CHAPTER III
RESULTS

3.1 Yield of crude kaffir lime leaf fractions extracted by organic solvents with variant polarities

Two kilograms of kaffir lime leaf were extracted by five organic solvents included ethanol, hexane, ethyl acetate, n-butanol, and methanol, which had relative polarity of 0.654, 0.009, 0.228, 0.602, and 0.762, respectively, compared to water (1.000), and showed the yields of 10.36, 3.51, 1.12, 2.78, and 10.31%, respectively.

3.2 Cytotoxicity of crude kaffir lime leaf fractional extracts on leukemic cell lines

After leukemic cell lines were cultured with crude kaffir lime leaf fractional extracts at various concentrations for 48 h, the cytotoxic effect of crude Kaffir lime leaf fraction extracts on leukemic cell lines were investigated by using MTT assay. Cytotoxicity of each crude fractional extracts was determined by an inhibitory concentration at 50% growth (IC_{50}). The results showed that ethanol, hexane, ethyl acetate and n-butanol fractions had cytotoxic effects on K562, Molt4, U937, and HL-60 cell lines whereas methanol fraction had no cytotoxicity on these leukemic cell lines. The IC_{50} values of ethanol, hexane, ethyl acetate, n-butanol, and methanol fraction on K562 cell line were 91.3±8.4, 40.1±2.8, 35.3±1.4, 97.0±5.3, and >100 µg/mL, respectively (Figure 4 and (Table 9). The IC_{50} values of fractions on Molt4 were 91.7±7.6, 34.9±5.1, 21.8±0.4,

94.9±4.5, and >100 µg/mL, respectively (Figure 5 and Table 9). The IC_{50} values of fractions on U937 were 85.8±6.3, 53.0±9.4, 19.8±1.0, 95.8±7.2, and >100 µg/mL, respectively (Figure 6 and Table 9). The IC_{50} values of fractions on HL60 were 55.6±3.6, 21.6±2.3, 19.0±0.6, 83.4±10.5, and >100 µg/mL, respectively (Figure 7 and Table 9). The IC_{50} values of five crude kaffir lime leaf fractional extracts on four leukemic cell lines were shown in Table 9. The non-cytotoxic concentration, concentration at IC_{20}, of all crude fractional extracts on four leukemic cell lines were used for further studies on the *WT1* gene and WT1 protein expression as shown in Table 10.

Table 9 The IC_{50} values of crude kaffir lime leaf fractional extracts determined from the plot of percent cytotoxicity on K562, Molt4, U937, and HL60 cell lines

Crude kaffir lime leaf fractional extracts	IC_{50} (µg/mL) (Mean ± 2SD)			
	K562	Molt4	U937	HL60
Ethanol	91.3 ± 8.4	91.7 ± 7.6	85.8 ± 6.3	55.6 ± 3.6
Hexane	40.1 ± 2.8[#]	34.9 ± 5.1[#]	53.0 ± 9.4[#]	21.6 ± 2.3[#]
Ethyl acetate	35.3 ± 1.4[*]	21.8 ± 0.4[*]	19.8 ± 1.0[*]	19.0 ± 0.6[*]
n-Butanol	97.0 ± 5.3	94.9 ± 4.5	95.8 ± 7.2	83.4 ± 10.5
Methanol	>100	>100	>100	>100

* The most effective fraction as compared to other fractions
[#] The minor effective fraction as compared to other fractions

The data is shown as the mean ± SD of three independent experiments. Asterisk (*) demonstrates the statistically significant difference between the value of vehicle control and sample ($p < 0.05$).

Table 10 The IC_{20} values of crude kaffir lime leaf fractional extracts determined from the plot of percent cytotoxicity on K562, Molt4, U937, and HL60 cell lines.

Crude kaffir lime leaf fractional extracts	IC_{20} (µg/mL) (Mean ± 2SD)			
	K562	**Molt4**	**U937**	**HL60**
Ethanol	40.9 ± 1.3	25 ± 3.8	10.2 ± 1.4	17.0 ± 5.8
Hexane	13.6 ± 6.9	2.8 ± 4.7	2.8 ± 3.1	3.7 ± 2.0
Ethyl acetate	11.9 ± 4.4	8.0 ± 0.6	3.2 ± 2.3	8.8 ± 1.4
n-Butanol	45.4 ± 1.6	37.5 ± 2.0	18.2 ± 5.3	25.0 ± 4.6

The data represents the mean value ± SD of three independent experiments.

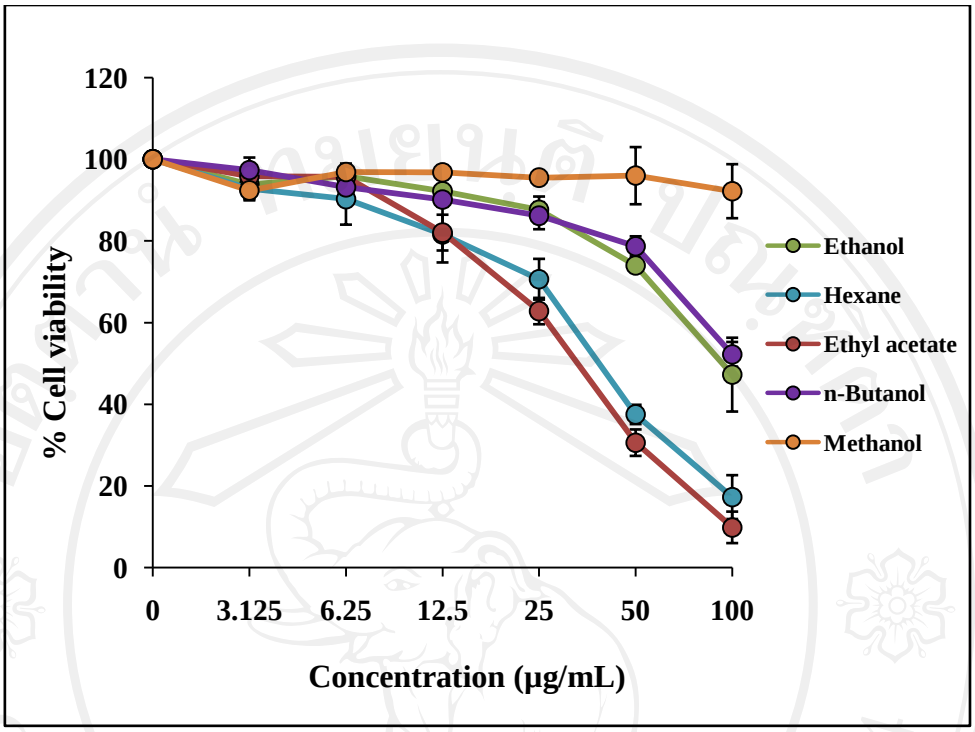

Figure 4 Cytotoxicity of crude kaffir lime leaf fractional extracts on K562 cell line at various concentrations. K562 cells were seeded at density of 1.0 x 10^5 cells/mL and treated with the various concentrations of crude kaffir lime leaf fractional extracts (ethanol, hexane, ethyl acetate, n-butanol, and methanol) for 48 h. The cell viability was determined by MTT assay. Each point represents the mean value ± SD of three independent experiments that are performed in triplicate.

