CHAPTER III
RESULTS

3.1 Yield of mangosteen peel fraction extracted by organic solvents of varied polarity

In this study, 1000 grams of dried mangosteen peels were extracted by organic solvents including ethanol (relative polarity = 0.654), methanol (relative polarity = 0.762), ethyl acetate (relative polarity = 0.228), butanol (relative polarity = 0.389), and hexane (relative polarity = 0.009). The percentage of yields after being extracted by ethanol, methanol, ethyl acetate, butanol, and hexane were 12.29, 5.10, 4.08, 4.04, and 4.14%, respectively.

3.2 Cytotoxicity of mangosteen peel fraction extracts on leukemic cell lines

In this study, the mangosteen peel extracts (using ethanol, hexane, ethyl acetate, butanol, and methanol), including ethanol with BSA precipitate fraction, pure xanthone and pure mangostin were tested for cytotoxicity in four leukemic cell lines (K562, Molt4, U937, and HL-60). Four cell lines were treated with various concentrations of mangosteen peel fraction extracts for 48 h. Cell viability was determined by the MTT assay. The cytotoxicity of the mangosteen peel extracts on leukemic cell lines are given by inhibitory concentration at 50% (IC_{50}). The IC_{50} values of the ethanol fraction in K562, HL-60, U937, and Molt4 were 43.6, 21.1, 11.1, and 11.1 μg/mL, respectively (Figure 9). The IC_{50} values for hexane were 11.9, 15.2, 8.9, and 8.1 μg/mL, respectively (Figrure 10). The IC_{50} values for ethyl acetate were 12.5, 8.9, 5.2, and 4.6 μg/mL, respectively (Figure 11). The IC_{50} values for butanol were 46.7, 35.8, 19.3, and 19.0 μg/mL, respectively (Figure 12). The IC_{50} of methanol was unable to be detected in all four cell lines (Figure 13). The IC_{50} of ethanol with BSA precipitate fraction was greater than 100 μg/mL in K562, U937, and HL-60 but 2.7μg/mL in Molt4 (Figure 14). The values for pure xanthone were greater than 100 μg/mL in all four cell lines (Figure 15). The IC_{50} values for pure mangostin were 17.7,

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5.8, 8.9, and 5.7 µg/mL, respectively (Figure 16). Noncytotoxic concentrations of 20% (IC_{20}) (Table 9) were used for further studies.

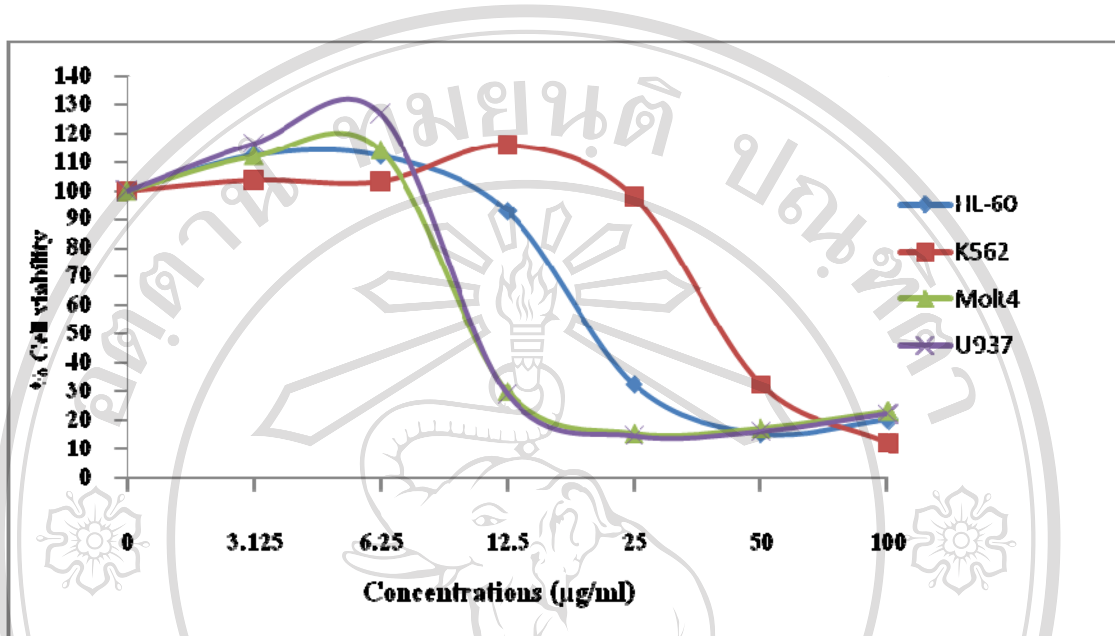

Figure 9 The effect of ethanol fraction on leukemic cell lines at various concentrations

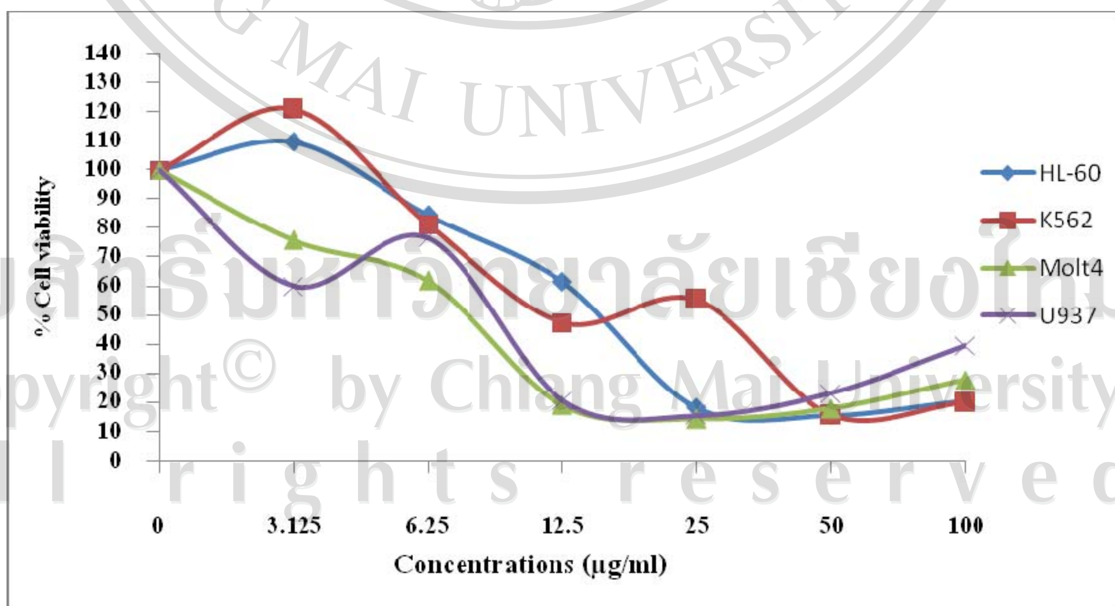

Figure 10 The effect of hexane fraction on leukemic cell lines at various concentrations

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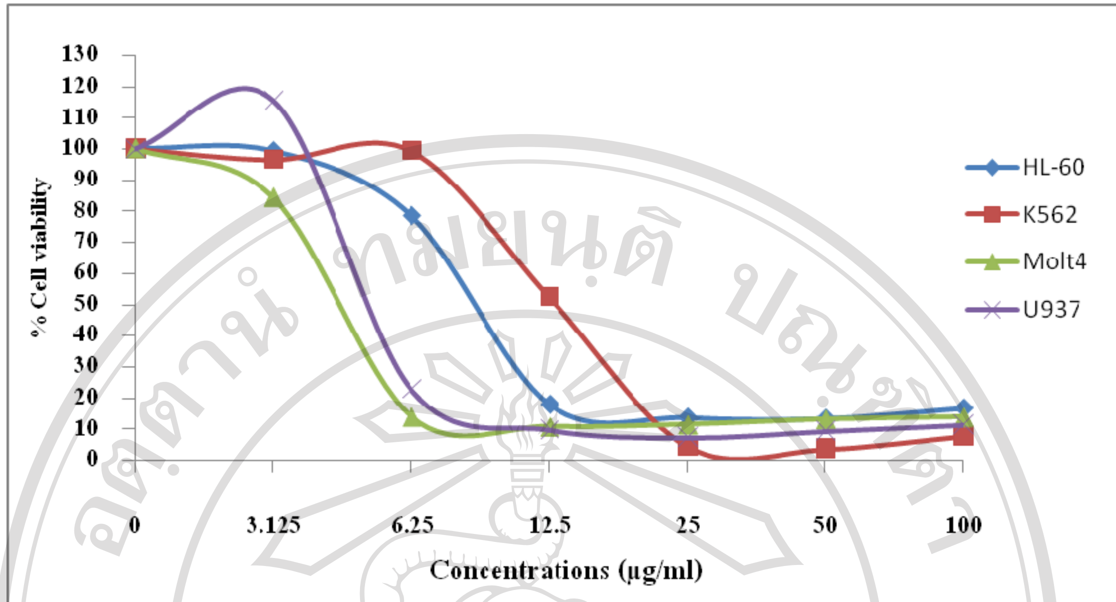

Figure 11 The effect of ethyl acetate fraction on leukemic cell lines at various concentrations

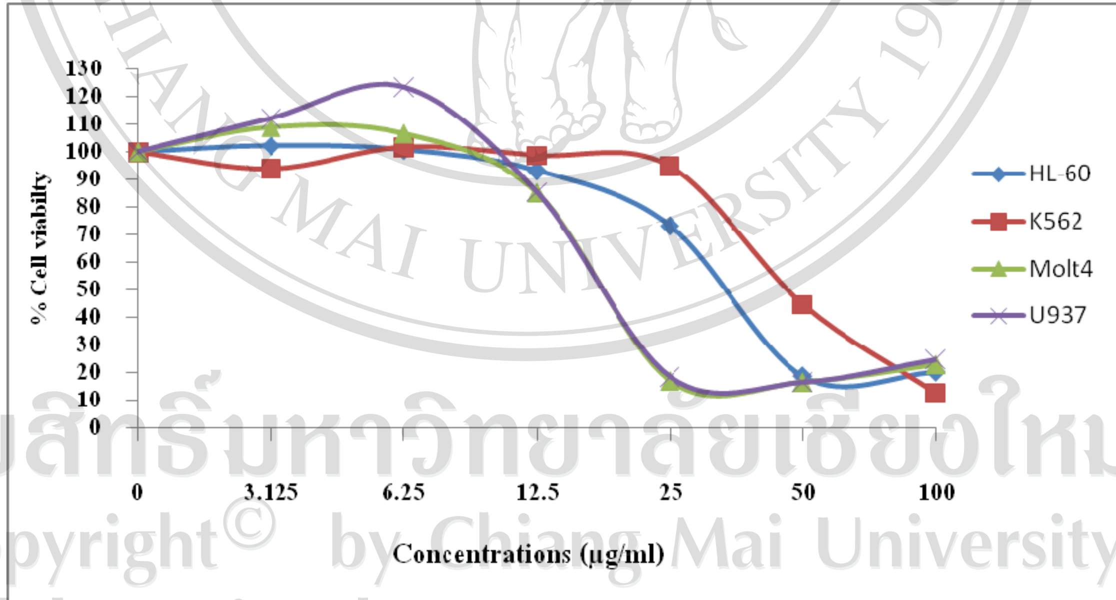

Figure 12 The effect of butanol fraction on leukemic cell lines at various concentrations

