CHAPTER III

RESULTS

3.1 Effect of pure curcumin on cell viability in K562 cells

To evaluate the activity of pure curcumin on cell viability in K562 cells, cells were cultured with various concentrations of pure curcumin (0-100 μM) for 48 h and then cell viability was measured by MTT assay as described in the Materials and Methods chapter. Pure curcumin showed a cytotoxic effect on K562 cells at an inhibitory concentration of 31.8 ± 1.1 μM (Mean ± SEM) at 50% (IC_{50} value). However, the non-cytotoxic concentration (IC_{20} value) of K562 was 16.5 ± 0.8 μM (Mean ± SEM). The result is shown in Table 9 and Figure 11. The IC_{20} value was used in this gene expression study.

3.2 WT1 overexpression reverses the effect of pure curcumin activity

To verify whether overexpression of WT1 could reverse the anti-proliferative effects of pure curcumin by over-expressing exogenous WT1 in K562 cells, four WT1 isoform constructs were transfected into K562 cells. WT1 protein overexpression was confirmed by immunoblotting of lysates collected at 48 h after 25 μM pure curcumin (IC_{50} value) treatment, as shown in Figure 12a and 12c. Next, the anti-proliferative effect of pure curcumin on K562 cells transfected with WT1 isoforms was determined by percentage of cell viability and proliferation using the MTT assay. The overexpression of all four WT1 isoforms in K562 showed resistance

to the anti-proliferative effects of pure curcumin activity when compared to transfected vector control, as shown in Figure 12b and 12d.

Table 9 Inhibitory concentration at 20% and 50% values of pure curcumin on K562 cells

Inhibitory concentration (IC) value	Pure curcumin concentration (µM)			
	1	2	3	Mean ± SEM
IC_{20}	16.7	17.9	15	16.7 ± 0.8
IC_{50}	31.8	33.5	29.8	31.8 ± 1.1