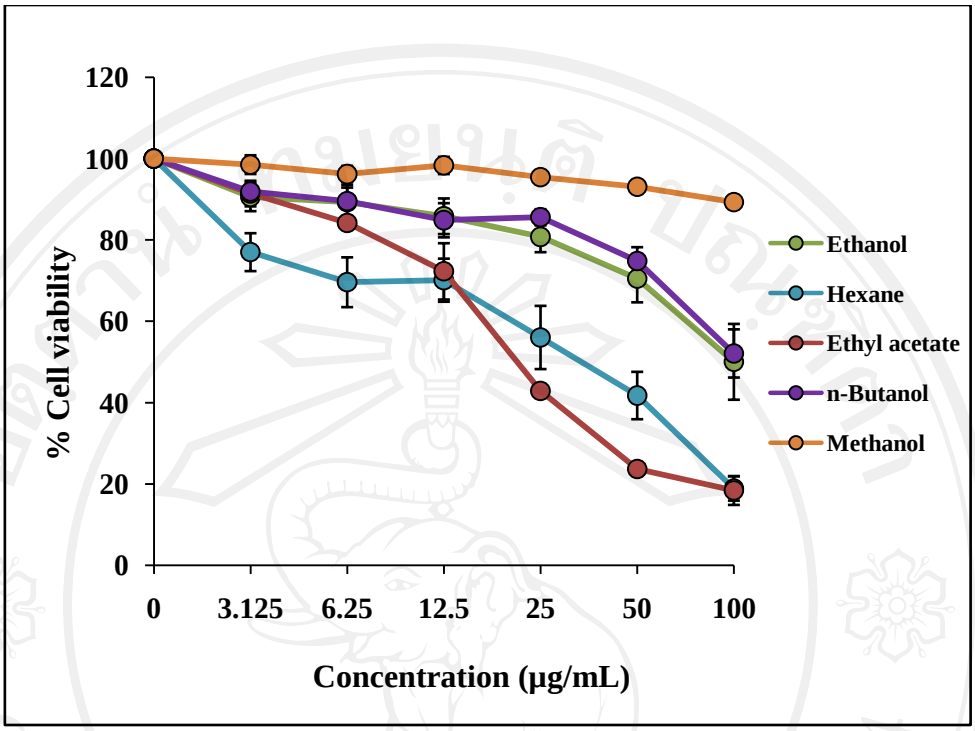

Figure 5 Cytotoxicity of crude kaffir lime leaf fractional extracts on Molt4 cell line at various concentrations. Molt4 cells were seeded at density of 1.0 x 10^5 cells/mL and treated with the various concentrations of crude kaffir lime leaf fractional extracts (ethanol, hexane, ethyl acetate, n-butanol, and methanol) for 48 h. The cell viability was determined by MTT assay. Each point represents the mean value ± SD of three independent experiments that are performed in triplicate.

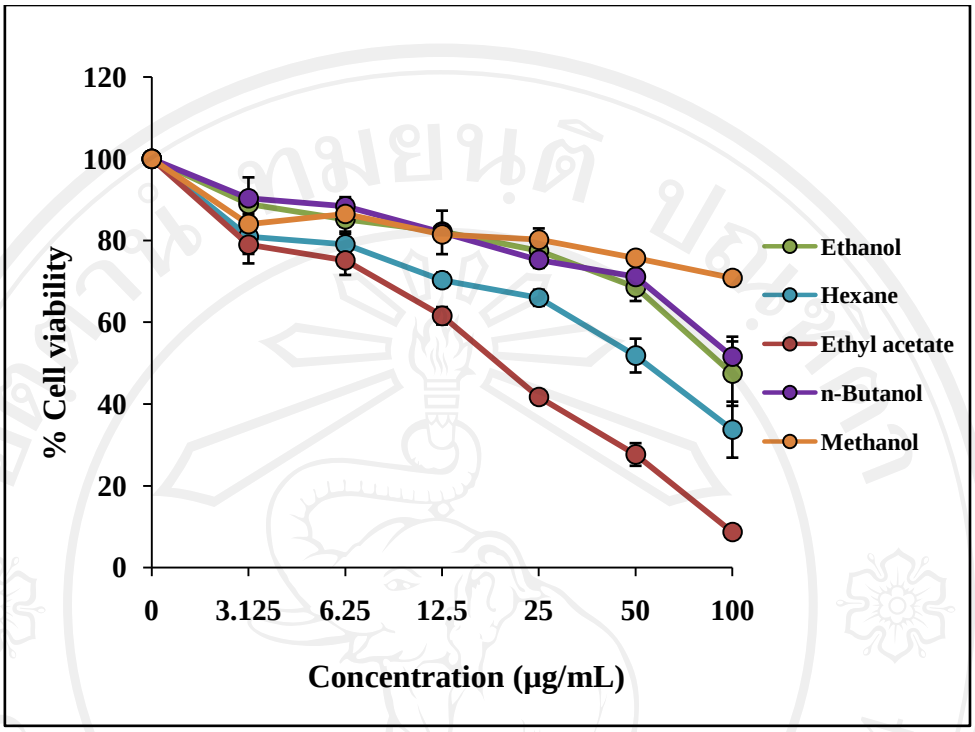

Figure 6 Cytotoxicity of crude kaffir lime leaf fractional extracts on U937 cell line at various concentrations. U937 cells were seeded at density of 1.0 x 10^5 cells/mL and treated with the various concentrations of crude kaffir lime leaf fractional extracts (ethanol, hexane, ethyl acetate, n-butanol, and methanol) for 48 h. The cell viability was determined by MTT assay. Each point represents the mean value ± SD of three independent experiments that are performed in triplicate.

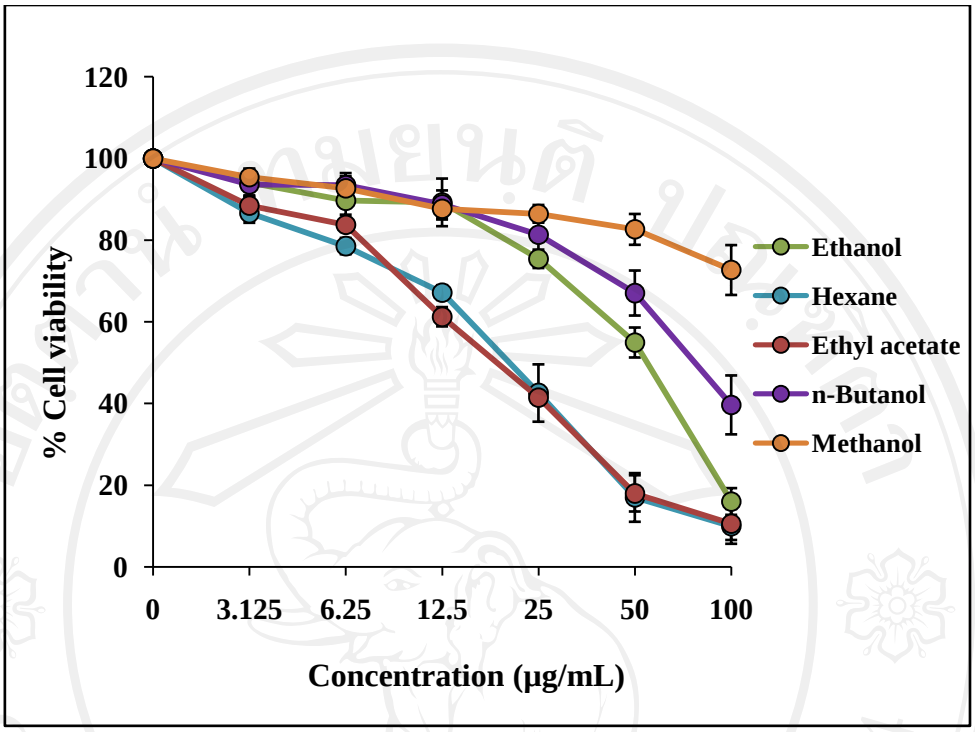

Figure 7 Cytotoxicity of crude kaffir lime leaf fractional extracts on HL60 cell line at various concentrations. HL60 cells were seeded at density of 1.0 x 10^5 cells/mL and treated with the various concentrations of crude kaffir lime leaf fractional extracts (ethanol, hexane, ethyl acetate, n-butanol, and methanol) for 48 h. The cell viability was determined by MTT assay. Each point represents the mean value ± SD of three independent experiments that are performed in triplicate.