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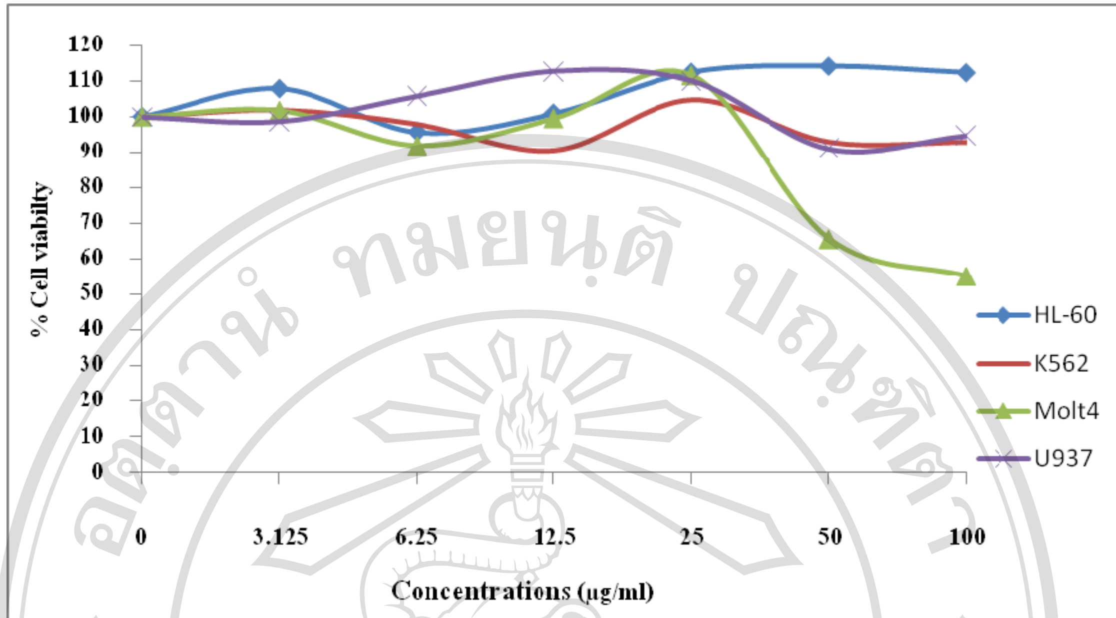

Figure 13 The effect of methanol fraction on leukemic cell lines at various concentrations

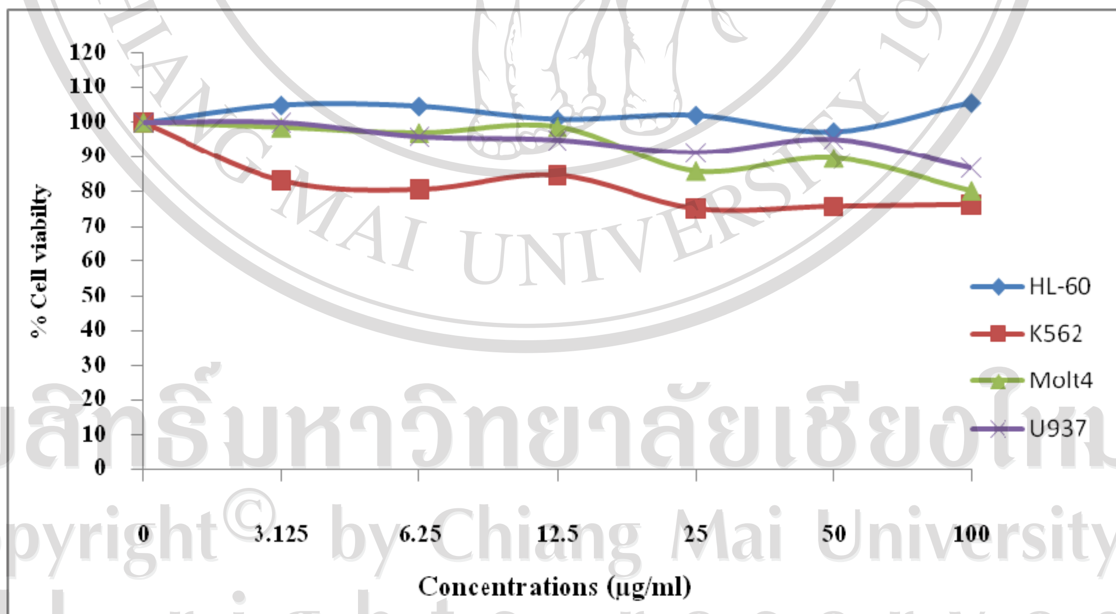

Figure 14 The effect of ethanol with BSA precipitate fraction on leukemic cell lines at various concentrations

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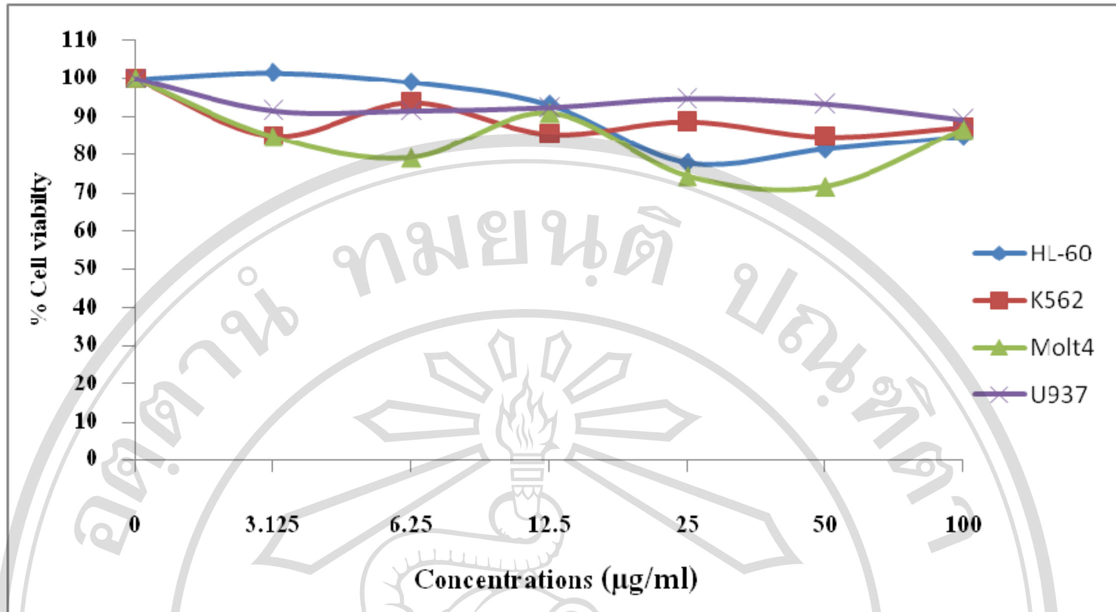

Figure 15 The effect of xanthone fraction on leukemic cell lines at various concentrations

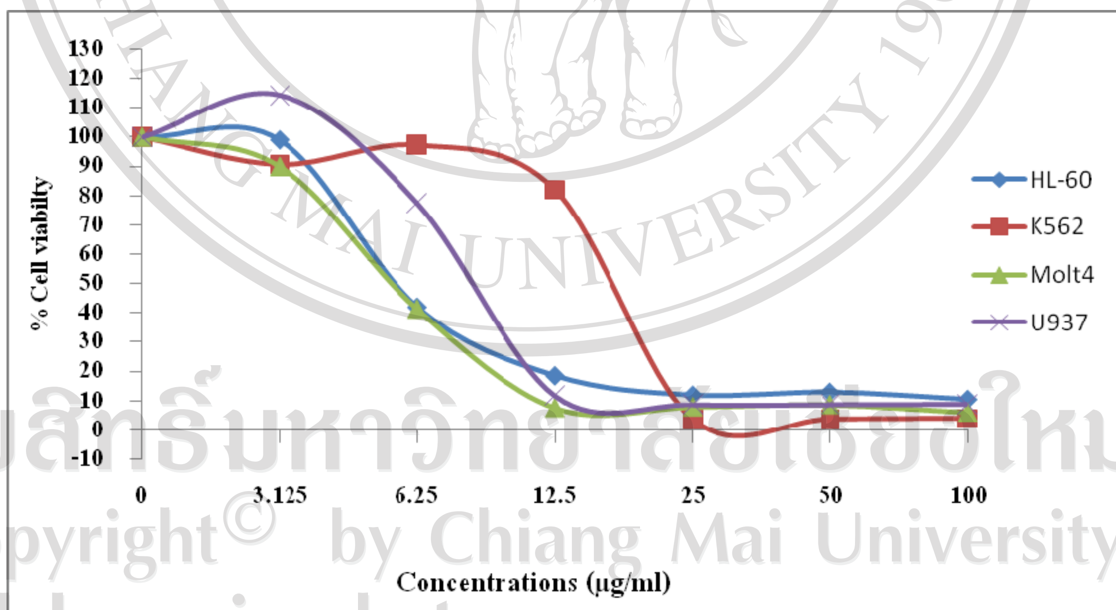

Figure 16 The effect of Mangostine fraction on leukemic cell lines at various concentrations

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Table 9 Summarize of IC_{50} and IC_{20} (µg/mL) of mangosteen peel fraction extract

Mangosteen Fraction	HL-60 (µg/mL)		K562 (µg/mL)		Molt4 (µg/mL)		U937 (µg/mL)	
	IC_{20}	IC_{50}	IC_{20}	IC_{50}	IC_{20}	IC_{50}	IC_{20}	IC_{50}
Butanol	20.1	35.8	32.6	46.7	13.5	19.0	13.6	19.3
Ethanol	15.7	21.1	31.5	43.6	8.9	11.1	9.1	11.1
Ethanol (BSA)	>100	>100	>100	>100	>100	>100	>100	>100
Ethyl Acetate	5.8	8.9	8.6	12.5	3.2	4.6	4.3	5.2
Methanol	>100	>100	>100	>100	41.6	>100	>100	>100
Hexane	7.3	15.2	6.5	11.9	2.7	8.1	1.5	8.9
Pure mangostine	4.2	5.8	12.5	17.7	3.8	5.7	6.1	8.9
Xanthone	>100	>100	>100	>100	>100	>100	>100	>100

3.3 Effect of mangosteen peel fraction extracts on *WT1* gene expression in leukemic cell lines

To determine the level of *WT1* gene expression in each leukemic cell line, the leukemic cell lines were treated with 0.05% DMSO (vehicle control) and mangosteen peel extracts at the concentration corresponding to the IC_{20}. Each treatement lasted for 2 days. Total RNA extractions were exmined by RT–PCR. The experiments were done in three times each WT1 mRNA levels of cells treated with mangosteen peel extracts were normalized by GAPDH mRNA levels and visualized using scan densitometry.

3.3.1 Effect of mangosteen peel fraction extracts on *WT1* gene expression in K562

After mangosteen peel extract treatment for 2 days, WT1 mRNA levels in K562 were decreased. The percentages of WT1 mRNA level were 47.0 ± 4.5, 61.8 ± 30.5, 49.8 ± 22, and 76.3 ± 22 in response to butanol, ethyl acetate, ethanol, and hexane fraction, respectively (Figure 17 and Table 10). In addition, treatment with butanol, ethanol, and ethyl acetate significantly decreased the WT1 mRNA levels when compared to the level in the vehicle control ($p<0.05$).

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3.3.2 Effect of mangosteen peel extracts on *WT1* gene expression in HL-60

WT1 mRNA levels of HL-60 after mangosteen peel extracts treatment were decreased for all fractions. The percentages of WT1 mRNA levels were 57±17.8, 89±6.7, 93±4.1, and 92±7.8 from butanol, ethyl acetate, ethanol, and hexane fractions, respectively (Figure 18 and Table 10). In addition, only treatment with butanol extract significantly decreased the WT1 mRNA levels as compared to the vehicle control (p<0.05).

3.3.3 Effect of mangosteen peel fractions on *WT1* gene expression in U937

The WT1 mRNA levels of U937 after mangosteen peel fraction extract treatment were decreased for all fractions. The percentagse of WT1 mRNA levels were 91± 10.9, 82 ± 7.8, 65 ± 6.0, and 94±7.2 on butanol, ethyl acetate, ethanol, and hexane fraction, respectively (Figure 19 and Table 10). In addition, treatment with ethanol and ethyl acetate fraction significantly decreased the WT1 mRNA levels when compared to the level in the vehicle control (p<0.05).

3.3.4 Effect of mangosteen peel fractions on *WT1* gene expression in Molt4

The WT1 mRNA level in Molt4 after mangosteen peel extracts treatment were decreased for all fractions. The percentages of WT1 mRNA levels were 91±12.9, 84±13.3, 56± 21.0, and 83±13.1 for butanol, ethyl acetate, ethanol, and hexane fraction, respectively (Figure 20 and Table 10). In addition, only treatment with ethanol fraction significantly decreased the WT1 mRNA levels when compared to the level in the vehicle control (p<0.05).