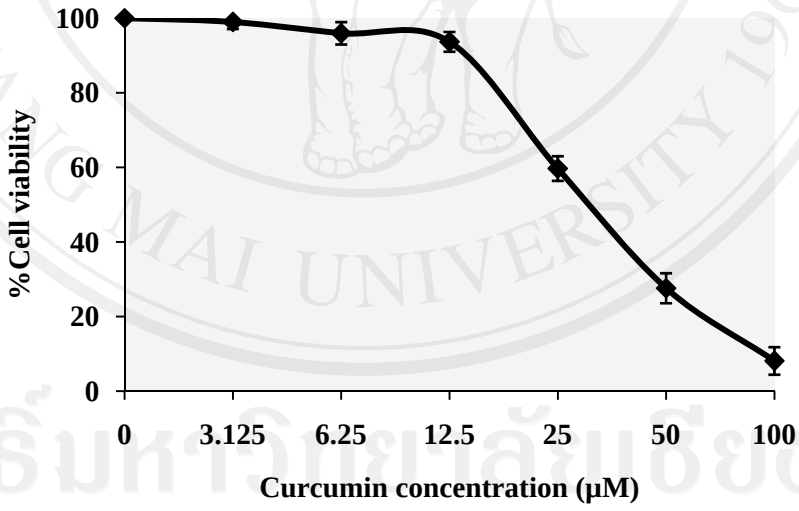
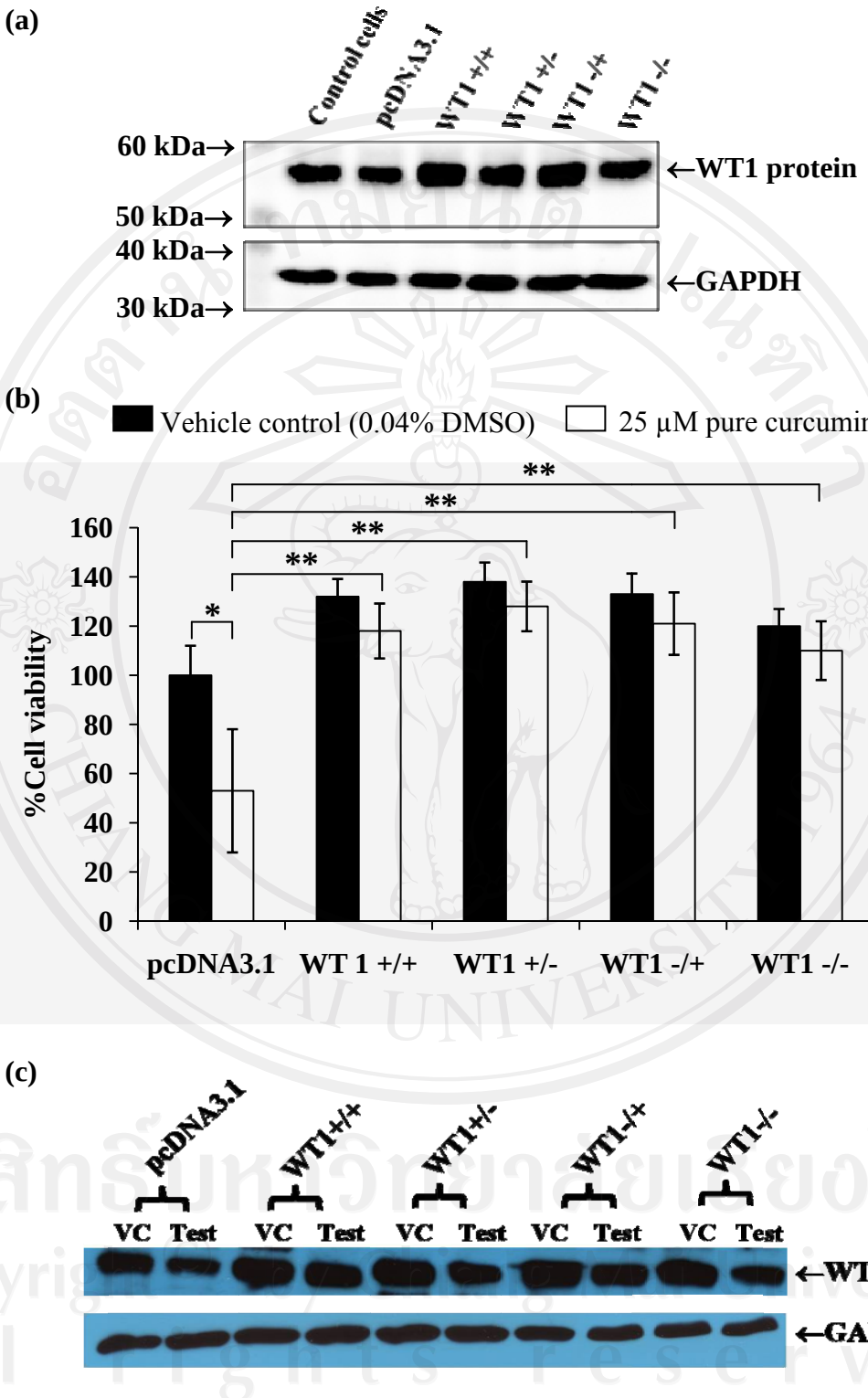


Figure 11 Cytotoxicity of pure curcumin on K562 cells. K562 cells (1 x 10^5 cells/mL) in 200 µL were grown in the presence of various concentration of pure curcumin for 48 h. Cell viabil ity was measured by MTT assay. Each point represents the mean value ± standard error of sample mean (SEM) of three independent experiments performed in triplicate.