3.3 Effect of crude kaffir lime leaf fractional extracts on WT1 mRNA levels in leukemic cell lines

3.3.1 Effect of crude kaffir lime leaf fractional extracts on WT1 mRNA levels in K562 cell line

To determine effect of crude kaffir lime leaf fractional extracts on the levels of WT1 mRNA in K562 cell line, the leukemic cell line was treated with 0.18% DMSO (vehicle control) and crude kaffir lime leaf fractional extracts, including ethanol, hexane, ethyl acetate, and butanol, at the concentration of 45.4, 13.6, 11.9, and 40.9 µg/mL, respectively. After 2 days of incubation, treated cells were harvested and extracted total RNA. The cDNA was synthesized and run through real-time PCR process. The experiment was done in three times. The results were shown in Ct (Cycle threshold) values. The obtained Ct values were used to calculate % WT1 mRNA level. The percentages of WT1 mRNA level were 51.3 ± 8.4, 40.3 ± 5.0, 76.3 ± 18.9, and 94.7±7.6 (Table 11) in the response to ethanol, hexane, ethyl acetate, and butanol fraction extracts, respectively. All of crude kaffir lime leaf fractional extracts decreased the WT1 mRNA levels in K562 cell line by 49, 60, 24, and 5%, when compared to vehicle control (Figure 8).

3.3.2 Effects of crude kaffir lime leaf fractional extracts on WT1 mRNA levels in Molt4 cell line

To determine effect of crude kaffir lime leaf fractional extracts on the levels of WT1 mRNA in Molt4 cell line, the leukemic cell line was treated with 0.15% DMSO (vehicle control) and crude kaffir lime leaf fractional extracts, including ethanol, hexane, ethyl acetate, and n-butanol, at the concentration of 25.0, 2.8, 8.0, and 37.5 µg/mL, respectively, for 48 h. The treated cells were extracted total RNA. The cDNA was synthesized and run through real-time PCR process. The experiment was done in three times. The results were shown in Ct (Cycle threshold) values. The obtained Ct values were used to calculate % WT1 mRNA level. The percentages of WT1 mRNA level were 77.6 ± 13.9, 48.8 ± 7.9, 74.0 ± 5.2, and 100 ± 11.0 in the response to ethanol, hexane, ethyl acetate, and n-butanol fraction extracts, respectively (Table 11). The crude ethanol, hexane, and ethyl acetate extracts decreased the WT1 mRNA levels in Molt4 cell line, when compared to vehicle control by 22, 51, and 26%, respectively, whereas butanol extract had no inhibitory effect on WT1 mRNA expression in Molt4 cell line, compared to vehicle control (Figure 9).

3.3.3 Effects of crude kaffir lime leaf fractional extracts on WT1 mRNA levels in U937 cell line

To determine effect of crude kaffir lime leaf fractional extracts on the levels of WT1 mRNA in U937 cell line, the leukemic cell line was treated with 0.07% DMSO (vehicle control) and crude kaffir lime leaf fractional extracts, including ethanol, hexane,

ethyl acetate, and n-butanol, at the concentration of 10.2, 2.8, 3.2, and 18.2 µg/mL, respectively, for 48 h. The treated cells were extracted total RNA. The cDNA was synthesized and run through real-time PCR process. The experiment was done in three times. The results were shown in Ct (Cycle threshold) values. The obtained Ct values were used for calculating % WT1 mRNA level. The percentages of WT1 mRNA level were 94.7 ± 9.2, 58.0 ± 8.9, 69.7 ± 6.0, and 91.0 ± 9.5 in the response to ethanol, hexane, ethyl acetate, and n-butanol fraction extracts, respectively (Table 11). All of crude kaffir lime leaf fractional extracts decreased the WT1 mRNA levels in U937 cell line by 5, 42, 30, and 9%, respectively, compared to vehicle control (Figure 10).

3.3.4 Effects of crude kaffir lime leaf fractional extracts on WT1 mRNA levels in HL60 cell line

To determine effect of crude kaffir lime leaf fractional extracts on the levels of WT1 mRNA in HL60 cell line, the leukemic cell line was treated with 0.1% DMSO (vehicle control) and crude kaffir lime leaf fractional extracts, included ethanol, hexane, ethyl acetate, and n-butanol, at the concentration of 17.0, 3.7, 8.8, and 25.0 µg/mL, respectively, for 48 h. The treated cells were extracted total RNA. The cDNA was synthesized and run through real-time PCR process. The experiment was done in three times. The results were shown in Ct (Cycle threshold) values. The obtained Ct values were used for calculating % WT1 mRNA level. The percentages of WT1 mRNA level were 45.2 ± 7.1, 44.3 ± 9.6, 66.0 ± 13.4, and 52.5 ± 10.5 in the response to ethanol, hexane, ethyl acetate, and n-butanol fraction extracts, respectively (Table 11). All of crude

kaffir lime leaf fractional extracts decreased the WT1 mRNA levels in U937 cell line by 55, 56, 34, and 48%, respectively, compared to vehicle control (Figure 11).

Table 11 The percentage of WT1 mRNA levels after crude kaffir lime leaf fractional extracts treatment compared to vehicle control.

Leukemic cell lines	% WT1 mRNA level				
	Vehicle control	Ethanol	Hexane	Ethyl acetate	n-Butanol
K562	100 ± 0	51.3 ± 8.4	40.3 ± 5.0	76.3 ± 18.9	94.7±7.6
Molt4	100 ± 0	77.6 ± 13.9	48.8 ± 7.9	74.0 ±5.2	100 ± 11.0
U937	100 ± 0	94.7 ± 9.2	58.0 ± 8.9	69.7 ± 6.0	91.0 ± 9.5
HL60	100 ± 0	45.2 ± 7.1	44.3 ± 9.6	66.0 ± 13.4	52.5 ± 10.5

The data represents the mean value ± SD of three independent experiments.