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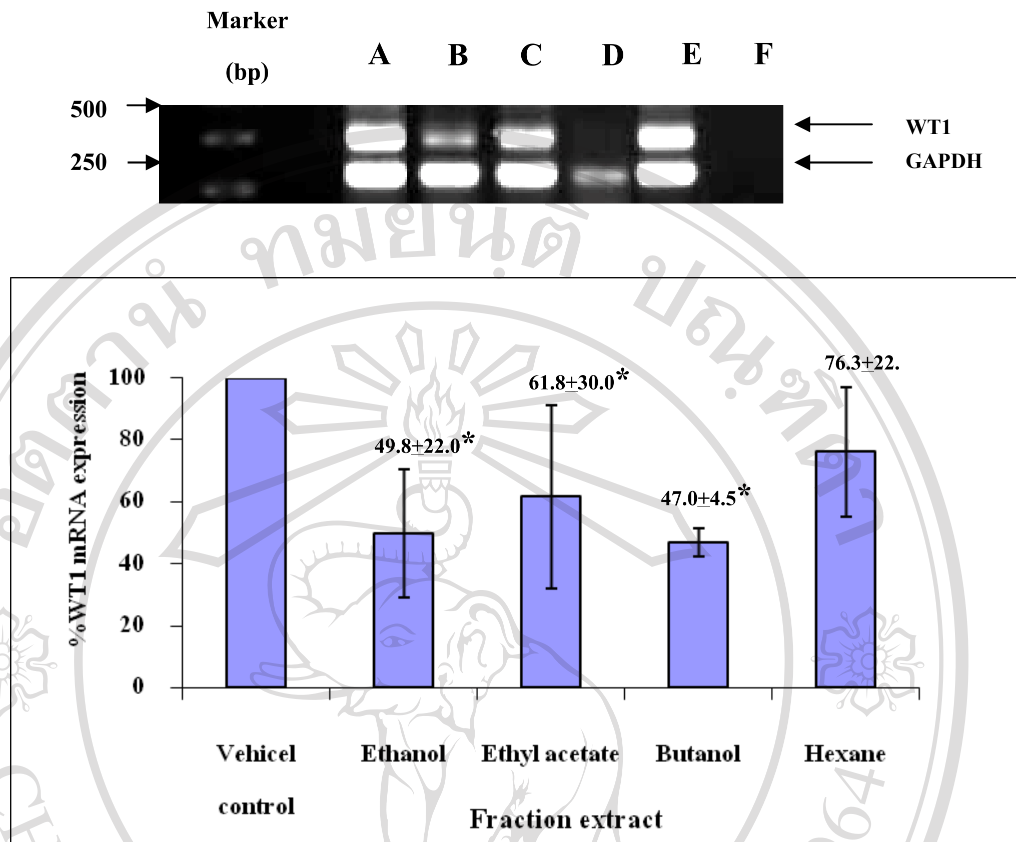

Figure 17 The effect of mangosteen peel extracts to WT1 mRNA expression on K562 by RT–PCR. The WT1 and GAPDH mRNA levels following treatment with (A) control, (B) ethanol fraction, (C) ethyl acetate fraction, (D) butanol fraction, (E) hexane fraction, and (F) deionized distilled water were detemined in K562 cells after 2 days by RT-PCR. The PCR products (474 bp for WT1 and 306 bp for GAPDH) were run on 0.75% agarose gel. The bands were quantified using a scan densitometer. Distilled water was used as a negative control. Data are the mean values±standard deviation (SD) of three independent experiments. Asterisks (*) denote value that were significantly different from the vehicle control (p<0.05).

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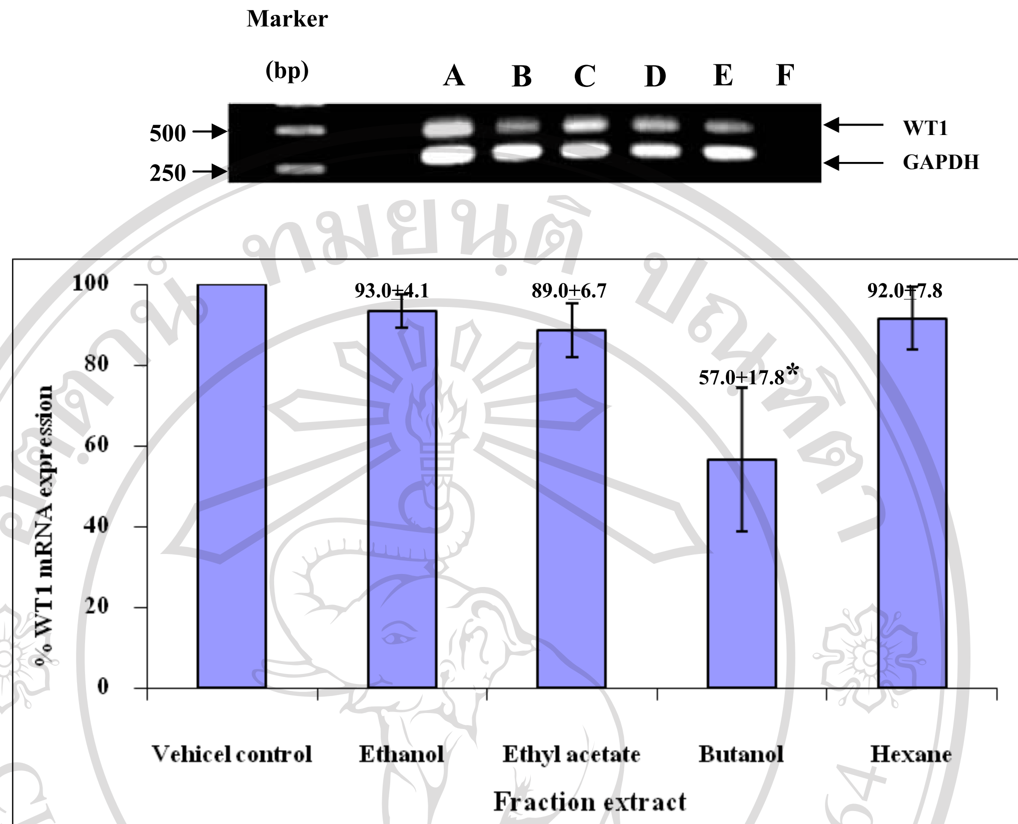

Figure 18 The effect of mangosteen peel extracts to WT1 mRNA expression on HL–60 by RT–PCR. The WT1 and GAPDH mRNA levels following treatment with (A) control, (B) ethanol fraction, (C) ethyl acetate fraction, (D) butanol fraction, (E) hexane fraction, and (F) deionized distilled water were detemined in HL-60 cells after 2 days by RT-PCR. The PCR products (474 bp for WT1 and 306 bp for GAPDH) were run on 0.75% agarose gel. The bands were quantified using a scan densitometer. Deionized distilled water was used as a negative control. Data are the mean values±standard deviation (SD) of three independent experiments. Asterisks (*) denote value that were significantly different from the vehicle control ($p<0.05$).

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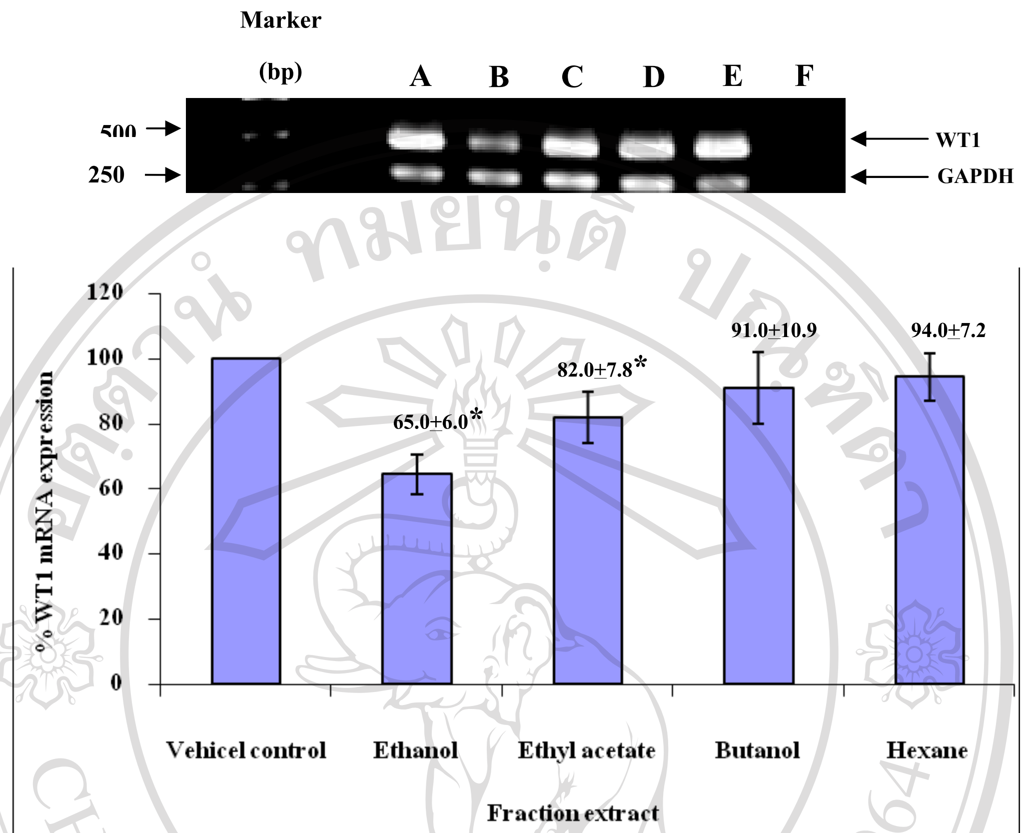

Figure 19 The effect of mangosten peel extracts on WT1 mRNA expression on U937 by RT–PCR. The WT1 and GAPDH mRNA levels following treatment with (A) control, (B) ethanol fraction, (C) ethyl acetate fraction, (D) butanol fraction, (E) hexane fraction, and (F) deionized distilled water were detemined in U937 cells after 2 days by RT-PCR. The PCR products (474 bp for WT1 and 306 bp for GAPDH) were run on 0.75% agarose gel. The bands were quantified using a scan densitometer. Deionized distilled water was used as a negative control. Data are the mean values±standard deviation (SD) of three independent experiments. Asterisks (*) denote value that were significantly different from the vehicle control (p<0.05).

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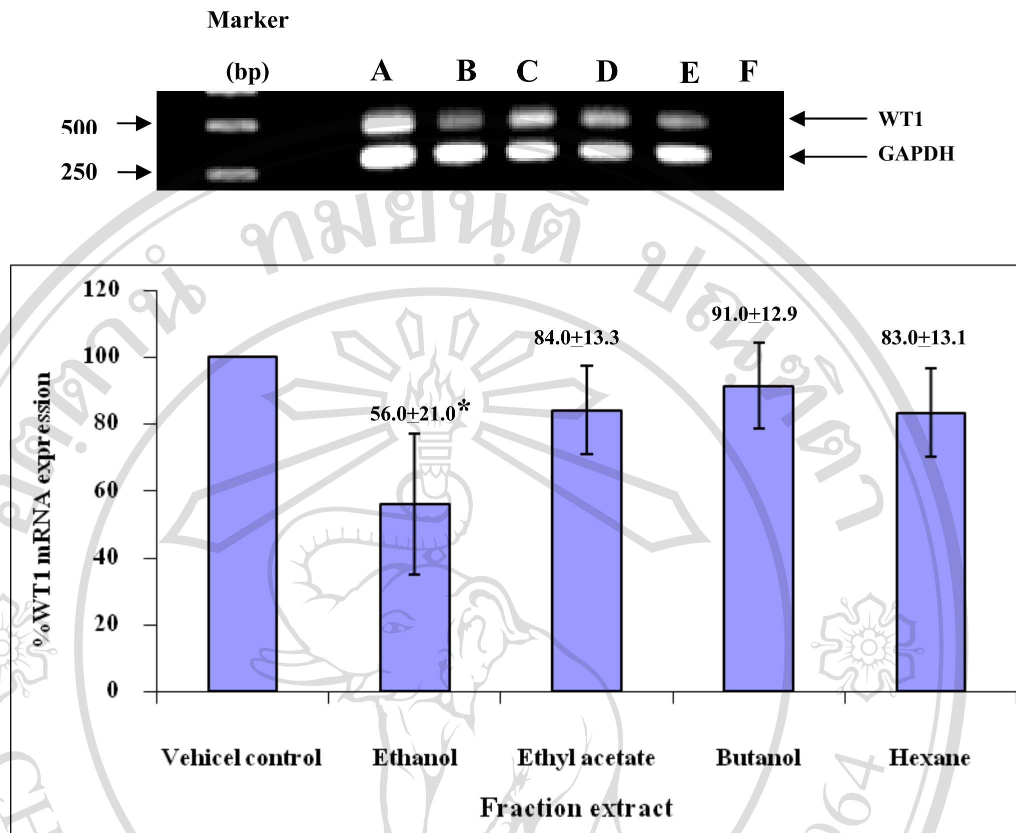

Figure 20 The effect of mangosteen peel extracts on WT1 mRNA expression on Molt4 by RT–PCR. The WT1 and GAPDH mRNA levels following treatment with (A) control, (B) ethanol fraction, (C) ethyl acetate fraction, (D) butanol fraction, (E) hexane fraction, and (F) deionized distilled water were detemined in Molt4 cells after 2 days by RT-PCR. The PCR products (474 bp for WT1 and 306 bp for GAPDH) were run on 0.75% agarose gel. The bands were quantified using a scan densitometer. Deionized distilled water was used as a negative control. Data are the mean values±standard deviation (SD) of three independent experiments. Asterisks (*) denote value that were significantly different from the vehicle control (p<0.05).

