CHAPTER IV
DISCUSSION

Leukemia is a cancerous disorder in the blood-forming tissues especially bone marrow. It is characterized by excessive production of immature or mature leukocytes and consequently a crowding-out of red blood cells and platelets. Possible causes of leukemia included chromosomal abnormalities, exposure to certain chemicals and radiation, certain drugs and infection with such microorganisms. These agents are suspected to cause overexpression or mutation of genes that involve in regulation of cell growth and division. *Wilms' tumor1* (*WT1*) gene is one of the genes that is involved in leukemogenesis. It was first described as tumor suppressor gene in Wilms' tumor or nephroblastoma and some of cancers. In the recent year, an oncogenic function of *WT1* was described in many types of cancer including leukemia [15]. In the past few decades, extensive studies have been performed with the aim of finding its mechanism on induction of turmerigenesis. The previous studies reported that the wild-type *WT1* gene is strongly expressed in leukemic blast cells with an increase in its expression levels at relapse and inverse correlation between its expression levels and prognosis [16, 286-288]. In addition, *WT1* gene was shown to overexpress in leukemic cells, K562 and HL-60 by RT-PCR [289]. These findings suggested that *WT1* plays an important role in leukemogenesis and may have an oncogenic function rather than a tumor-suppressive function in hematopoietic progenitor cells and leukemic blast cells.

In anticancer drug research, dietary and/or medicinal plants, such as turmeric, ginger, garlic, chili, and pepper are become popular for chemotherapeutic drugs. Especially turmeric (*Curcuma longa* Linn.), a member of Zingiberaceae or ginger family, is a spice grown in tropical regions of Asia. It has a long history of use in herbal remedies, particularly in Thailand, China, India, and Indonesia. Turmeric's rhizome contains the major phenolic coloring compound called curcuminoids. It has been identified as the major active yellow pigment in turmeric. Curcuminoids consist of three major active ingredients; pure curcumin (curcumin I), demethoxycurcumin (curcumin II), and bisdemethoxycurcumin (curcumin III). These three curcuminoid

compounds are always mixed together in turmeric powder. It is also called curcuminoid mixture. In addition, commercial grade curcuminoids, commonly known as "Curcumin" due to the main content in curcuminoid mixture. Curcuminoids especially pure curcumin, the major biological active phytochemical compound, have a wide range of biological and pharmacological activities, including antioxidant and anti-inflammatory effects, as well as anti-mutagen and anticancer properties [25-27, 235]. The anticancer properties of curcuminoids have been described by many investigators. The inhibitory effect of curcuminoids regulated a wide variety of genes that require assembly protein 1 (AP1) and nuclear factor kappa B (NFκB) activation which promote cell proliferation and cell differentiation. These transcription factors are generally known as protein kinase C (PKC) regulators that also found to regulate WT1 protein by phosphorylation at C-terminal domain [290], and in turn regulate cell proliferation in leukemic cells. In addition, Duvoix, *et al*. [291] found that curcumin inhibits glutathione S-transferase P1-1 (GSTP1-1) mRNA as well as protein which is correlated to the apoptotic effect on K562 cell line. The expression of STAT5 mRNA and protein in K562 cells were inhibited by curcumin and the curcumin could also inhibit K562 cell proliferation [292]. Recently commercial grade curcuminoids (Sigma-Aldrich) have shown suppressive effect on the expression of WT1 mRNA and WT mRNA levels in human leukemic cells, K562 and patient leukemic cells [31, 32]. However, there was no report demonstrate which component of curcuminoids showed the most effective suppressive effect on *WT1* gene and protein expression of leukemic cell lines. Thus, this study aims to evaluate the modulatory effect of three curcuminoids on *WT1* gene expression in the human leukemic cell lines, K562, U937, HL-60 and Molt4.

The cytotoxicity of curcuminoids including commercial grade curcuminoids, inhouse curcuminoids mixture, pure curcumin, demethoxycurcumin, and bisdemethoxycurcumin on leukemic cell lines was assessed by MTT assay. The curcuminoids showed the cytotoxic effect on K562 cell line with the inhibitory concentration at 50% (IC_{50}) less toxic than the cytotoxic effect of curcuminoids on HL-60, U937, and Molt4 cell line, respectively. This result was similar to the data reported by Anuchapreeda, *et al*. [30, 278]. From this result, Molt4 appeared to be more sensitive to curcuminoid treatment. This result was similar to the result in