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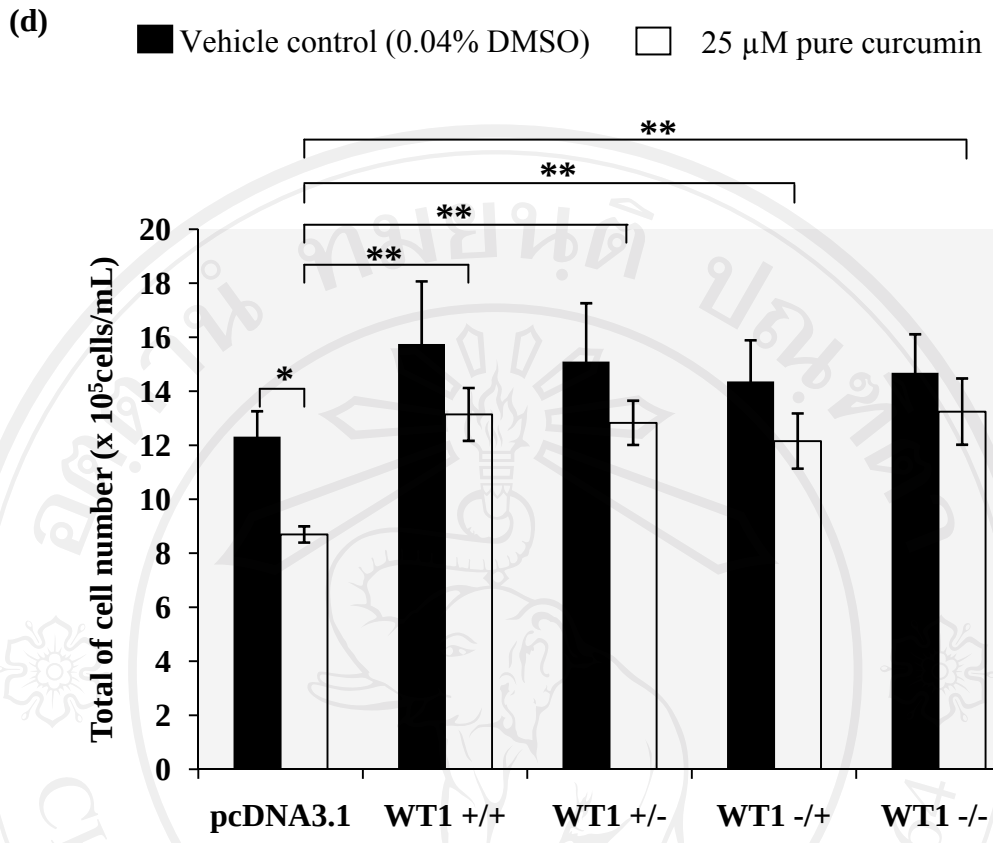


Figure 12 Effect of WT1 isoform overexpression on pure curcumin activity. K562 cells were transiently transfected with different isoforms: WT1 +/+, WT1 +/-, WT1 -/+, WT1 -/-, or the pcDNA 3.1 vector for 48 h, followed by treatment with 25 μM pure curcumin for 48 h. (a) Western blot analysis of WT1 isoform expression in the transfected K562 cells. GAPDH was used as a loading control. (b) Cell viability of K562 cells was measured with the MTT assay. (c) Western blot analysis of WT1 isoforms after treatment with 25 μM pure curcumin. (d) K562 total cell number was assayed by the trypan blue exclusion method. Data is the mean value ± SEM of three independent experiments. Single asterisks (*) denote values that were significantly different from the vehicle control (P<0.05). Double asterisks (**) denote values that were significantly different from pcDNA3.1 transfection cell experiment (P<0.05).

3.3 Effect of pure curcumin on *WT1* gene expression in K562 cells

3.3.1 Effect of pure curcumin on WT1 mRNA expression in K562 cells

3.3.1.1 Effect of various concentrations of pure curcumin on WT1 mRNA expression at 24 h of incubation time

To evaluate the activity of pure curcumin on WT1 mRNA levels in K562 cells, cells were cultured with various concentrations of pure curcumin for 24 h. The levels of WT1 mRNA expression were measured using qRT-PCR. The study consisted of three independent experiments, and mRNA was normalized by GAPDH. The levels of WT1 mRNA expression after treatment with 5, 10, and 15 μM of pure curcumin were 97, 85, and 38% respectively. The results are shown in Table 10 and Figure 13. The concentration of pure curcumin at 10 and 15 μM significantly decreased WT1 mRNA expression (P<0.05).

3.3.1.2 Effect of 15 μM pure curcumin on WT1 mRNA expression at various periods of time

To study whether pure curcumin at the concentration of 15 μM could reduce WT1 mRNA expression in K562 cells in a time-dependent manner, cells were cultured with 15 μM of pure curcumin for 0, 3, 6, 12, and 24 h. The levels of WT1 mRNA expression were measured using qRT-PCR. The experiment was performed 3 times and the mRNA was normalized by GAPDH. The levels of WT1 mRNA expression after treatment with 15 μM of pure curcumin for 0, 3, 6, 12, and 24 h were 100, 63, 49, 34, and 19% respectively (Table 11 and Figure 14). The incubation times of 3, 6, 12, and 24 h significantly decreased WT1 mRNA expression (P<0.05).