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Table 10 Summarized of percentage of WT1 mRNA levels when compared to vehicle control

Cell lines	Percentage of WT1 mRNA levels			
	Ethanol	Ethyl acetate	Butanol	Hexane
K562	49.8±22.0*	61.8±30.0*	47.0±4.5*	76.3±22.0
HL– 60	93.0±4.1	89.0±6.7	57.0±17.8*	92.0±7.8
U937	65.0±6.0*	82.0±7.8*	91.0±10.9	94.0±7.2
Molt4	56.0±21.0*	84.0±13.3	91.0±12.9	83.0±13.1

3.4 Effect of concentrations of mangosteen peel extracts on *WT1* gene expression in each leukemic cell line.

In previous studies, *WT1* gene expression in leukemic cell lines was inhibited by mangosteen peel extracts. However, the results were varied in different cell lines. Butanol fraction had the strongest inhibitory effect on WT1 mRNA levels in HL–60 cell lines, while the ethyl acetate fraction had the strongest inhibitory effect in K562 cells. But the ethanol fraction had the strongest inhibitory effect in Molt 4 and U937 cells. These three extracts were tested for dosage response using three non-toxic doses. The leukemic cell lines were cultured with 0.05% DMSO (vehicle control) or non-toxic doses of each fraction for 2 days. Then, total RNA extraction and RT–PCR was performed. The PCR products were electrophoresed and visualized by a scan densitometer. The experiment was done three times, and the WT1 mRNA levels were normalized by the GAPDH mRNA levels.

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3.4.1 Effect of concentrations of ethyl acetate fraction on *WT1* gene expression in K562 cell line

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Although the butanol extracts had the strongest inhibitory effect on WT1 mRNA level in K562, the concentration (32.6 µg/mL) was higher than for the ehthyl acetate fraction (8.6 µg/mL). Thus, the ethyl acetate fraction was used for further experiments. To study its effect in a dosage dependent manner, non–toxic doses of ethyl acetate (5, 10 and 15 µg/mL) were used. Ethyl acetate fractions with the concentrations of 5, 10, and 15 µg/mL decreased the WT1 mRNA levels in a dosage dependent manner. The

percentages of WT1 mRNA levels were 52±5.6, 40±6.2, and 34±8.5%, respectively when compared to the level of vehicle control (Figure 21 and Table 11). In addition, all concentrations of the ethyl acetate fraction significantly decreased the WT1 mRNA levels in a dose dependent maner (p<0.05) when compared with the vehicle control.

3.4.2 Effect of concentrations of butanol fraction on *WT1* gene expression in H-60 cell line

Buthanol fractions with concentrations of 15, 20 amd 25 µg/mL decreased the WT1 mRNA level in a dose dependent manner. The percentages of WT1 mRNA levels were 93±6.2, 49±5.1 and 38±2.2%, respectively when compared to the level of the vehicle control (Figure 22 and Table 11). In addition, all concentrations of the butanol fraction significantly decreased the WT1 mRNA levels in a dose dependent maner (p<0.05) when compared with the vehicle control.

3.4.3 Effect of concentrations of ethanol fraction on *WT1* gene expression in U937 cell line

Ethanol fractions with concentrations of 3, 5 and 10 µg/mL decreased the WT1 mRNA level in a dose dependent manner. The percentages of WT1 mRNA levels were 82±2.9, 74±5 and 66±13.5%, respectively when compared to the level of the vehicle control (Figure 23 and Table 11). In addition, all concentrations of the ethanol fraction significantly decreased the WT1 mRNA levels in a dose dependent maner (p<0.05) when compared with the vehicle control.

3.4.4 Effect of concentrations of ethanol fraction on *WT1* gene expression in Molt4 cell line

Ethanol fractions with concentrations of 3, 7 and 10 µg/mL decreased the WT1 mRNA level in a dose dependent manner. The percentages of WT1 mRNA levels were 85±10.2, 80±7.1 and 44±21.7%, respectively when compared to the level of the vehicle control (Figure 24 and Table 11). In addition, 7 and 10 µg/mL of the ethanol fraction significantly decreased the WT1 mRNA levels in a dose dependent maner (p<0.05) when compared with the vehicle control.

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Table 11 Summarized of percentage of WT1 mRNA levels due to concentration effect of mangosteen peel fraction extracts when compared with vehicle control

Type of leukemic cell line	K562			HL–60			U937			Molt4		
Fraction selection	Ethyl acetate			Butanol			Ethanol			Ethanol		
Concentration (µg/mL)	5	10	15	15	20	25	3	5	10	3	7	10
Percentage of RT-PCR products	52* ± 5.6	40* ± 6.2	34* ± 8.5	93* ± 6.2	49* ± 5.1	38* ± 2.2	82* ± 2.9	74* ± 5	66* ± 13.5	85 ± 10.2	80* ± 7.1	44* ± 21.7

3.5 Effect of duration of mangosteen peel extracts on *WT1* gene expression in each leukemic cell lines

The leukemic cell lines were treated with 0.05% DMSO (vehicle control). The doses of mangosteen peel fraction extracts; 10 µg/mL of ethyl acetate for K562, 20 µg/mL of butanol for HL-60, 5 µg/mL of ethanol for U937, and 7 µg/mL of ethanol for Molt4 were treated for 1, 2, and 3 days. Total RNA extractions were determined by RT–PCR. The experiments were done in three times. WT1 mRNA levels of mangosteen peel extracts were normalized by GAPDH mRNA levels and visualized using scan densitometry.

3.5.1 Effect of duration of ethyl acetate fraction on *WT1* gene expression in K562 cell line

K562 cells were treated with 10 µg/mL of the ethyl acetate fraction for 1, 2, and 3 days. Ethyl acetate fraction extract decreased the WT1 mRNA levels in a time dependent manner. The percentages of WT1 mRNA levels were 95±2.9, 92±6.6, and 83±4.5%, respectively as compared to the level of the vehicle control (Figure 25 and Table 12). In addition, treatment with ethyl acetate for 2 and 3 days of incubation significantly decreased the WT1 mRNA levels in a time dependent manner (p<0.05) when compared with the vehicle control.

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3.5.2 Effect of duration of ethanol fraction on *WT1* gene expression in Molt4 cell line

Molt4 cells were treated with 7 µg/mL of the ethanol fraction for 1, 2, and 3 days. The ethanol extract decreased the WT1 mRNA level in a time dependent manner. The percentages of WT1 mRNA levels were 92±1.0, 86±2.0, and 72±7.5%, respectively as compared to the level in the vehicle control (Figure 26, and Table 12). In addition, treatment with ethyl acetate for 2 and 3 days of incubation times significantly decreased the WT1 mRNA levels in a time dependent manner ($p<0.05$) when compared with the vehicle control.

3.5.3 Effect of duration of ethanol fraction on *WT1* gene expression in U937 cell line

U937 cells were treated with 5 µg/mL of the ethanol fraction for 1, 2, and 3 days. The ethanol extract decreased the WT1 mRNA level in a time dependent manner and the percentages of WT1 mRNA levels were 96±2.1, 87±8.4 and 79±10.2%, respectively as compared to the level in the vehicle control (Figure 27 and Table 12). In addition, treatment with ethyl acetate for 2 and 3 days of incubation significantly decreased the WT1 mRNA levels in a time dependent manner ($p<0.05$) when compared with the vehicle control.

3.5.4 Effect of duration of buthanol fraction on *WT1* gene expression in HL-60 cell line

HL-60 cells were treated with 20 µg/mL of the buthanol fraction for 1, 2, and 3 days. The butanol fraction extract decreased the WT1 mRNA level in a time dependent manner and the percentages of WT1 mRNA levels were 85±9.0, 76±9.1 and 68±5.1%, respectively when compared to the level in the vehicle control (Figure 28 and Table 12). In addition, treatment with ethyl acetate for 2 and 3 days of incubation significantly decreased the WT1 mRNA levels in a time dependent manner ($p<0.05$) when compared with the vehicle control.

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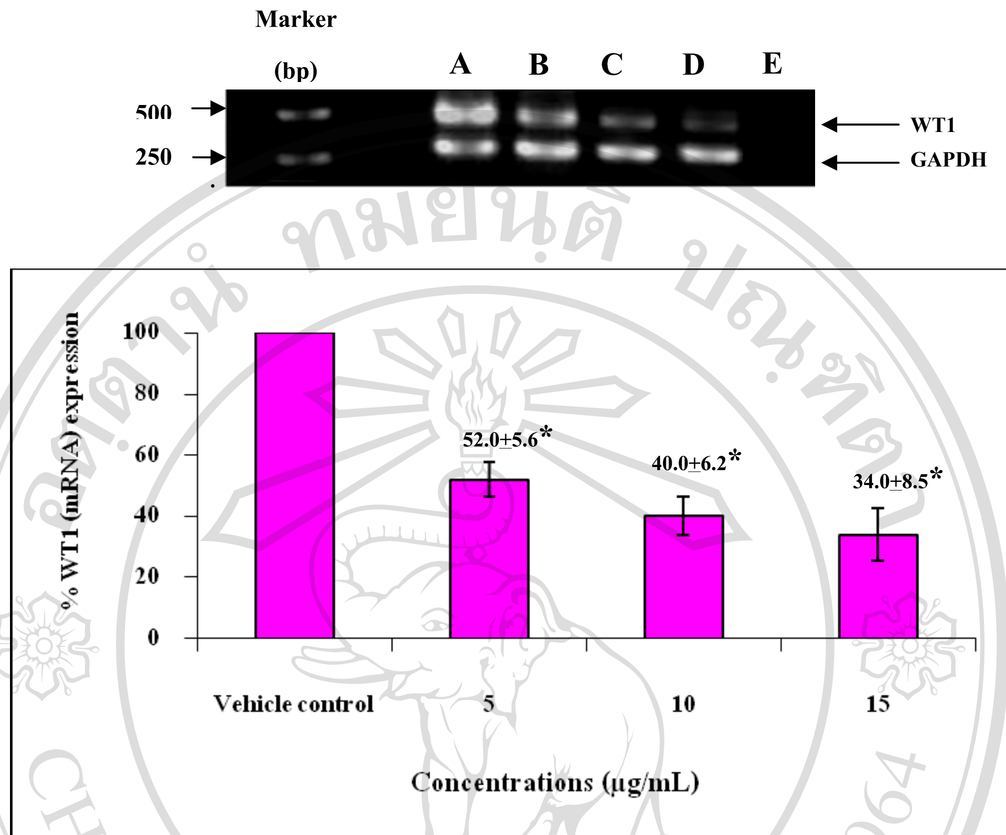

Figure 21 The WT1 mRNA levels in K562 cell line after being treated with 5, 10 and 15 µg/mL of ethyl acetate fraction for 2 days. The WT1 and GAPDH mRNA levels following treatment with (A) 0.05% DMSO, (B) 5 µg/mL, (C) 10 µg/mL, (D) 15 µg/mL, and (E) deionized distilled water were determined in K562 cells after 2 days by RT-PCR. The PCR products were electrophoresed on 0.75% agarose gel. The bands were quantified using a scan densitometer. WT1 mRNA level was measured and normalized with GAPDH mRNA level. Deionized distilled water (DW) was used as a negative control. Data are the mean value ± SD of three independent experiments. Asterisk (*) denote values that were significantly different from the vehicle control ($p<0.05$).

