trunked with very hard wood with thin, smooth, and gray-brown bark with short spines [73]. The leaves of the kaffir lime tree are a dark green color with a glossy sheen. They come in two parts; the top leaflet is lightly pointed at its tip and is attached to another leaflet beneath that is broader on its upper edge [73]. The fruits are dark green and round with a distinct nipple on the stem end. They have a thick rind, knobby, and wrinkled. As the fruit becomes older, the color fades to a lighter, yellowish green. Kaffir lime is commonly used in Southeast Asian cuisine, especially in Thailand. The kaffir lime leaves and peels are aromatic and used as a spice for various flavoring purposes such as seasoning or savory curry paste. Kaffir lime has great potential in research and commercialization [74]. The usages of kaffir lime include aromatherapy and spa practices and making shampoos, cosmetics and beauty products [75, 76]. The key element for such application is the oil contained in kaffir lime. Lawrence *et al*. reported that the main chemical constituents in kaffir lime peel were β-pinene (30.6%), limonene (29.2%) and sabinene (22.6%), and the main compound in leaves is citronellal (65.4%) [78]. However, reports concerning the amount of each chemical contents are slightly different, for example, Chantaphon *et al.* reported that β-pinene (30.48%), sabinene (22.75%), and citronellal (15.66%) as major components of the hydrodistillated essential oil of kaffir lime peel [79], and Kasuan *et*

al. showed that the steam distilled essential oil of kaffir lime included limonene (27.97%), citronellal (15.31%), and α/β-pinene (9.82%) for the peel and citronellal (71.55%) for the leaf [74]. Weikedre *et al*. found that the main constituents of essential oil of kaffir lime leaf growing in New Caledonia were also monoterpenes with terpinen-4-ol (13.0%), β-pinene (10.9%) as principal compounds [80]. Many studies have demonstrated various biological activities of kaffir lime. An anti-oxidative property of kaffir lime was reported by Tachakittirungrod *et al.* [81]. The kaffir lime peel and leaf is a source of phenolic compound [82], antioxidative substance [83]. Furthermore, the extracts from leaf and peel of the kaffir lime exert the strongest effect on protection of deoxyribose from OH·, suggesting the free radical scavenging and anti-inflammatory activities of kaffir lime [84].

In regard to cancer research, bioactive compound in many kinds of citrus fruits are capable of inhibiting cancer cell proliferation. Limonoids, a family of triterpenoids with putative anticancer properties in citrus fruits, exert a strong multifaceted lethal action against human neoblastoma and colon cancer cells [85]. The volatile oil of citrus aurantifolia containing two major compounds, D-limonene and D-hydrocarvone, can inhibit proliferation of colon cancer cells by apoptosis mediation [86]. It has been demonstrated that D-limonene and the oils isolated from citrus fruits can inhibit the formation of pulmonary adenoma and the occurrence of forestomach tumors induced by carcinogens such as 4-(methylnitrosamino)-I-(3-pyridyl)-1-butanone (NNK) [87]. Murakami *et. al*. reported that two glyceroglycolipids, l,2-di-o-a-linolenoyl-3-o-galacto-pyranosyl-sn-glycerol and a mixture of two compounds, l-o-a-linolenoyl-2-o-palmitoyl-3-o-galactopyranosylsn-glycerol and its counterpart, extracted from kaffir lime leaves were potent inhibitors of tumor promoter-induced Epstein-Barr virus (EBV) activation and 12-o-tetradecanoylphorbol 13-acetate (TPA), a skin carcinogen, activities in mice [88]. The essential oils of kaffir lime leaf by steam distillation had been shown to have anti-proliferative activity on KB (cervical cancer) and P388 (mouse leukemia) cell lines [8].

This study focuses on the biological effects of partially purified fractional hexane extracts of kaffir lime leaf on cell cycle arrest in leukemic K562 and Molt4 cell lines models. However, cytotoxic effects of all fractional extracts from kaffir lime leaf (ethanol, hexane, ethyl acetate, and *n*-butanol fraction) on leukemic cells have been reported in previous studies [12]. To investigate the possible effective compound in

hexane fractional extract of kaffir lime leaf, the 13 partially purified fractions by reverse phase column chromatography were studied. The goal of this study is to obtain new basic knowledge of active compounds in kaffir lime leaf extracts which have anti-leukemic activity to stop cell cycle progression.