Table 10 Percentage of relative WT1 mRNA expression in K562 after treatment with various concentrations of pure curcumin

Pure curcumin treatment (μM)	**%Relative level of WT1 mRNA expression**			
	1	**2**	**3**	**Mean ± SEM**
Vehicle control (0.02% DMSO)	100	100	100	100 ± 0
5	97	99	95	97 ± 1.2
10	85	89	80	85 ± 2.6*
15	35	37	41	38 ± 1.8*

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

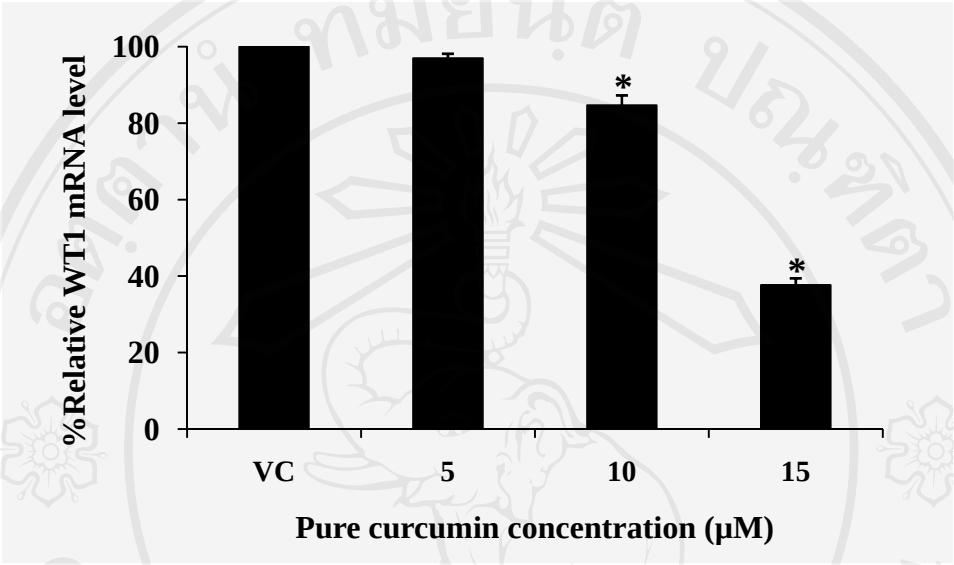


Figure 13 Effect of various concentrations of pure curcumin on WT1 mRNA expression in K562 cells. Cells were cultured with different concentrations of pure curcumin (5, 10, and 15 µM) for 24 h. The level of WT1 mRNA expression was measured by qRT-PCR. Each treatment was compared to the vehicle control (0.02% DMSO alone without the pure curcumin in the culture medium). Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

Table 11 Percentage of relative WT1 mRNA levels in K562 cells after treatment with 15 μM pure curcumin for 0, 3, 6, 12, and 24 h

Incubation time (h)	%Relative level of WT1 mRNA expression			
	1	2	3	Mean ± SEM
0	100	100	100	100 ± 0
3	67	58	63	63 ± 2.6*
6	47	51	49	49 ± 1.2*
12	24	43	34	34 ± 5.5*
24	10	28	19	19 ± 5.2*