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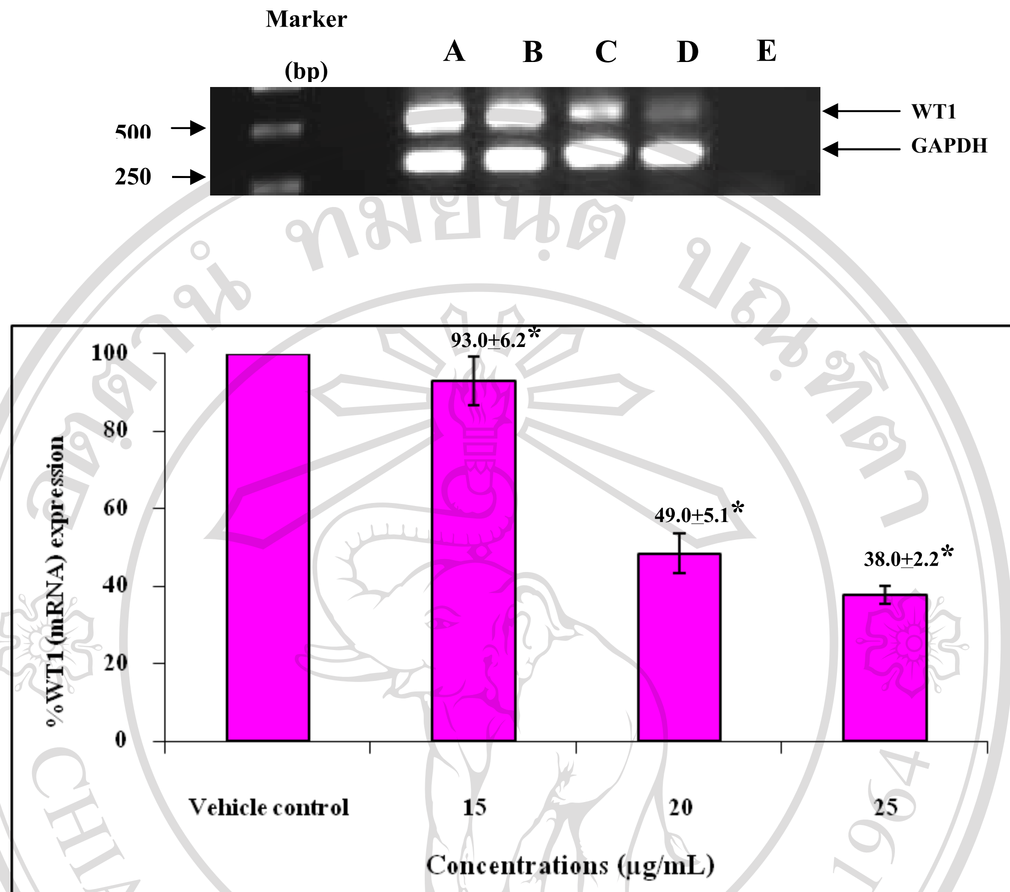

Figuren 22 The WT1 mRNA levels in HL-60 cell line with 15, 20 and 25 µg/mL of the butanol fraction for 2 days. The WT1 and GAPDH mRNA levels following treatment with (A) 0.05% DMSO, (B) 15 µg/mL, (C) 20 µg/mL, (D) 25 µg/mL, and (E) deionized distilled water were determined in HL-60 cells after 2 days by RT-PCR. The PCR products were electrophoresed on 0.75% agarose gel. The bands were quantified using a scan densitometer. WT1 mRNA level was measured and normalized with GAPDH mRNA level. Deionized distilled water (DW) was used as a negative control. Data are the mean value±SD of three independent experiments. Asterisk (*) denote values that were significantly different from the vehicle control (p<0.05).

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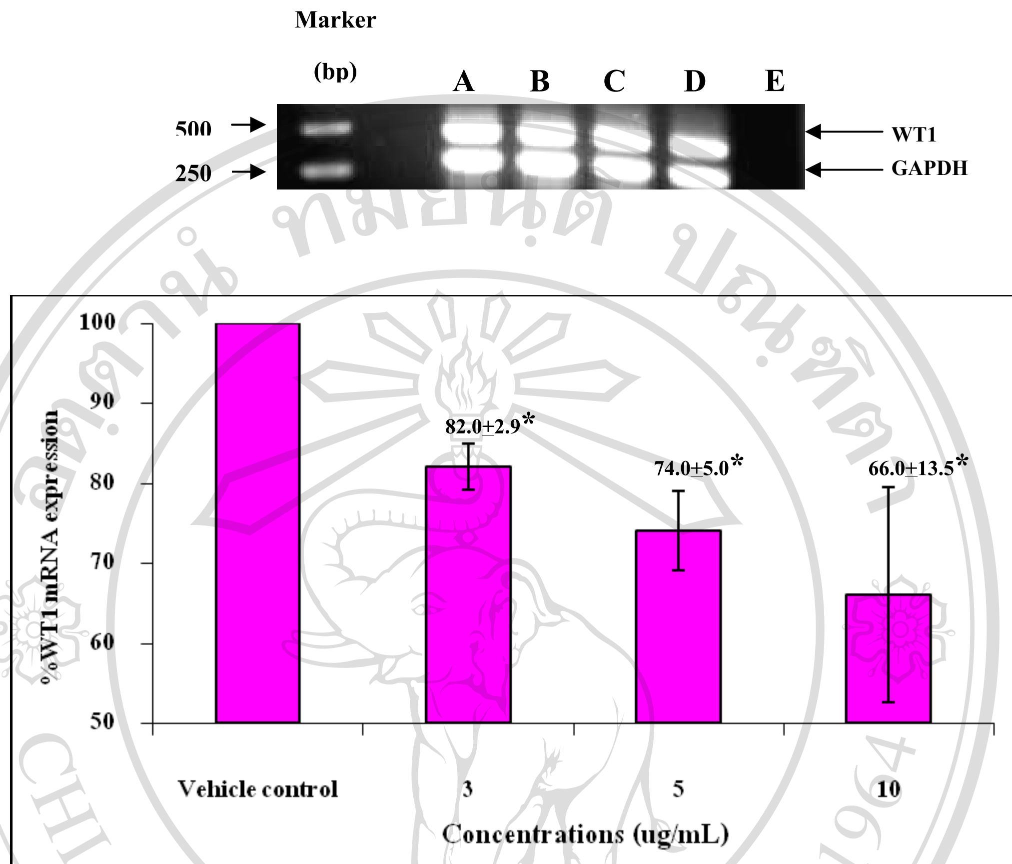

Figure 23 The WT1 mRNA levels in U937 cell line in 3, 5 and 10 µg/mL of the ethanol fraction for 2 days. The WT1 and GAPDH mRNA levels following treatment with (A) 0.05% DMSO, (B) 3 µg/mL, (C) 5 µg/mL, (D) 10 µg/mL, and (E) deionized distilled water were determined in U937 cells after 2 days by RT-PCR. The PCR products were electrophorresd on 0.75% agarose gel. The bands were quantified using a scan densitometer. WT1 mRNA level was measured and normalized with GAPDH mRNA level. Deionized distilled water (DW) was used as a negative control. Data are the mean value±SD of three independent experiments. Asterisk (*) denote values that were significantly different from the vehicle control (p<0.05).

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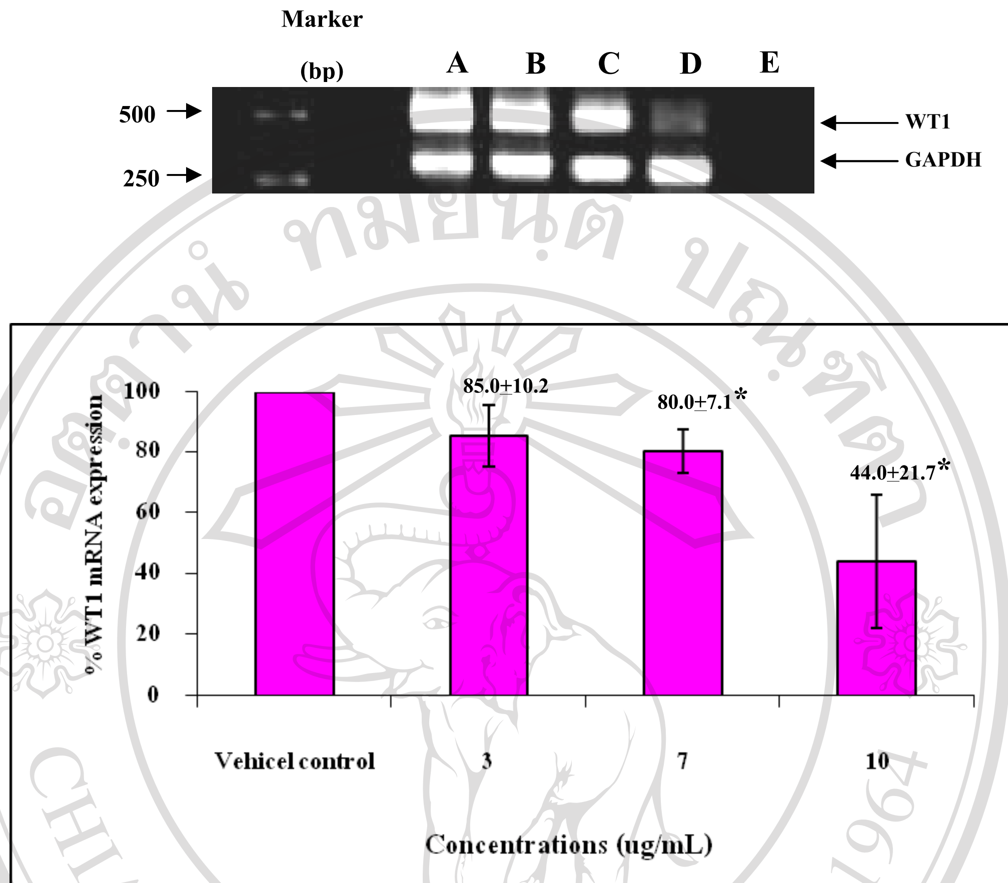

Figure 24 The WT1 mRNA levels in Molt4 cell line in 3, 7 and 10 µg/mL of the ethanol fraction for 2 days. The WT1 and GAPDH mRNA levels following treatment with (A) 0.05% DMSO, (B) 3 µg/mL, (C) 7 µg/mL, (D) 10 µg/mL, and (E) deionized distilled water were determined in Molt4 cells after 2 days by RT-PCR. The PCR products were electrophoresed on 0.75% agarose gel. The bands were quantified using a scan densitometer. WT1 mRNA level was measured and normalized with GAPDH mRNA level. Deionized distilled water (DW) was used as a negative control. Data are the mean value ± SD of three independent experiments. Asterisk (*) denote values that were significantly different from the vehicle control (p<0.05).