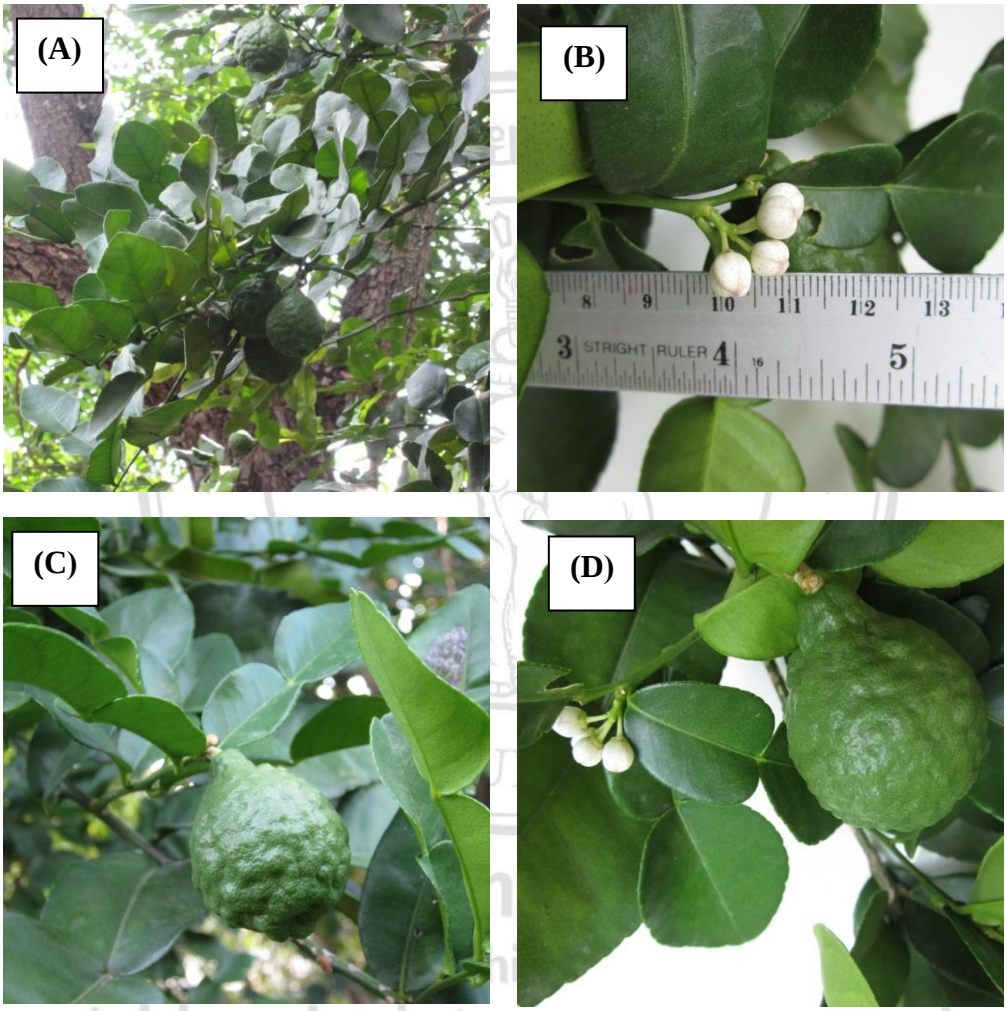

Figure 1.3 Kaffir lime botanical appearance. (A) Kaffir lime is a small hard wood with thorns on branches, dark green leaf on top but light green underside, smooth skin, varicose, thick, and fragrant from oily gland around the leaf. It looks like two leaves joined to be one leaf. (B) Cluster flowers with white petal, yellow pollen, fall easily, fragrant. (C) and (D) Dark green fruit like lime, rough skin, calyx looks like cock, green fruit is dark green (Photos by Methee Rungrojsakul).