previous study which demonstrated that Jurkat cell line, one of human T-cell lymphoblastic cell line, appeared to be more sentitive to curcumin than K562 cell line [28]. However, the IC_{50} of each curcuminoid derivative on leukemic cell line was different in each type of leukemic cell. However, there were not significantly different among the IC_{50} of pure curcumin, demethoxycurcumin, and bisdemethoxycurcumin on K562, U937, and HL-60. The significantly different of IC_{50} in each curcuminoid was found in Molt4 cell line. Pure curcumin strongly exhibited the cytotoxicity followed by demethoxycurcumin and bisdemethoxycurcumin, respectively. Moreover, the concentration of curcuminoids at 17 μM (5.2 μg/mL) and higher showed the cytotoxic effect on all leukemic cell lines. This result is correlated with Kuo's report, that the curcumin induced cell apoptosis in HL-60 cell line at the concentration as low as 3.5 μg/mL [29]. The non-cytotoxic concentration (less than 17 μM) of curcuminoid derivatives was used to investigate their effect on *WT1* gene expression in leukemic cell lines in this study.

According to the study of WT1 gene expression (WT1 mRNA and WT protein) in leukemic cell lines, the WT1 mRNA was detected in 4 types of leukemic cell lines. It was shown that *WT1* gene was overexpressed in all types of leukemic cells. These finding was supported by the previous studies, that *WT1* gene plays in leukemogenesis and may concern an oncogenic function rather than a tumor-suppressor gene function in leukemic cell [15, 20, 293]. On the contrary, the high level of WT1 protein was detected in K562 and Molt4 cell lines. It can be suggested that the WT1 protein levels in U937 and HL-60 are too low amount to be detected by this method. However, different mechanisms of *WT1* gene expression at the translational level of each type of leukemic cell will be investigated further.

After treated with 10 μM curcuminoids (non-cytotoxic dose), the leukemic cell morphology and viability did not change when compared to those of the vehicle control. These finding suggested that all curcuminoid extracts at a concentration of 10 μM did not alter the morphology and viability of leukemic cell lines. In addition, WT1 mRNA and WT1 protein level in leukemic cell lines were decreased by all curcuminoid derivatives including commercial grade curcuminoids, in-house curcuminoid mixture, pure curcumin, demethoxycurcumin, and bisdemethoxycurcumin after normalization. Moreover, the results clearly showed that

pure curcumin, the major component of curcuminoid powder, was the most effective component in decreasing WT1 mRNA and WT1 protein levels followed by bisdemethoxycurcumin, and demethoxycurcumin, respectively. These result is similar to what Nagabhushan has reported [25]. The molecular structure of curcuminoids was discussed. The unique molecular structure of the curcuminoids has been shown to be endowed with beneficial biological activities including antioxidant action, anti-inflammatory action, anticarcinogenic action and anti-mutagen activity.

Curcumin has been shown to be endowed with several biological activities potentially useful for clinical use. These biological activities are attributed from their unique molecular structure: a) Parahydroxyl groups: antioxidant activity, b) Keto groups: anti-inflammatory, anticancer, and anti-mutagen activity, c) Double bonds: anti-inflammatory, anticancer, and anti-mutagen activity [Figure 44].

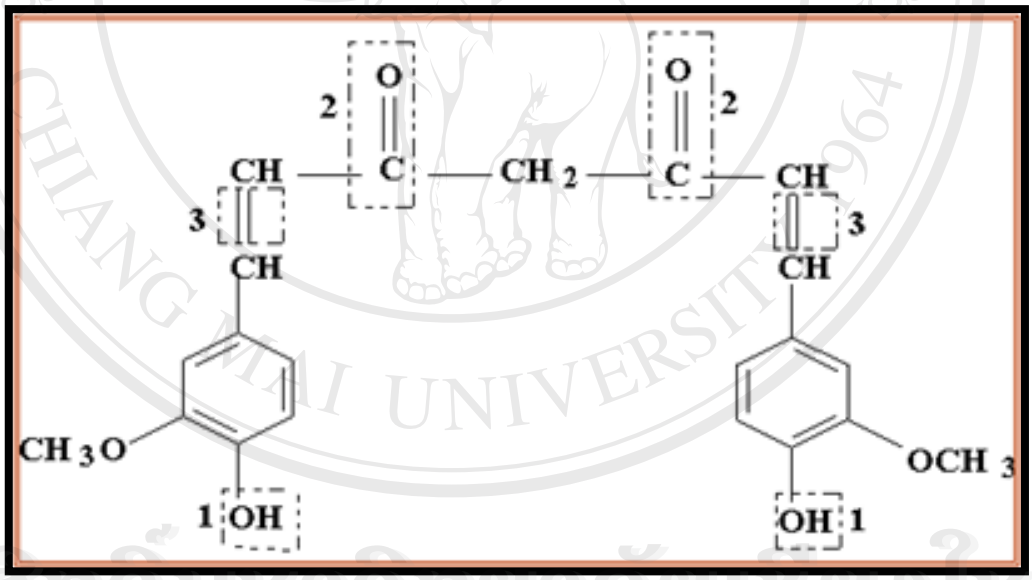

Figure 44. Molecular structure of curcumin [294]