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

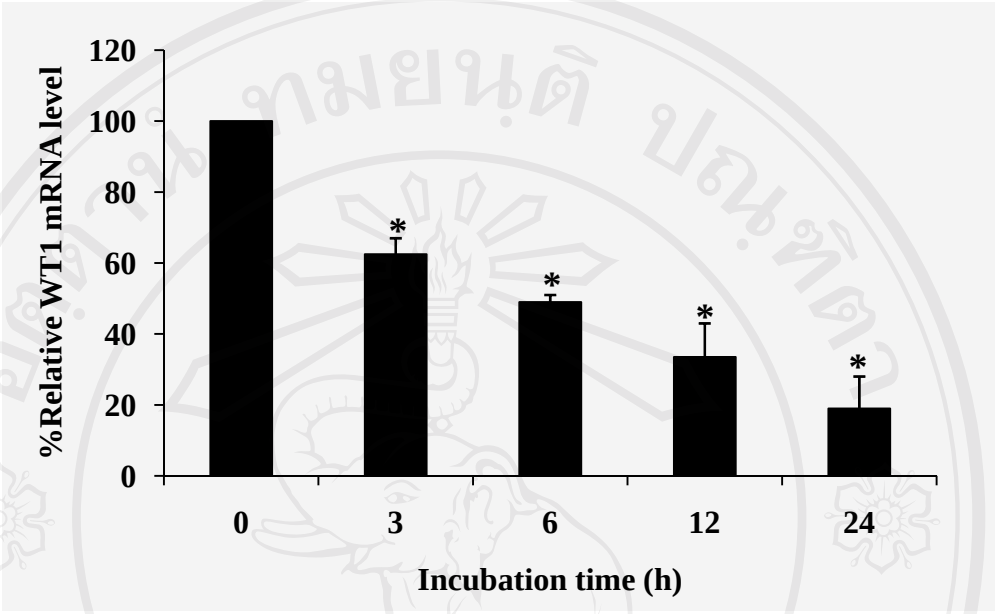


Figure 14 Effect of pure curcumin on WT1 mRNA expression of K562 cells in a time-dependent manner. Cells were cultured with different concentrations of pure curcumin (5, 10, and 15 μM) for 24 h. The level of WT1 mRNA expression was measured by qRT-PCR. Each treatment was compared to the vehicle control (0.02% DMSO alone without the pure curcumin in the culture medium). Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

3.3.2 Effect of pure curcumin on WT1 protein expression in K562 cells

3.3.2.1 Effect of various concentrations of pure curcumin on WT1 protein expression in K562 cells at 24 h of incubation time

To examine whether various concentrations of pure curcumin affect WT1 protein expression in K562 cells in a dose-dependent manner, cells were cultured with various concentrations of pure curcumin for 24 h. The levels of WT1 protein expression were measured using Western blot analysis. The study consisted of three independent experiments, and the mRNA was normalized by GAPDH. The levels of WT1 protein expression after treatment with 5, 10, and 15 μM of pure curcumin were 100, 86, 68, and 50% respectively. The results are shown in Table 12 and Figure 15. Pure curcumin concentrations of 10 and 15 μM significantly decreased WT1 protein expression (P<0.05).

3.3.2.1 Effect of 15 μM pure curcumin on WT1 protein expression in K562 cells at different time points

To test whether pure curcumin at 15 μM could reduce the level of WT1 protein in K562 cells in a time-dependent manner, cells were cultured with 15 μM of pure curcumin for 0, 3, 6, 12, and 24 h. The levels of WT1 protein expression were measured using Western blotting. The study consisted of three independent experiments, and the protein levels were normalized by GAPDH. The levels of WT1 protein expression after treatment with 15 μM of pure curcumin for 0, 3, 6, 12, and 24 h were 100, 71, 60, 53, and 44% respectively (Table 13 and Figure 16). Incubation times of 6, 12, and 24 h significantly decreased WT1 protein expression (P<0.05).

Table 12 Percentage of relative WT1 protein levels in K562 cells after treatment with various concentrations of pure curcumin

Pure curcumin treatment (μM)	**%Relative level of WT1 protein expression**			
	1	**2**	**3**	**Mean ± SEM**
Vehicle control (0.02% DMSO)	100	100	100	100 ± 0
5	79	81	99	86 ± 6.36
10	64	69	70	68 ± 1.86*
15	41	51	57	50 ± 4.67*