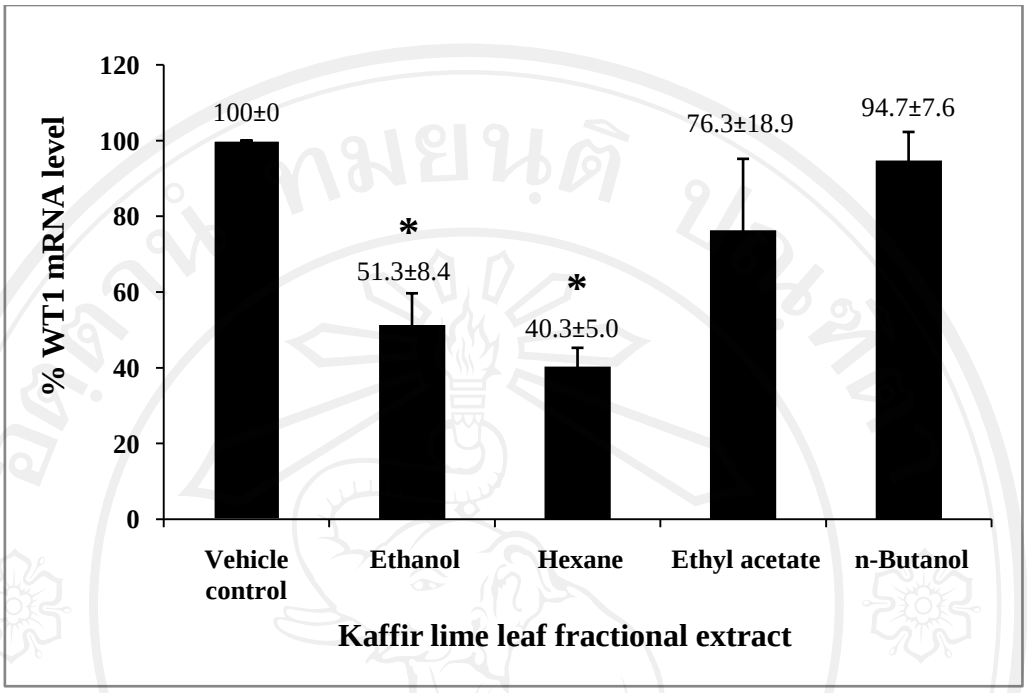

Figure 8 The effects of crude kaffir lime leaf fractional extracts on WT1 mRNA levels in K562 cell line. The leukemic cell line in density of 2.0 x 10^5 cells/mL was grown in complete RPMI 1640 medium with 0.18% DMSO and crude kaffir lime leaf ethanol, hexane, ethyl acetate, and n-butanol fractional extracts with the concentrations at IC_{20} for 48 h. The levels of the WT1 mRNA were determined by real-time PCR and represented as % WT1 mRNA level. The expression levels of WT1 mRNA were normalized by using the expression of β-actin mRNA as an internal control. The data is shown as the mean ± standard deviation (SD) of three independent experiments. Asterisk (*) demonstrates the statistically significant difference between the value of vehicle control and sample ($p < 0.05$).

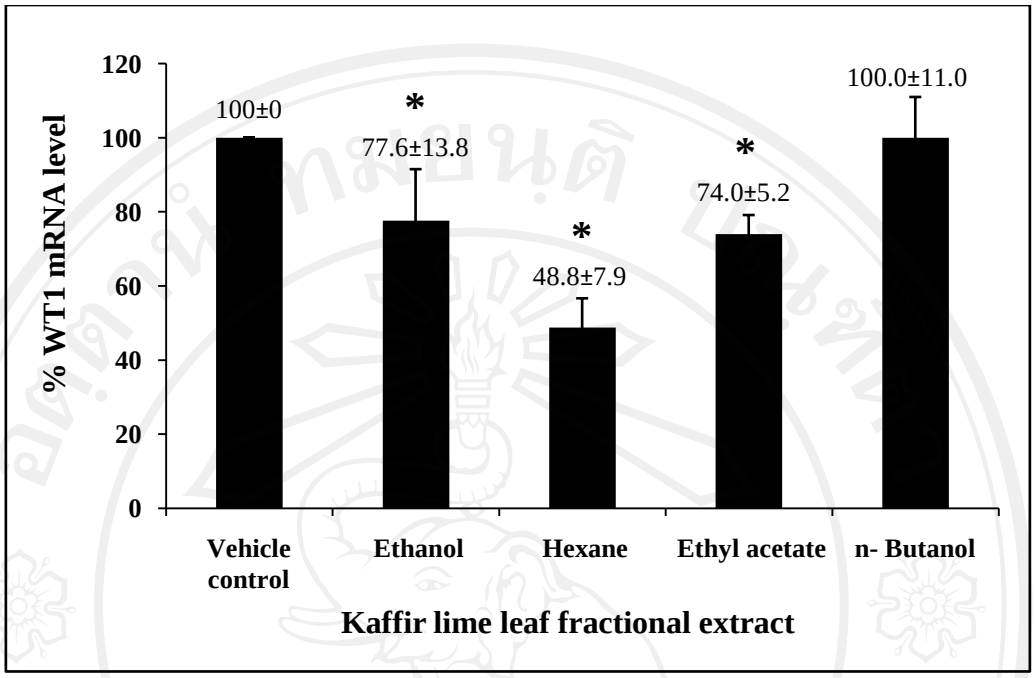

Figure 9 The effects of crude kaffir lime leaf fractional extracts on WT1 mRNA levels in Molt4 cell line. The leukemic cell line in density of 2.0 x 10^5 cells/mL was grown in complete RPMI 1640 medium with 0.15% DMSO and crude kaffir lime leaf ethanol, hexane, ethyl acetate, and n-butanol fractional extracts with the concentration at IC_{20} for 48 h. The levels of the WT1 mRNA were determined by real-time PCR and represented as % WT1 mRNA level. The expression levels of WT1 mRNA were normalized by using the expression of β-actin mRNA as an internal control. The data is shown as the mean ± standard deviation (SD) of three independent experiments. Asterisk (*) demonstrates the statistically significant difference between the value of vehicle control and sample ($p < 0.05$).

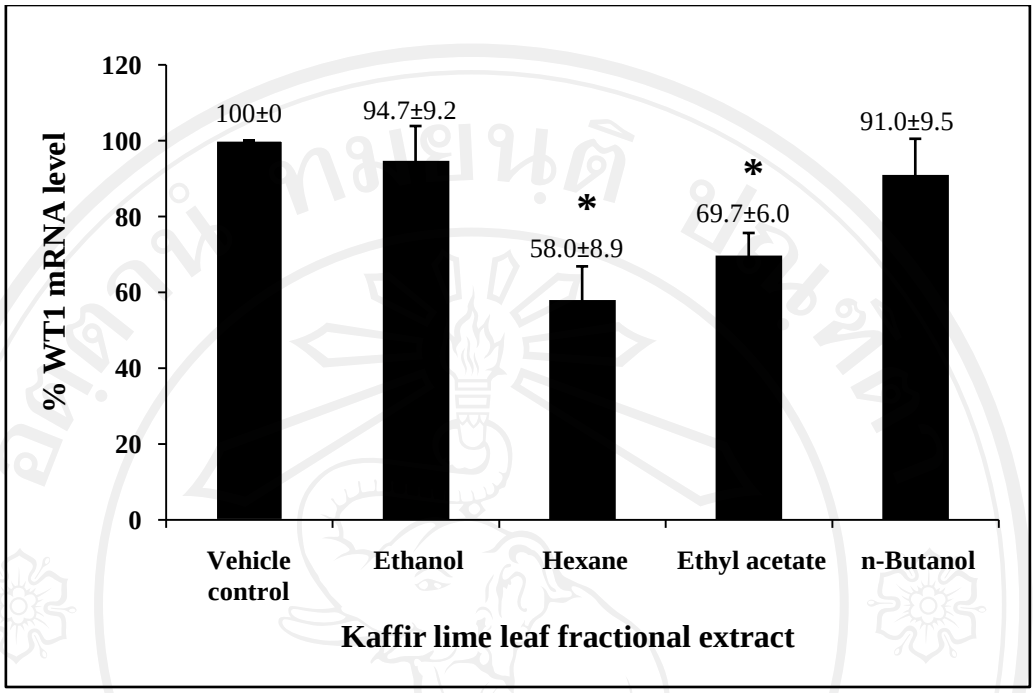

Figure 10 The effects of crude kaffir lime leaf fractional extracts on WT1 mRNA levels in U937 cell line. The leukemic cell line in density of 2.0 x 10^5 cells/mL was grown in complete RPMI 1640 medium with 0.07% DMSO and crude kaffir lime leaf ethanol, hexane, ethyl acetate, and n-butanol fractional extracts with the concentrations at IC_{20} for 48 h. The levels of the WT1 mRNA were determined by real-time PCR and represented as % WT1 mRNA level. The expression levels of WT1 mRNA were normalized by using the expression of β-actin mRNA as an internal control. The data is shown as the mean ± standard deviation (SD) of three independent experiments. Asterisk (*) demonstrates the statistically significant difference between the value of vehicle control and sample ($p < 0.05$).