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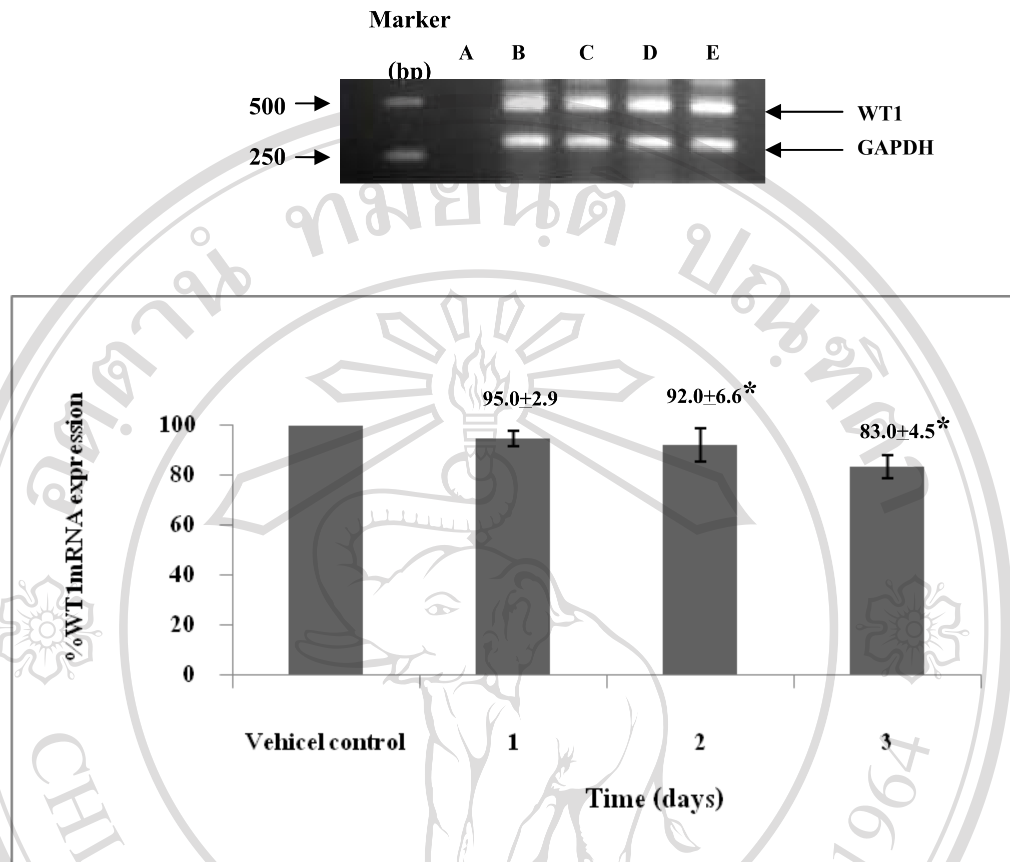

Figure 25 The WT1 mRNA levels in K562 cell line cultured 10 µg/mL of the ethyl acetate extract for 1, 2, and 3 days. The WT1 and GAPDH mRNA levels following treatment with (A) deionized distiled water (B) 0.05% DMSO, (C) 1 day, (D) 2 days and (E) 3 days were determined in K562 by RT-PCR. The PCR products were electrophoresed on 0.75% agarose gel. The bands were quantified using a scan densitometer. WT1 mRNA level was measured and normalized with the levels of GAPDH mRNA. Deionized distilled water was used as a negative control. Data are the mean value±SD of three independent experiments. Asterisk (*) denote values that were significantly different from the vehicle control ($p<0.05$).

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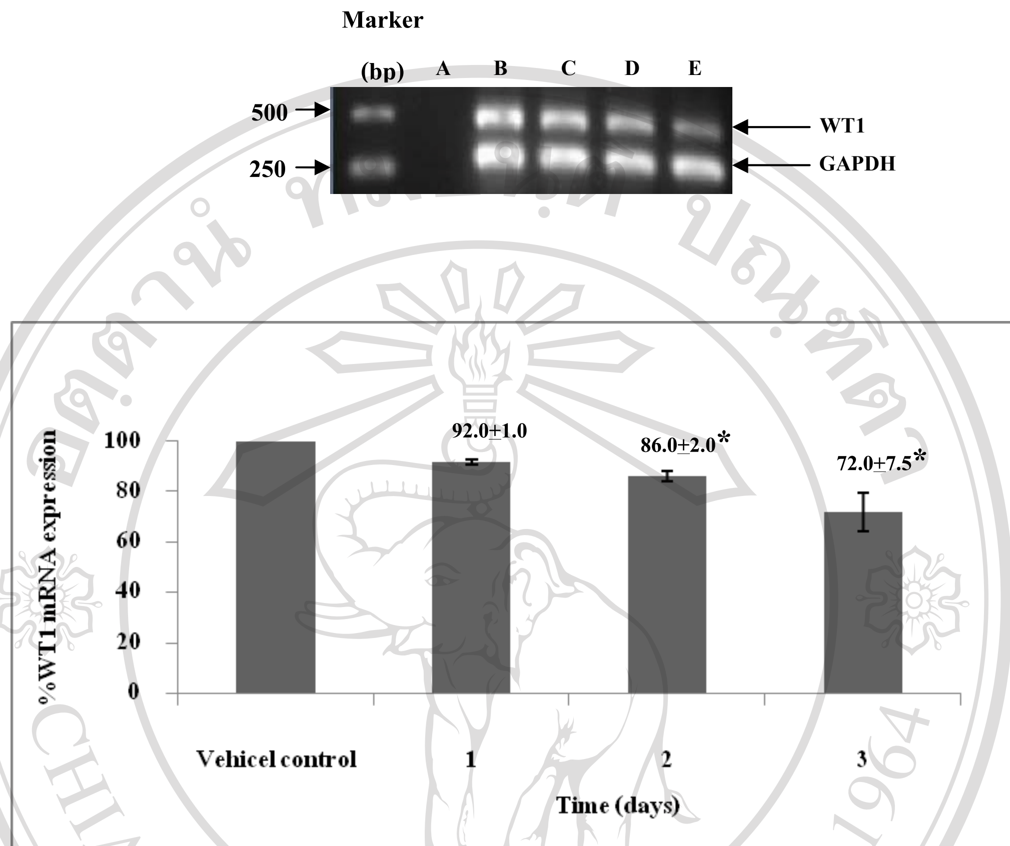

Figure 26 The WT1 mRNA levels in Molt4 cell line cultured with 7 μg/mL of the ethanol fraction extract for 1, 2, and 3 days. The WT1 and GAPDH mRNA levels following treatment with (A) deionized distiled water (B) 0.05% DMSO, (C) 1 day, (D) 2 days and (E) 3 days were determined in Molt4 by RT-PCR. The PCR products were electrophoresed on 0.75% agarose gel. The bands were quantified using a scan densitometer. WT1 mRNA level was measured and normalized with levels of GAPDH mRNA. Deionized distilled water was used as a negative control. Data are the mean value ± SD of three independent experiments. Asterisk (*) denote values that were significantly different from the vehicle control (p<0.05).

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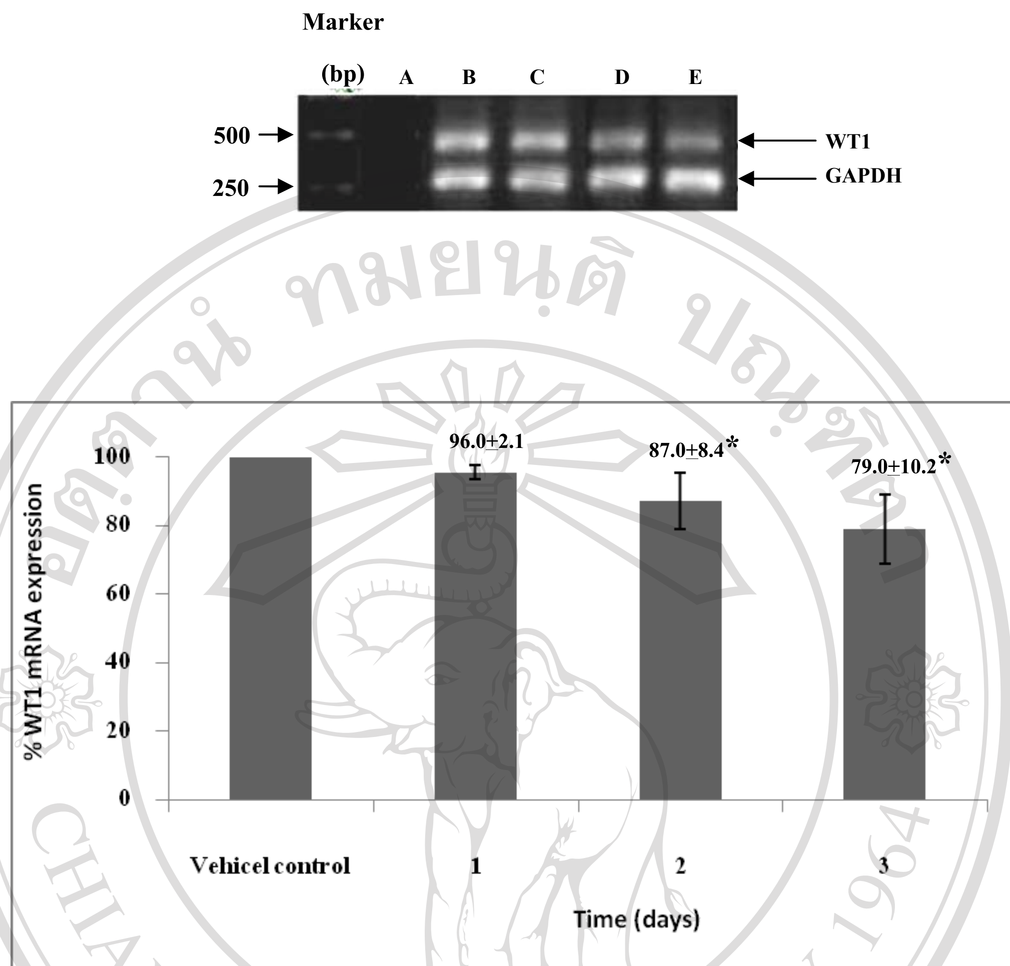

Figure 27 The WT1 mRNA levels in U937 cell line cultured with 5 µg/mL of the ethanol extract for 1, 2, and 3 days. The WT1 and GAPDH mRNA levels following treatment with (A) deionized distiled water (B) 0.05% DMSO, (C) 1 day, (D) 2 days and (E) 3 days were determined in U937 by RT-PCR. The PCR products were electrophoresed on 0.75% agarose gel. The bands were quantified using a scan densitometer. WT1 mRNA level was measured and normalized with the levels of GAPDH mRNA. Deionized distilled water was used as a negative control. Data are the mean value±SD of three independent experiments. Asterisk (*) denote values that were significantly different from the vehicle control (p<0.05).

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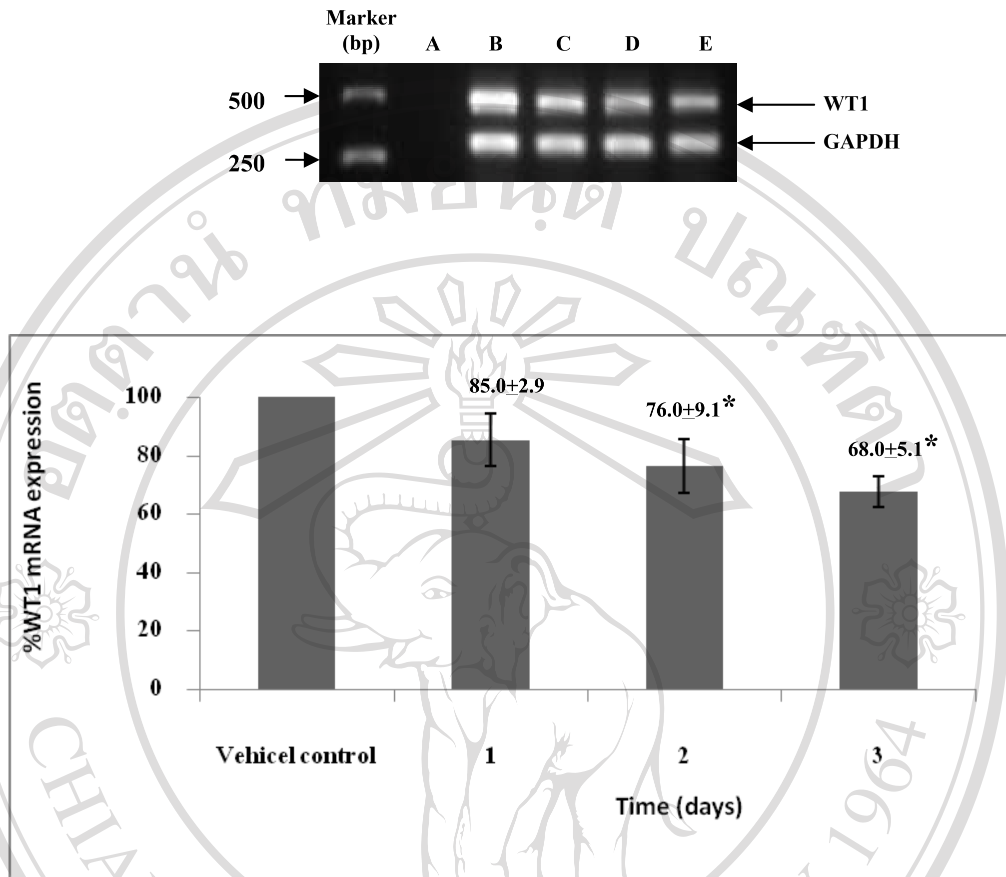

Figure 28 The WT1 mRNA levels in HL-60 cell line cultured with 20 µg/mL of butanol extract for 1, 2, and 3 days. The WT1 and GAPDH mRNA levels following treatment with (A) deionized distiled water (B) 0.05% DMSO, (C) 1 day, (D) 2 days and (E) 3 days were determined in HL-60 by RT-PCR. The PCR products were electrophoresed on 0.75% agarose gel. The bands were quantified using a scan densitometer. WT1 mRNA level was measured and normalized with the levels of GAPDH mRNA. Deionized distilled water was used as a negative control. Data are the mean value ± SD of three independent experiments. Asterisk (*) denote values that were significantly different from the vehicle control (p<0.05).