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

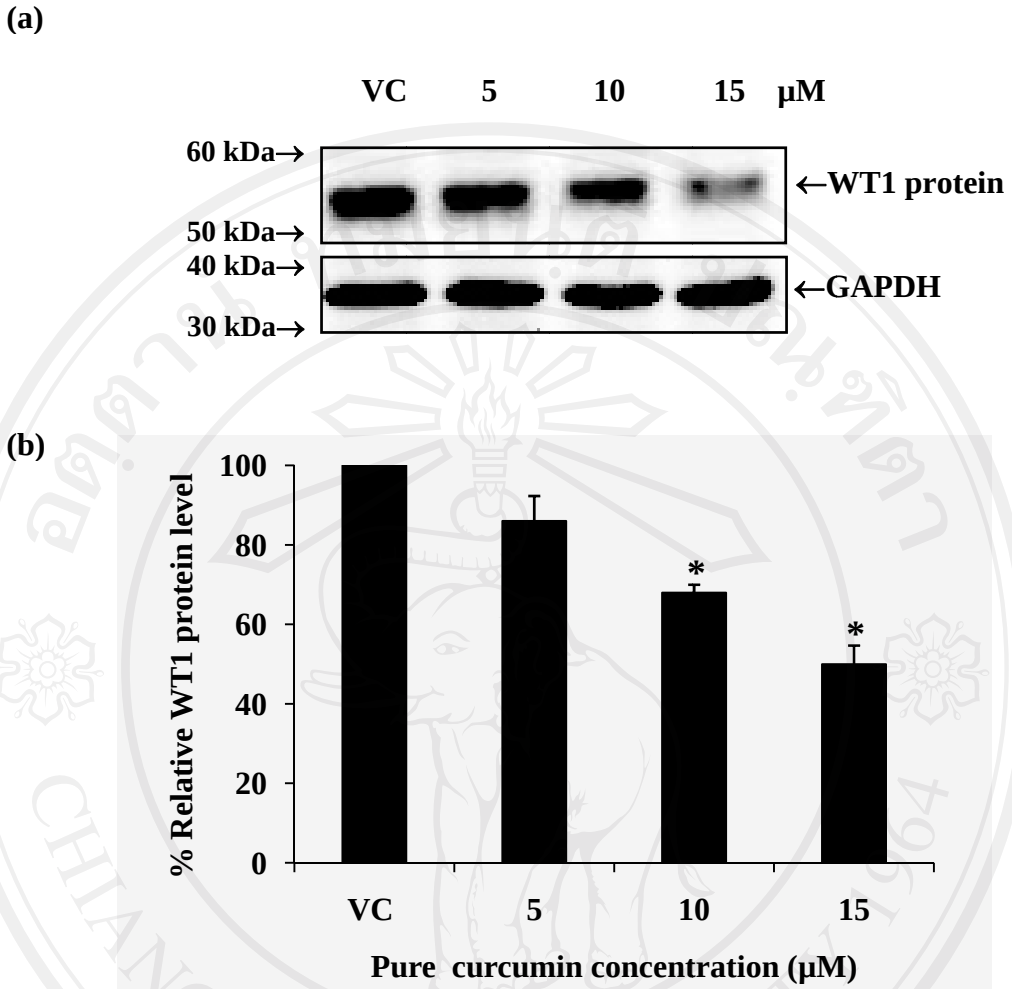


Figure 15 Effect of various concentrations of pure curcumin on WT1 protein expression in K562 cells by Western blot analysis. Cells were cultured with different concentrations of pure curcumin (5, 10, and 15 μM) for 24 h. (a) WT1 protein expression level was measured by Western blotting. GAPDH was used as loading control. (b) Densitometry was used to quantitate the protein levels and graph as the percentage of vehicle control (0.02% DMSO alone without pure curcumin in the culture medium). Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

Table 13 Percentage of relative WT1 protein levels in K562 cells after treatment with 15 μM pure curcumin at different time points

Incubation time (h)	%Relative level of WT1 protein expression			
	1	**2**	**3**	**Mean ± SEM**
0	100	100	100	100 ± 0
3	57	78	80	71 ± 7.4
6	52	54	74	60 ± 7.2*
12	51	55	52	53 ± 1.2*
24	45	47	40	44 ± 2.0*