The vanillyl (4-hydroxy-3-methoxyphenyl) moiety and a ketone functional group in the structure of [6]-Gingerol and [6]-paradol, which share common structural features found in curcuminoid, induce HL-60 cell death via cell apoptosis [295]. Moreover, the diketone system of curcuminoids appears to be the part of the molecule involved in the antiproliferative effect of curcuminoids [296]. Both phenolic hydroxyl

groups, phenolic methoxyl groups, and the diketone moiety could be responsible for inhibitory effect of *WT1* gene expression. The present study indicated that hydroxyl groups on the benzene rings are not essential for this activity because curcuminoids derivatives have the same number of hydroxyl groups but showed a different inhibitory effect. This study proposed that the functional group which plays a role on inhibitory effect of *WT1* gene expression is phenolic methoxyl groups of each curcuminoid derivative. Pure curcumin possesses two methoxyl groups and exhibited the maximum inhibitory effect, while demethoxycurcumin and bisdemethoxycurcumin possess one and no methoxyl group, respectively, and are less inhibitory. In addition, an imbalance between the methoxyl groups on the structure of demethoxycurcumin may influences this activity. This study suggested that the diketone groups and the imbalance of the phenolic methoxyl groups of curcuminoids may contribute to an inhibitory effect of curcuminoids on *WT1* gene expression.

The effect of pure curcumin (non-cytotoxic doses) on *WT1* gene expression in leukemic cell lines was subsequent evaluated. It was found that pure curcumin significantly decreased the WT1 mRNA and WT1 protein levels in dose and time-dependent manners. A decrease in *WT1* gene expression with increasing pure curcumin concentrations (5-15 μM), demonstrated that pure curcumin inhibited the level of immunoreactive WT1 protein observed in K562 and Molt4 cells and also reduced the level of WT1 mRNA under the same conditions in 4 types of leukemic cell lines used in this study. Furthermore, the experiments also showed that treatment of cells with pure curcumin at 10 μM for 1-3 days inhibited the *WT1* gene expression in a time-dependent manner. According to the function of WT1 in hematopoietic progenitor cells and leukemic blast cells, Inoue, *et al*. demonstrated that WT1 expression competed with the differentiation-inducing signal mediated by granulocyte colony-stimulating factor receptor (G-CSFR) and constitutively activated Stat3 protein, resulting in the blocking of differentiation and subsequent proliferation [293]. Down-regulation of the *WT1* gene in some leukemic cell lines occurs during differentiation along the myeloid and erythroid lineage pathways [227, 297], and WT1 antisense oligonucleotides inhibited the proliferation of these leukemic cells [298]. These findings suggested that WT1 was important for the malignant proliferation of leukemic cells. Furthermore, WT1 is a transcription factor that has been implicated in the

regulation of target genes related to apoptosis, genitourinary differentiation, and cell cycle progression. The induction of WT1 leads indirectly to increase the cyclin-dependent kinase inhibitor p21 expression [299] and down-regulates cyclin E protein levels at G1/S phase of cell cycle [300]. Moreover, WT1 gene induces G1 arrest and cell apoptosis in myeloblastic leukemia, M1 cells [301]. Previous studies suggested that compounds with antioxidant or anti-inflmmatory activites also inhibit tumor promotion and cell proliferation. Pure curcumin is one of the compound exhibited remarkable antioxidant and free radical-scavenging activities [302-305]. Thus, pure curcumin also exhibited the inhibitory effect on tumor promotion and cell proliferation. In the previous study pure curcumin was found to be the most effective in the DNA cleavage reaction and reduction of Cu (II) [306]. Mechanistic roles of pure curcumin on *WT1* gene promoter activities and its signaling control are under extensively investigation in many laboratories. However, an antiproliferative activity of pure curcumin on MCF-7 cell line at G2/M phase has been explained. The presence of the diketone moiety in the pure curcumin molecule seems to be essential for the inhibitory activity [296].

Taken together, this result indicated that treatment of human leukemic cell lines with non-cytotoxic concentrations (low doses) of pure curcumin inhibited *WT1* gene expression whereas pure curcumin, at high dose, induced cell cytotoxicity (IC_{50} = 44 μM). In additional study, the three curcuminoid compounds also showed good cytotoxicity in K562, U937, and HL-60 cell lines [30]. The current studies suggested that pure curcumin can potentially be used as a promising chemotherapeutic agent in human leukemic cancer. This research may lead to clinical trials in the future.