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Table 12 Summarized of percentage of WT1 mRNA levels due to duration effect of mangosteen peel fraction extracts when compared with vehicle control

Leukemic cell lines	**K562**			**HL – 60**			**U937**			**Molt4**		
Fractions	**Ethyl acetate**			**Butanol**			**Ethanol**			**Ethanol**		
Concentrations (µg/mL)	**10**			**20**			**5**			**7**		
Time (Days)	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Percentage of RT-PCR products	95 ± 2.9	92* ± 6.6	83* ± 4.5	85 ± 9.0	76* ± 9.1	68* ± 5.1	96 ± 2.1	87* ± 8.4	79* ± 10.2	92 ± 1.0	86* ± 2.0	73* ± 7.5

3.6 Effect of mangosteen peel fraction extracts on WT1 protein expression in leukemic cell lines

According to a study of protein expression in four leukemic cell lines (K562, Molt4, U937, and HL-60) as described by Anuchapreeda *et. al*. [189], the WT1 protein is highly expressed K562 and Molt4 cell lines. Here the effect of mangosteen peel extracts (ethanol, butanol, ethyl acetate and hexane fractions) on WT1 protein expression was further investigated in K562 and Molt4 cell lines. The leukemic cell lines were cultured in RPMI 1640 medium with 0.05% DMSO as a vehicle control, and with mangosteen peel extracts at the concentration of IC_{20}. After treatement, nuclear protein extraction and Western blot analysis were carried out to monitor the WT1 protein levels. After WT1 detection, the nitrocellulose membrane was stripped and the GAPDH protein was detected in the same nitrocellulose membrane.The experiment was done three times.

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3.6.1 Effect of mangosteen peel extracts on WT1 protien expression in K562 cells

After mangosteen peel fraction extract treatment for 2 days, WT1 protein levels in K562 were decreased. The percentages of WT1 protein levels were 99±1.7, 77±13.9, 66±28.3 and 41±10.8 in response to hexane, ethyl acetate, ethanol and butanol fractions, respectively (Figure 29 and Table 13). In addition, treatment with ethyl acetate, butanol and ethanol fractions significantly decreased the WT1 protein levels when compared with the vehicle control (p<0.05)

3.6.2 Effect of mangosteen peel extracts on WT1 protien expression in Molt4 cells

After mangosteen peel extract treatment for 2 days, WT1 protein levels in K562 were decreased. The percentages of WT1 protein levels were 98±2.1, 98±1.9, 77±28.0 and 96±4.8 in response to hexane, ethyl acetate, ethanol and butanol fractions, respectively (Figure 30 and Table 13). Only thr treatment with ethanol significantly decreased the WT1 protein levels when compared with the vehicle control (p<0.05)

Table 13 Summarized of percentage of WT1 protein levels when compared with vehicle control

Cell lines	Percentage of WT1 mRNA levels			
	Ethanol	Ethyl acetate	Butanol	Hexane
K562	66±28.3*	77±13.9*	41±10.8 *	99±1.7
Molt4	77±28.0 *	98±1.9	96±4.8	98±2.1

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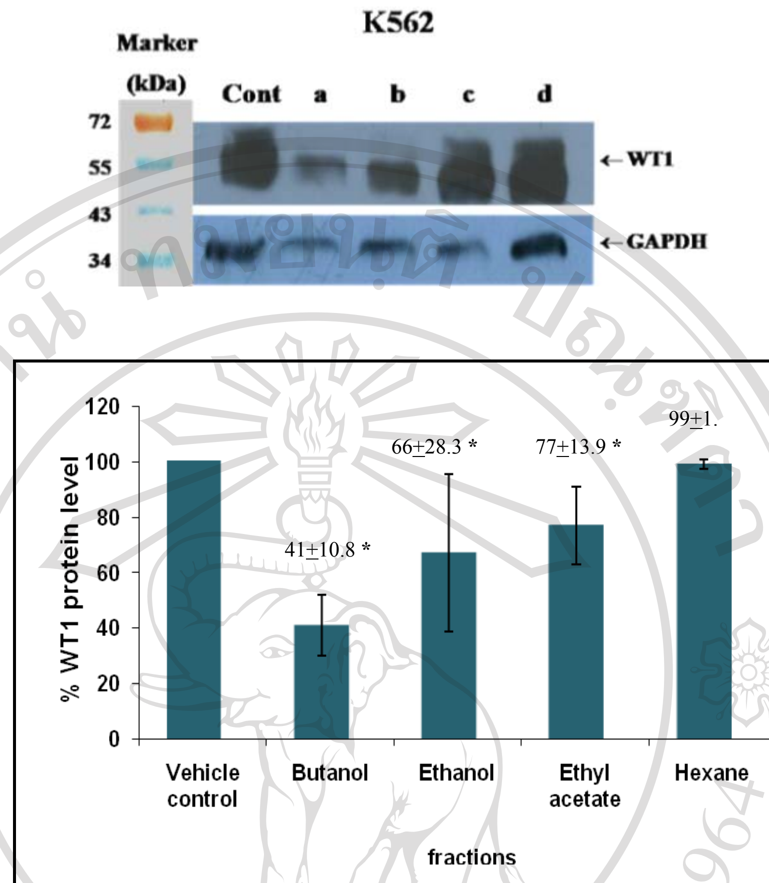

Figure 29 The effect of mangosteen peel extracts on WT1 protein levels in K562 cell line cultured for 2 days. The WT1 and GAPDH protein levels following treatment with 0.05% DMSO and mangosten peel extracts with concentration at IC_{20}. The bands (48 to 54 kDa for WT1 and 37 kDa for GAPDH) were quantified using a scan densitometer. WT1 protein levels (A) butanol (B) ethanol (C) ethyl acetate and (D) hexane. Data are the mean value±SD of three independent experiments. Asterisk (*) denote values that were significantly different from the vehicle control (p<0.05).

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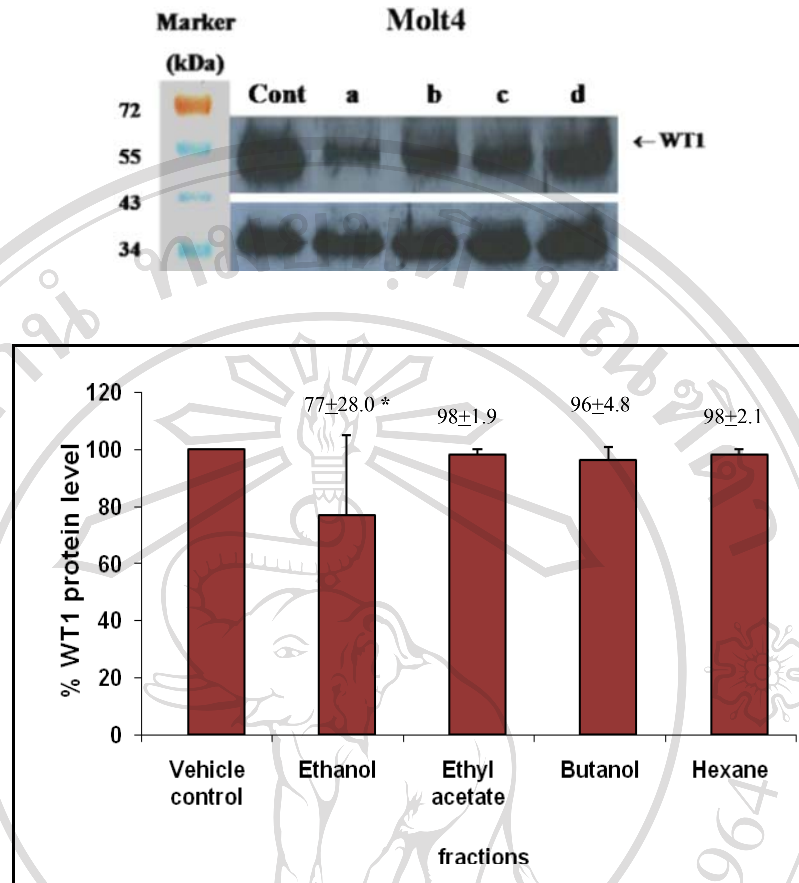

Figure 30 The effect of mangosteen peel extracts on WT1 protein levels in Molt4 cell line cultured for 2 days. The WT1 and GAPDH protein levels following treatment with 0.05% DMSO and mangosten peel extracts with concentration at IC_{20}. The bands (48 to 54 kDa forWT1 and 37 kDa for GAPDH) were quantified using a scan densitometer. WT1 protein levels (A) ethanol (B) ethyl acetate (C) butanol and (D) hexane. Data are the mean value±SD of three independent experiments. Asterisk (*) denote values that were significantly different from the vehicle control (p<0.05).

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3.7 Effect of concentration of ethyl acetate fraction on WT1 protein level in K562 cell line

To study the effect of the ethyl acetate fraction on WT1 protein levels in a dose dependent manner, non–toxic doses of the ethyl acetate fraction (5, 10, and 15 μg/mL) were used in this experiment. K562 cells were cultured in complete RPMI 1640 medium with 0.05% DMSO (vehicle control) and the three concentrations of ethyl acetate for 2 days. After treatment, nuclear protein extraction and Western blot analysis were carried out to monitor the WT1 protein levels. The ethyl acetate fraction decreased the WT1 protien levels in a dose dependent manner. The percentages of WT1 protein levels were 84$\pm$9.9, 58$\pm$10.9 and 33$\pm$11.9%, respectively when compared to the level in the vehicle control (Figure 31). The inhibitory percentages were 16, 42, and 66%, respectively. 10 and 15 μg/mL of ethyl acetate significantly decreased WT1 protien when compared with the level of the vehicle control.

3.7.1 Effect of duration of ethyl acetate fraction on WT1 protein level in K562 cell line

To study the effect of the ethyl acetate fraction on WT1 protein levels in a time dependent manner, the leukemic cells were treated with 10 μg/mL of the ethyl acetate fraction for 1, 2, and 3 days. After treatment, nuclear protein extraction and Western blot analysis were carried out to monitor the WT1 protein levels. The ethyl acetate fraction decreased the WT1 protien levels in a time dependent manner. The percentages of WT1 protein levels were 88$\pm$9.2, 74$\pm$5.2, and 57$\pm$4.7%, respectively when compared to the level of the vehicle control (Figure 32). The inhibitory percentages were 12, 26, and 43%, respectively. All of the incubation times significantly decreased WT1 protien when compared with the level of the vehicle control.

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3.7.2 Effect of concentration of ethanol fraction on WT1 protein level in Molt4 cell line

To study the effect of the ethanol fraction on WT1 protein levels in a dose dependent manner, non–toxic doses of the ethanol fraction (3, 7, and 10 μg/mL) were used in this experiment. Molt4 cells were cultured in complete RPMI 1640 medium with 0.05% DMSO (vehicle control) and the three concentrations of ethanol for 2 days. After treatment, nuclear protein extraction and Western blot analysis were carried out to monitor the WT1 protein levels. The ethanol fraction in Molt4 decreased the WT1 protien levels in a dose dependent manner. The percentages of WT1 protein levels were 84±11.5, 65±15.5, and 56±10.1%, respectively when compared to the level of the vehicle control (Figure 33). The inhibitory percentages were 16, 35, and 44%, respectively. 7 and 10 μg/mL of ethanol significantly decreased WT1 protien when compared with the level of the vehicle control.