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

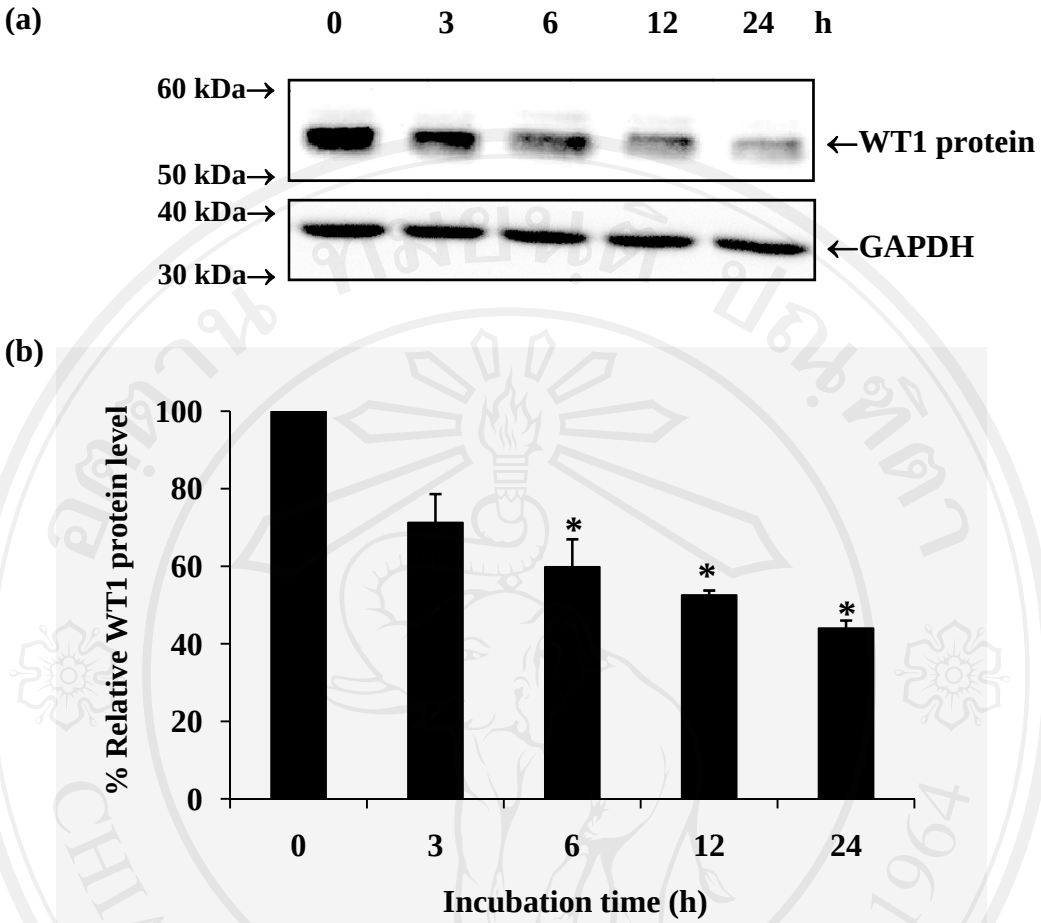


Figure 16 Effect of pure curcumin on WT1 protein expression in K562 cells by Western blot analysis. Cells were treated with 15 μM pure curcumin for different time courses. (a) The levels of WT1 protein expression were assessed by immunoblotting; GAPDH was used as loading control. (b) Densitometry was used to quantitate the protein levels and graph as the percentage of vehicle control (0.02% DMSO alone without the pure curcumin in the culture medium). Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

3.4 Effect of pure curcumin on *WT1* gene stability in K562 cells

3.4.1. Effect of pure curcumin on WT1 mRNA stability in K562 cells

To verify whether pure curcumin-reduced WT1 mRNA expression in K562 cells involved the stability of WT1 mRNA, cells were treated with actinomycin D together with or without 15 μM pure curcumin for 0, 3, and 6 h. Then total RNAs were measured by qRT-PCR. Then the WT1 mRNA levels were used to quantify mRNA half-lives. The WT1 mRNA half-lives after pure curcumin treatment were not different than those in the untreated condition. Half-lives of WT1 mRNA after 15 μM pure curcumin treatment and non-treatment were 6.7 and 6.1 h, respectively. The results are shown in Table 14 and Figure 17.

3.4.2. Effect of pure curcumin on WT1 protein stability in K562 cells

To verify whether pure curcumin-reduced WT1 protein expression in K562 cells involved the stability of WT1 protein, cells were treated with 50 μg/mL cyclohexamide in the presence or absence of 15 μM pure curcumin for 0, 3, 6, 12, and 24 h. Then WT1 protein levels were measured by Western blot analysis. GAPDH was used as loading control. Then the relative WT1 levels were used to quantify protein half-life. The levels of WT1 protein half-life after treatment were not different than those in the untreated condition. Half-lives of WT1 protein after 15 μM pure curcumin treatment and non-treatment were 6.7 and 6.1 h, respectively. The results are shown in Table 15 and Figure 18.