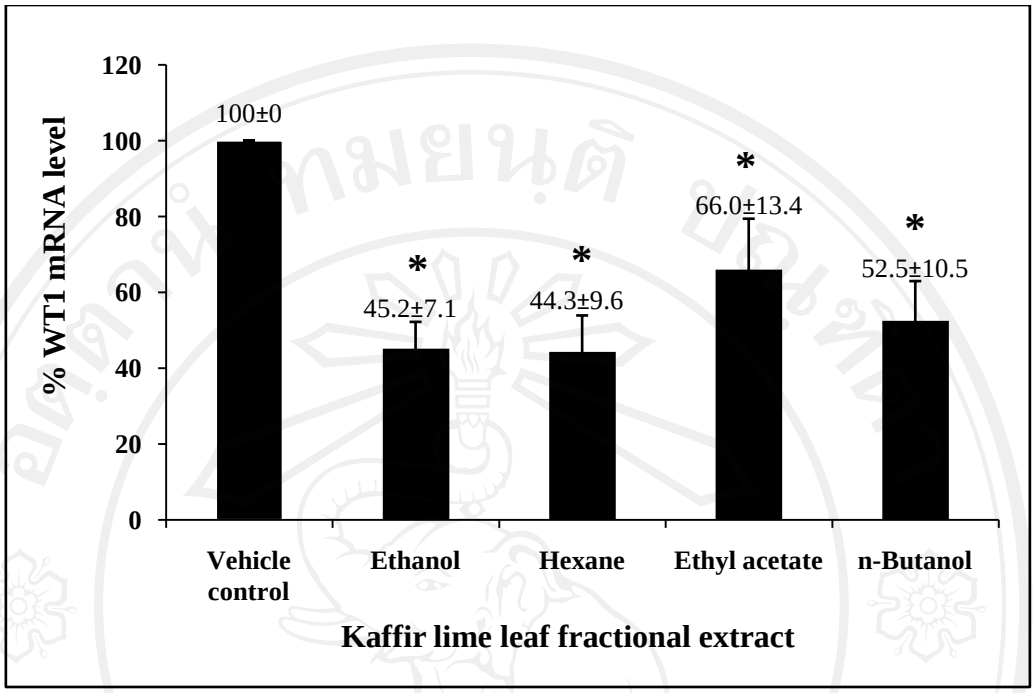

Figure 11 The effects of crude kaffir lime leaf fractional extracts on WT1 mRNA levels in HL60 cell line. The leukemic cell line in density of 2.0 x 10^5 cells/mL was grown in complete RPMI 1640 medium with 0.1% DMSO and crude kaffir lime leaf ethanol, hexane, ethyl acetate, and n-butanol fractional extracts with the concentrations at IC_{20} for 48 h. The levels of the WT1 mRNA were determined by real-time PCR and represented as % WT1 mRNA level. The expression levels of WT1 mRNA were normalized by using the expression of β-actin mRNA as an internal control. The data is shown as the mean ± standard deviation (SD) of three independent experiments. Asterisk (*) demonstrates the statistically significant difference between the value of vehicle control and sample ($p < 0.05$).

3.4 Effect of concentrations of crude hexane fractional extract from kaffir lime leaf on WT1 mRNA levels in K562 cell line

According to the results from section 3.3, the crude kaffir lime leaf hexane fractional extract had the strongest inhibitory effect on *WT1* gene expression in K562, Molt4, U937, and HL60 leukemic cell lines. In addition, the different concentrations of hexane fractional extract were used to study on *WT1* gene expression. To study its effect in a dose dependent manner of crude effective fractional extract on leukemic cell lines, the hexane fractional extract was used. K562 cell line was chosen as the representative of all leukemic cell lines. The leukemic cell line was treated with 5, 10, 15, and 20 µg/mL (non-cytotoxic doses) of crude hexane fractional extract and 0.08% DMSO was used as vehicle control. After 2 days of incubation, treated cells were harvested and extracted total RNA. Then, the cDNA was synthesized and run through real-time PCR process. The experiment was done in three times. The results were shown in Ct (Cycle threshold) values. The obtained Ct values were used for calculating % WT1 mRNA level. The percentages of WT1 mRNA level were 74.7 ± 11.4, 64.3 ± 4.0, 57.7 ± 2.5, and 52.0 ± 4.4 in the response to the treatment with 5, 10, 15, and 20 µg/mL, respectively, and the hexane fractional extract could decrease the WT1 mRNA levels in a dose dependent manner by 25, 36, 42, and 48%, respectively, as compared to vehicle control (Figure 12).

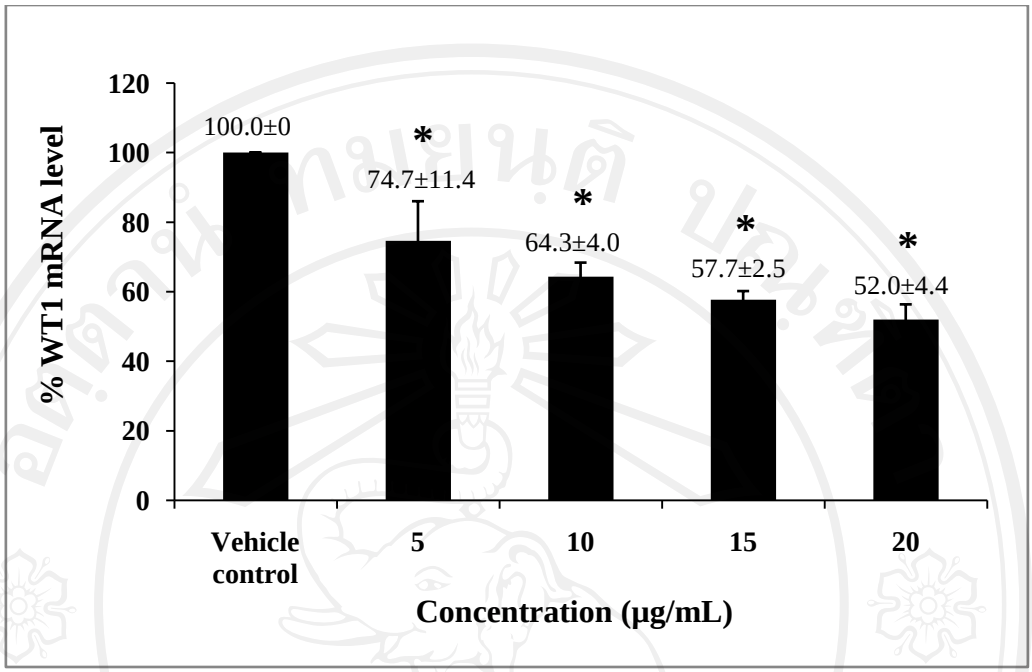

Figure 12 The effects of concentrations of hexane fractional extract on WT1 mRNA levels in K562 cell line. The leukemic cell line in density of 2.0 x 10^5 cells/mL was grown in medium with 0.08% DMSO and 5, 10, 15, and 20 µg/mL of hexane fractional extract for 48 h. The levels of the WT1 mRNA were determined by real-time PCR and represented as % WT1 mRNA level. The expression levels of WT1 mRNA were normalized by using the expression of β-actin mRNA as an internal control. The data is shown as the mean ± standard deviation (SD) of three independent experiments. Asterisk (*) demonstrates the statistically significant difference between the value of vehicle control and sample ($p < 0.05$).

3.5 Effect of different time points of crude hexane fractional extract from kaffir lime leaf on WT1 mRNA levels in K562 cell line

The crude hexane fractional extract from kaffir lime leaf had the strongest inhibitory effect on *WT1* gene expression. To study the effect in a time dependent manner of crude hexane extract on leukemic cell lines, leukemic cell was treated with 13.6 µg/mL (IC_{20}) of hexane fractional extract for 1, 2, and 3 days. The vehicle control (0.05% DMSO) was treated for 3 days. After incubation, treated cells were harvested and extracted total RNA. Then, the cDNA was synthesized and run through real-time PCR process. The experiment was done three times. The results were shown in Ct (Cycle threshold) values. The obtained Ct values were used for calculating % WT1 mRNA level. The percentages of WT1 mRNA level were 81.7 ± 11.9, 62.0 ± 4.4, and 57.3 ± 4.9 in the response to 1, 2 and 3 days, respectively. The hexane fractional extract could decrease the WT1 mRNA levels in a time dependent manner by 17, 38, and 43%, respectively, as compared to vehicle control (Figure 13).