3.7.3 Effect of duration of ethanol fraction on WT1 protein level in Molt4 cell line

To study the effect of the ethanol fraction on WT1 protein levels in a time dependent manner, the leukemic cells were treated with 7 μg/mL of ethanol fraction for 1, 2, and 3 days. After treatment, nuclear protein extraction and Western blot analysis were carried out to monitor the WT1 protein levels. The ethanol fraction in Molt4 decreased the WT1 protien levels in a time dependent manner. The percentages of WT1 protein levels were 76±20.8, 66±16.8, and 48±9.5%, respectively when compared to the level of the vehicle control (Figure 34). The inhibitory percentages were 24, 33, and 52%, respectively. 2 and 3 days incubation time significantly decreased WT1 protien when compared with the level of the vehicle control.

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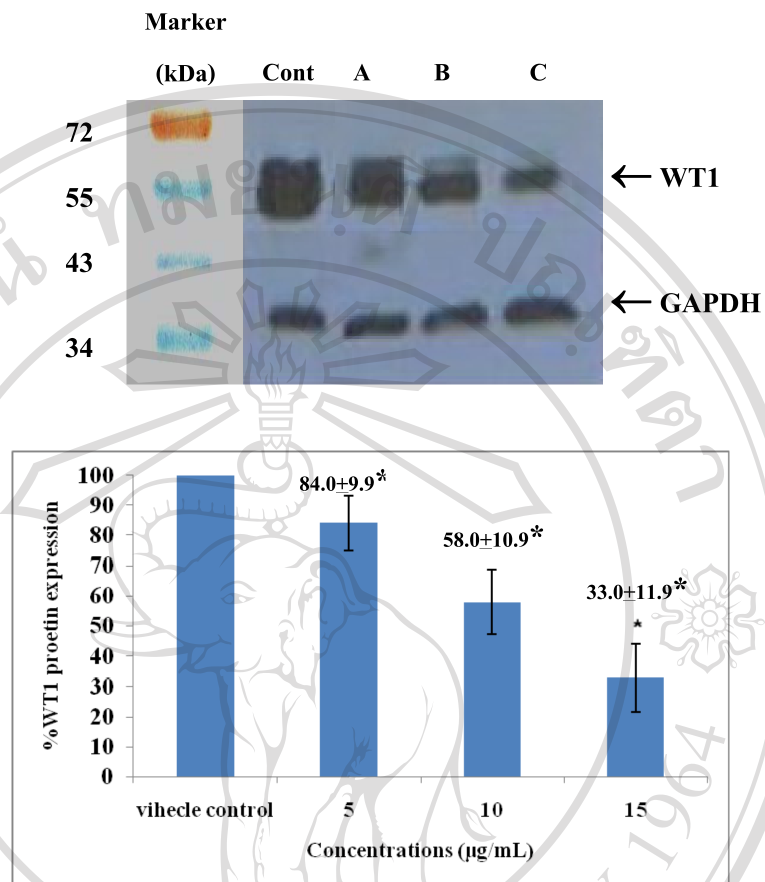

Figure 31 The WT1 levels in K562 cell line cultured with 5, 10, and 15 µg/mL of the ethyl acetate extract for 2 days. The WT1 and GAPDH protein levels following treatment with 0.05% DMSO and ethyl acetate fraction at 5, 10, and 15 µg/mL were determined in K562 cells after 2 days by Western blot analysis. The bands (48 to 54 kDa for WT1 and 37 kDa for GAPDH) were quantified using a scan densitometer. WT1 protein levels (A) 5 µg/mL (B) 10 µg/mL and (C) 15 µg/mL. Data are the mean value±SD of three independent experiments. Asterisk (*) denote values that were significantly different from the vehicle control ($p<0.05$).

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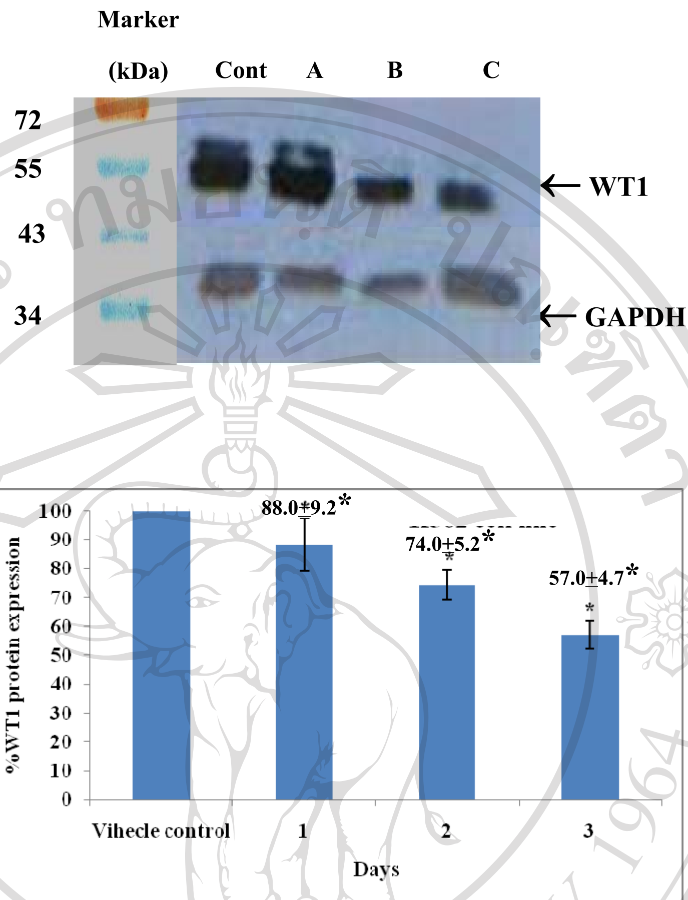

Figure 32 The WT1 levels in K562 cell line cultured in 10 µg/mL of ethyl acetate fraction extract for 1, 2, and 3 days. The WT1 and GAPDH protein levels following treatment with 0.05% DMSO and 10 µg/mL of ethyl acetate fraction for 1, 2, and 3 days were determined in K562 cells by Western blot analysis. The bands (48 to 54 kDa for WT1 and 37 kDa for GAPDH) were quantified using a scan densitometer. WT1 protein levels (A) 1 day, (B) 2 days, and (C) 3 days. Data are the mean value±SD of three independent experiments. Asteriks (*) denote values that were significantly different from the vehicle control (p<0.05).

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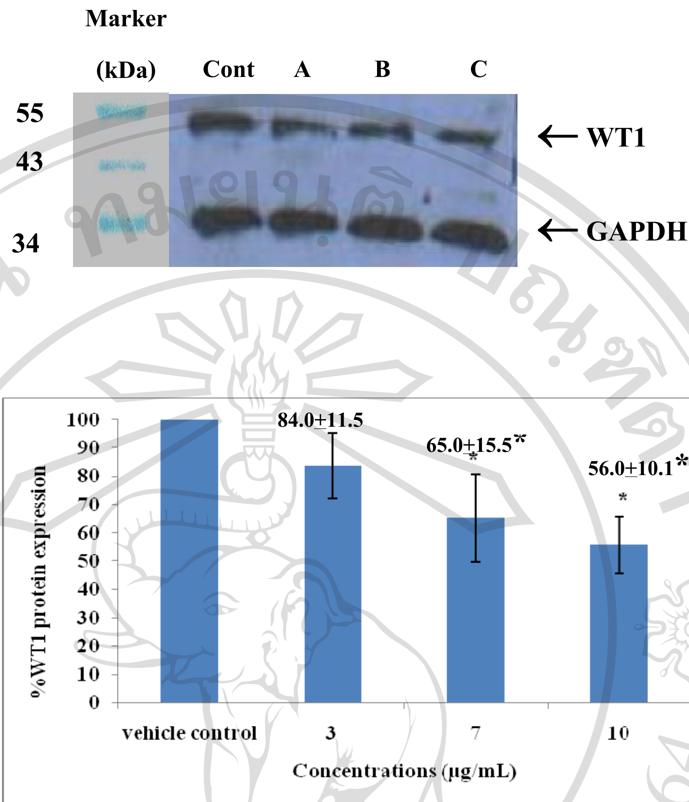

Figure 33 The WT1 levels in Molt4 cell line cultured with 3, 7, and 10 μg/mL of the ethanol fraction extract for 2 days. The WT1 and GAPDH protein levels following treatment with 0.05% DMSO and ethanol fraction at 3, 7, and 10 μg/mL were determined in Molt4 cells after 2 days by Western blot analysis. The bands (48 to 54 kDa forWT1 and 37 kDa for GAPDH) were quantified using a scan densitometer. WT1 protein levels (A) 3 μg/mL (B) 7 μg/mL and (C) 10 μg/mL. Data are the mean value±SD of three independent experiments. Asteriks (*) denote values that were significantly different from the vehicle control (p<0.05).

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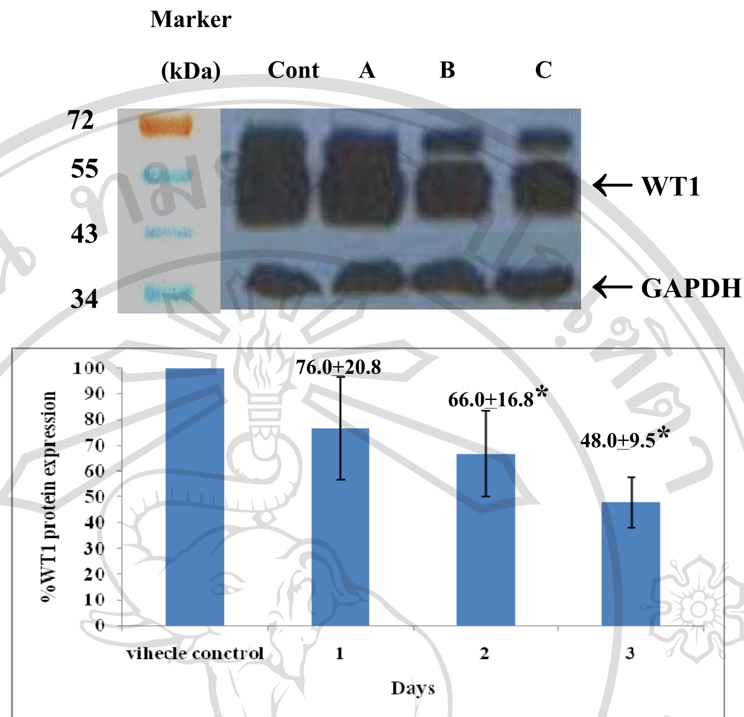

Figure 34 The WT1 levels in Molt4 cell line cultured with 7 μg/mL of the ethanol extract for 1, 2, and 3 days. The WT1 and GAPDH protein levels following treatment with 0.05% DMSO and 7 μg/mL of ethanol fraction for 1, 2, and 3 days were determined in K562 cells by Western blot analysis. The bands (48 to 54 kDa for WT1 and 37 kDa for GAPDH) were quantified using a scan densitometer. WT1 protein levels (A) 1 day, (B) 2 days, and (C) 3 days. Data are the mean value± SD of three independent experiments. Asterisk (*) denote values that were significantly different from the vehicle control (p<0.05).

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Table 14 Summary of percentage of WT1 protein levels due to concentration effect of mangosteen peel extracts when compared with vehicle control

Leukemic cell lines	**K562**			**Molt4**		
Fractions	**Ethyl acetate**			**Ethanol**		
Concentration (µg/mL)	**5**	**10**	**15**	**3**	**7**	**10**
Percentage of WT1 protien	84±9.9	58±10.9*	33±11.9*	84±11.5	65±15.5*	56±10.1*

Table 15 Summary of percentage of WT1 protein levels due to duration effect of mangosteen peel extracts when compared with vehicle control

Leukemic cell lines	**K562**			**Molt4**		
Fractions	**Ethyl acetate**			**Ethanol**		
Concentrations (µg/mL)	**10**			**7**		
Time (Days)	**1**	**2**	**3**	**1**	**2**	**3**
Percentage of WT1 protein	88±9.2*	74±5.2*	57±4.7*	76±20.8	66±16.8*	48±9.5*

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