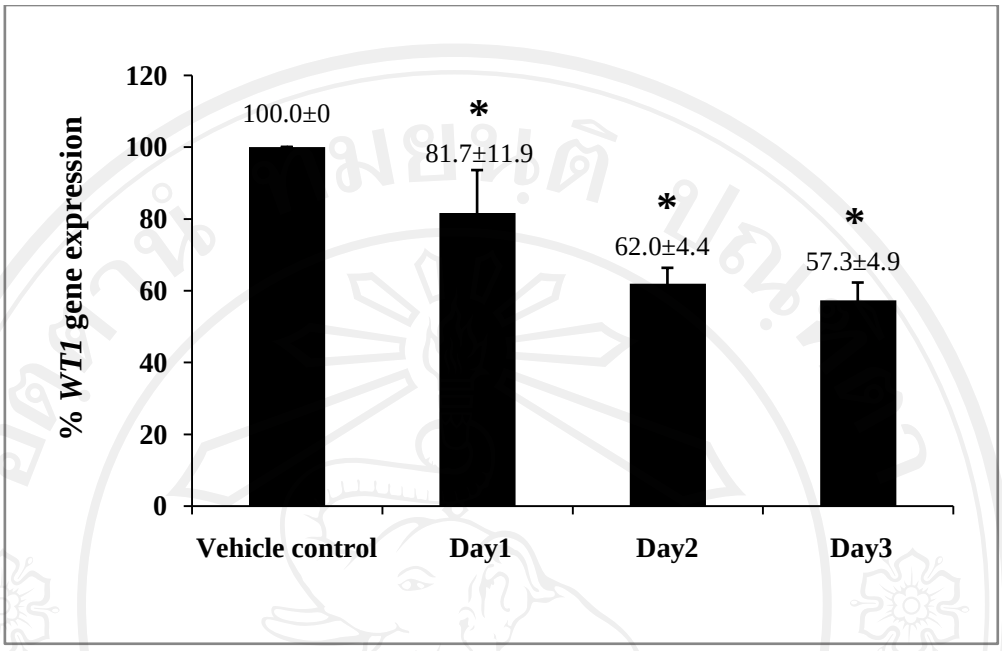

Figure 13 The effects of different time points of crude kaffir lime leaf hexane fractional extract on WT1 mRNA levels in K562 cell line. The leukemic cell line in density of 2.0 x 10^5 cells/mL was grown in complete RPMI 1640 medium with 0.05% DMSO for 3 days (vehicle control) and 13.6 µg/mL of crude kaffir lime leaf hexane fractional extract for 1, 2 and 3 days. The levels of the WT1 mRNA were determined by Real-time PCR and represented as % WT1 mRNA level. The expression levels of WT1 mRNA were normalized by using the expression of β-actin mRNA as an internal control. The data is shown as the mean ± standard deviation (SD) of three independent experiments. Asterisk (*) demonstrates the statistically significant difference between the value of vehicle control and sample ($p < 0.05$).

3.6 Effect of crude kaffir lime leaf fractional extracts on WT1 protein levels on leukemic cell lines

According to the study of Anuchapreeda *et al.* (2007) [196], the WT1 protein was detected in only K562 and Molt4 cell lines whereas U937 and HL60 could not be detected the expression of WT1. However, GAPDH, used as an internal control, could be detected in all leukemic cell lines. K562 and Molt4 cell lines were used for study the effect crude kaffir lime leaf fractional extracts on levels of WT1 protein expression. Leukemic cell lines were treated with DMSO (vehicle control) and crude kaffir lime leaf fractional extracts, which had no cytotoxic effect on the tested cells, with the concentration at IC_{20} value for 2 days. Then treated cells were extracted nuclear protein and Western blot analysis was performed as described in section 2.9 and 2.10.

3.6.1 Effect of crude kaffir lime leaf fractional extracts on WT1 protein levels in leukemic cell lines

To determine effect of crude kaffir lime leaf fractional extracts on the levels of WT1 protein expression in K562 cell line, the leukemic cell line was cultured in medium containing 0.18% DMSO (vehicle control) and crude kaffir lime leaf fractional extracts, including ethanol, hexane, ethyl acetate, and n-butanol, at the IC_{20} concentrations of 45.4, 13.6, 11.9, and 40.9 µg/mL, respectively, for 2 days. Then, the nuclear protein extraction and Western blot analysis was performed to investigate the WT1 levels. The levels of WT1 protein was normalized by using GAPDH protein and calculated percentage of WT1 protein level (%WT1 protein level). The percentages of WT1 protein level were 58.7 ±

6.2, 38.9 ± 10.4, 83.4 ± 8.6, and 99.2 ± 19.5% in the response to ethanol, hexane, ethyl acetate, and n-butanol fractions, respectively (Table 12). All of crude kaffir lime leaf fractional extracts could decrease the WT1 protein levels in K562 cell line by 41, 17, 61, and 1%, respectively, when compared to vehicle control (Figure 14).

3.6.2 Effects of crude kaffir lime leaf fractional extracts on WT1 protein levels in Molt4 cell line

To determine effect of crude kaffir lime leaf fractional extracts on the levels of WT1 mRNA expression in Molt4 cell line, the leukemic cell line was treated with 0.15% DMSO (vehicle control) and crude kaffir lime leaf fractional extracts, including ethanol, hexane, ethyl acetate, and n-butanol, at the concentrations of 25.0, 2.8, 8.0, and 37.5 µg/mL, respectively, for 2 days. Then, the nuclear protein extraction and Western blot analysis was performed to investigate WT1 level. The level of WT1 protein was normalized by using GAPDH protein and calculated percentage of WT1 protein level (%WT1 protein level). The percentages of WT1 protein level were 96.1 ± 9.7, 67.5 ± 16.6, 79.1 ± 13.0, and 91.6 ± 2.9% in the response to ethanol, hexane, ethyl acetate, and n-butanol fractions, respectively (Table 12). All of crude kaffir lime leaf fractional extracts could decrease the WT1 levels in Molt4 cell line by 8, 21, 4, and 32%, respectively when compared to vehicle control (Figure 15).

Table 12 The percentage of WT1 protein levels after crude kaffir lime leaf fractional extract treatments compared to vehicle control.

Leukemic cell lines	% WT1 protein level				
	Vehicle control	Ethanol	Hexane	Ethyl acetate	n-Butanol
K562	100 ± 0	58.7 ± 6.2	38.9 ± 10.45	83.4 ± 8.6	99.2 ± 19.5
Molt4	100 ± 0	96.1 ± 9.7	67.5 ± 16.6	79.1 ± 13.0	91.6 ± 2.9

The data represents the mean value ± SD of three independent experiments.

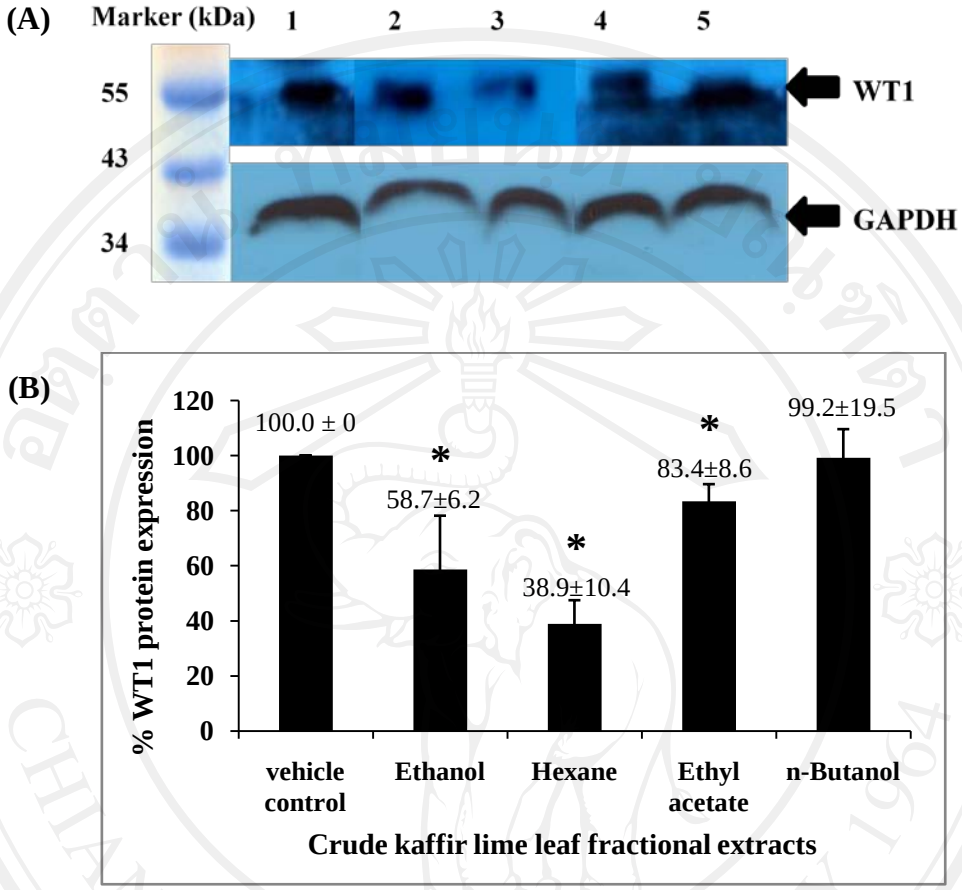

Figure 14 The effects of crude kaffir lime leaf fractional extracts on WT1 protein levels in K562 cell line. The WT1 and GAPDH proteins following the treatment of (No.1) 0.18% DMSO and crude kaffir lime leaf fractional extracts including (No.2) ethanol, (No.3) hexane, (No.4) ethyl acetate, and (No.5) n-butanol fractions with the concentration at IC_{20} values for 2 days were determined by Western blot analysis. (A) The WT1 (48 to 54 kDa) and GAPDH (37 kDa) bands were quantified using densitometer. WT1 protein levels were measured and normalized using GAPDH protein level. (B) The data is shown as the mean ± standard deviation (SD) of three independent experiments. Asterisk (*) demonstrates the statistically significant difference between the value of vehicle control and sample ($p < 0.05$).

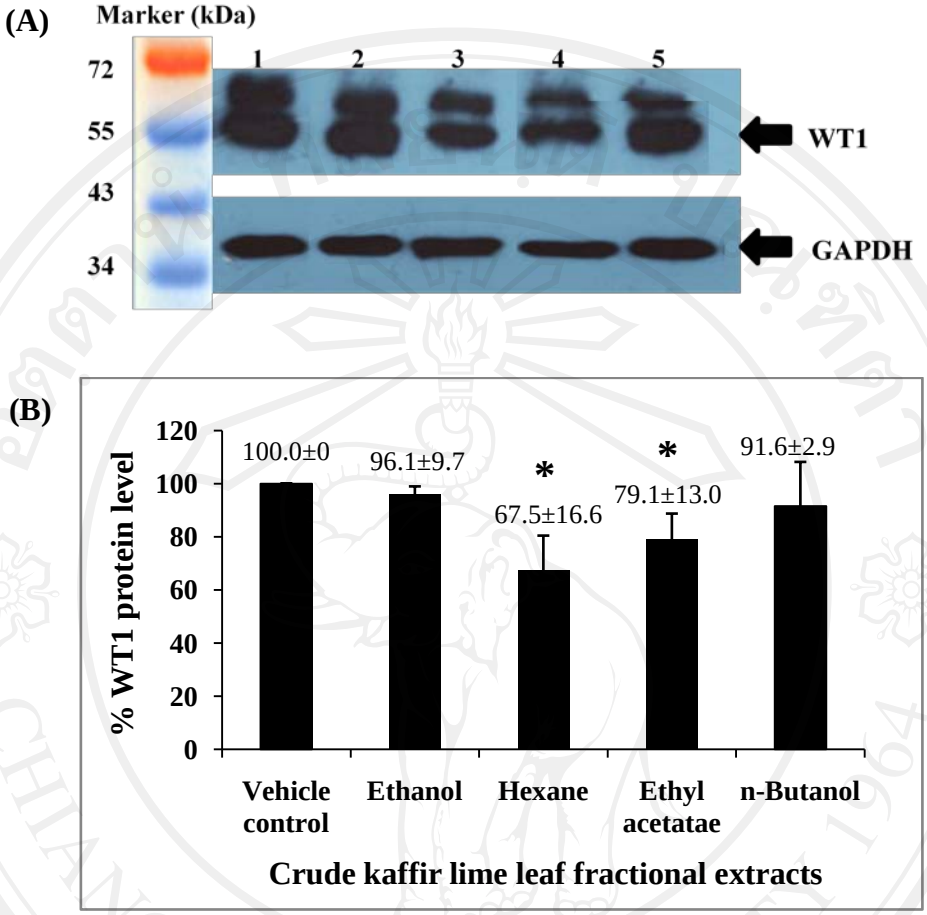

Figure 15 The effects of crude kaffir lime leaf fractional extracts on WT1 protein levels in Molt4 cell line. The WT1 and GAPDH proteins following the treatment of (No.1) 0.15% DMSO and crude kaffir lime leaf fractional extracts including (No.2) ethanol, (No.3) hexane, (No.4) ethyl acetate, and (No.5) n-butanol, fractions with the concentration at IC_{20} values for 2 days were determined by Western blot analysis. (A) The WT1 (48 to 54 kDa) and GAPDH (37 kDa) bands were quantified using densitometer. WT1 protein levels were measured and normalized using GAPDH protein level. (B) The data is shown as the mean ± standard deviation (SD) of three independent experiments. Asterisk (*) demonstrates the statistically significant difference between the value of vehicle control and sample ($p < 0.05$).

3.7 Effect of concentrations of crude kaffir lime leaf hexane fractional extracts on WT1 protein levels in K562 cell line

According to the results from section 3.6, the crude kaffir lime leaf hexane fractional extract had the strongest inhibitory effect on WT1 protein expression in K562 and Molt4 leukemic cell lines. In addition, the different concentrations of hexane fractional extract were used to study on WT1 protein expression. To study its effect in a dose dependent manner of crude effective fractional extract on leukemic cell lines, the hexane fractional extract was used. K562 cell line was chosen as the representative of all leukemic cell lines. The leukemic cell line was treated with 5, 10, 15, and 20 µg/mL (non-cytotoxic doses) of crude hexane fractional extract and 0.08% DMSO was used as vehicle control. After 2 days of incubation, treated cells were harvested, and then the nuclear protein extraction and Western blot analysis were performed to investigate WT1 level. The level of WT1 protein was normalized by using GAPDH protein and calculated percentage of WT1 protein level (%WT1 protein level). The percentages of WT1 protein level were 93.3 ± 12.9, 79.1 ± 5.7, 61.4 ± 9.2, and 36.8 ± 9.7 in the response to 5, 10, 15, and 20 µg/mL, respectively. The hexane fractional extract could decrease the WT1 protein level in a dose dependent manner by 7, 21, 39, and 63%, respectively, compared to vehicle control (Figure 16).

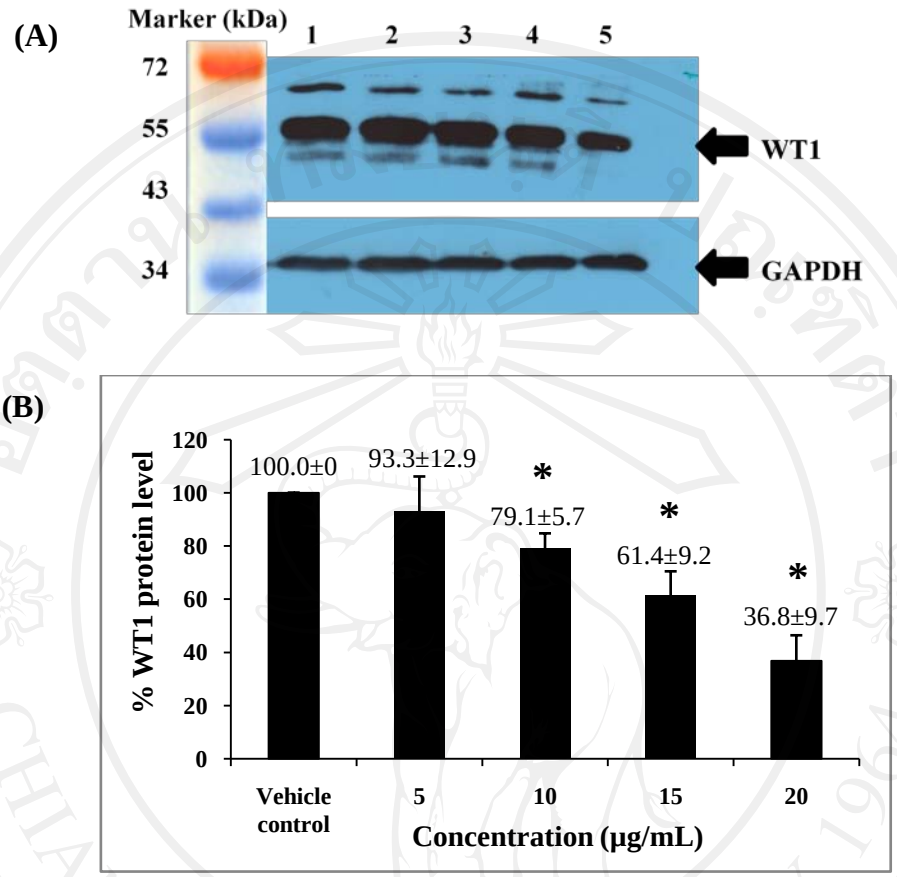

Figure 16 The effect of concentrations of crude kaffir lime leaf hexane fractional extract on WT1 protein levels in K562 cell line. leukemic cell in density of 2.0 x 10^5 cells/mL was grown in medium with (No.1) 0.08% DMSO and (No.2) 5, (No.3) 10, (No.4) 15, and (No.5) 20 µg/mL of hexane fractional extract for 48 h. The WT1 and GAPDH proteins were determined by Western blot analysis. The WT1 and GAPDH proteins were determined by Western blot analysis. (A) The WT1 (48 to 54 kDa) and GAPDH (37 kDa) bands were quantified using densitometer. WT1 protein levels were measured and normalized using GAPDH protein level. (B) The data is shown as the mean ± standard deviation (SD) of three independent experiments. Asterisk (*) demonstrates the statistically significant difference between the value of vehicle control and sample ($p < 0.05$).

3.8 Effect of different time points of crude kaffir lime leaf hexane fractional extracts on WT1 protein levels in K562 cell line

The crude hexane fractional extract from kaffir lime leaf had the strongest inhibitory effect on *WT1* gene expression. To study the effect in a time dependent manner of crude hexane extract on leukemic cell lines, leukemic cell was treated with 13.6 µg/mL (IC_{20}) of hexane fractional extract for 1, 2, and 3 days. The vehicle control (0.05% DMSO) was treated for 3 days. After incubation, treated cells were harvested, and then the nuclear protein extraction and Western blot analysis were performed to investigate WT1 protein level. The level of WT1 protein was normalized by using GAPDH protein and calculated percentage of WT1 protein level (%WT1 protein level). The percentages of WT1 protein level were 92.9 ± 8.0, 65.9 ± 15.8, and 34.1 ± 28.4 in the response to 1, 2 and 3 days, respectively. The crude hexane fractional extract could decrease the WT1 protein level in a time dependent manner by 7, 34, and 66%, respectively, compared to vehicle control (Figure 17).

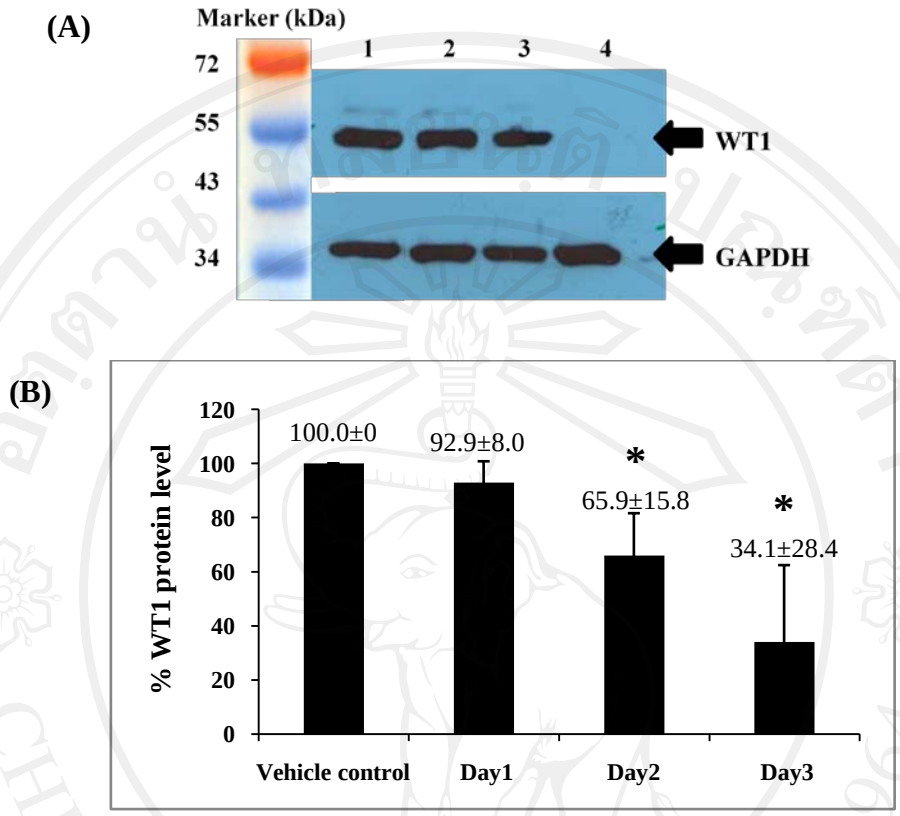

Figure 17 The effect of different time points of crude kaffir lime leaf hexane fractional extract on WT1 protein levels in K562 cell line. The leukemic cell line in density of 2.0 x 10^5 cells/mL was grown in complete RPMI 1640 medium with (No.1) 0.05% DMSO for 3 days (vehicle control) and 13.6 µg/mL of crude kaffir lime leaf hexane fractional extract for (No.2) 1, (No.3) 2 and (No.4) 3 days. The vehicle control (0.05% DMSO) was treated for 3 days. The WT1 and GAPDH proteins were determined by Western blot analysis. (A) The WT1 (48 to 54 kDa) and GAPDH (37 kDa) bands were quantified using densitometer. WT1 protein levels were measured and normalized using GAPDH protein level. (B) The data is shown as the mean ± standard deviation (SD) of three independent experiments. Asterisk (*) demonstrates the statistically significant difference between the value of vehicle control and sample ($p < 0.05$).