Table 14 Percentage of relative WT1 mRNA levels and half-lives in K562 cells after treatment with actinomycin D in the presence or absence of 15 μM pure curcumin at different time points

Pure curcumin treatment (μM)	**Incubation time (h)**	**%Relative level of WT1 mRNA**			
		1	**2**	**3**	**Mean ± SEM**
Vehicle control (0.02% DMSO)	0	100	100	100	100 ± 0
	6	24	48	51	41 ± 8.5
	12	11	22	50	28 ± 11.6
Half-life = 6.1 h					
15 μM pure curcumin	0	100	100	100	100 ± 0
	6	55	76	83	71 ± 8.4
	12	12	28	35	25 ± 6.8
Half-life = 6.7 h					

Data is the mean ± standard error of sample mean of three independent experiments.

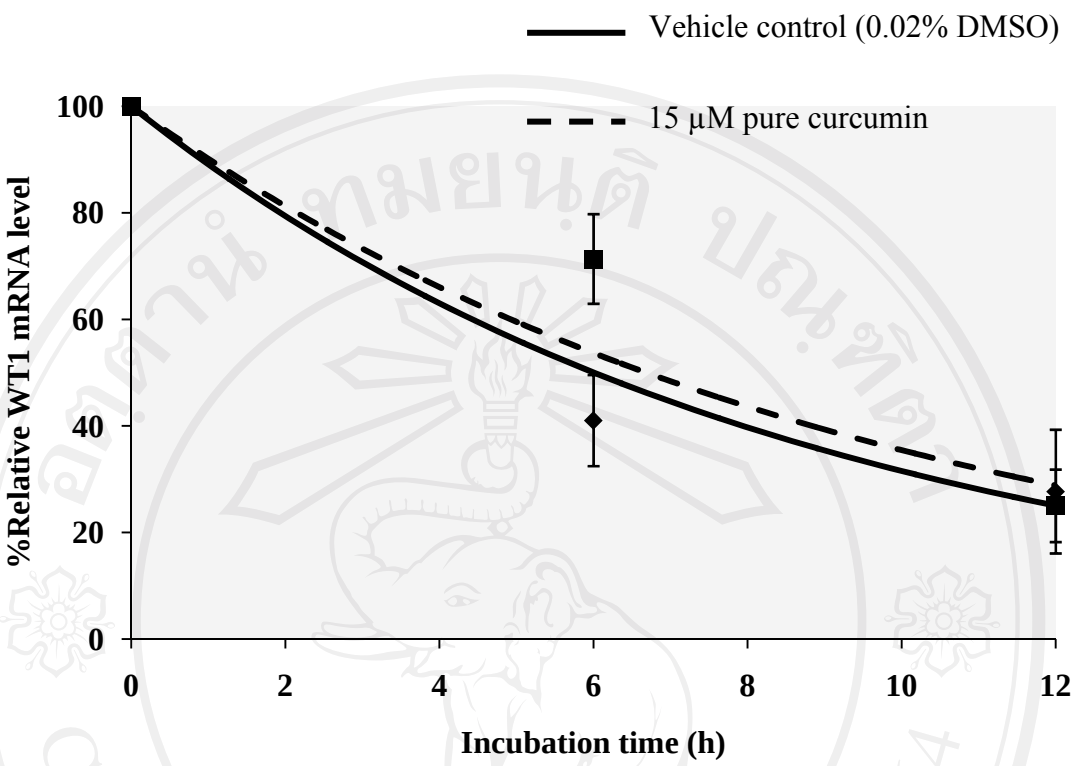


Figure 17 Effect of pure curcumin on WT1 mRNA stability using actinomycin D. To investigate the effect of pure curcumin on mRNA stability, K562 cells were treated with 4 µM actinomycin D in the presence or absence of 15 µM pure curcumin for 0, 6, and 12 h. The WT1 mRNAs were assayed by TaqMan qRT-PCR and *GAPDH* gene was used as housekeeping gene. Data is the mean value ± SEM of three independent experiments.

Table 15 Percentage of relative WT1 protein levels and half-lives in K562 cells after treatment with cycloheximide in the presence or absence of 15 μM pure curcumin at different time points

Pure curcumin treatment (μM)	**Incubation time (h)**	**%Relative level of WT1 protein**			
		1	**2**	**3**	**Mean ± SEM**
Vehicle control (0.02% DMSO)	0	100	100	100	100 ± 0
	3	91	62	50	73 ± 10.4
	6	73	44	65	64 ± 6.8
	12	44	64	30	46 ± 8.5
	24	48	40	30	30 ± 5.4
Half-life = 12.6 h					
15 μM pure curcumin	0	100	100	100	100 ± 0
	3	79	56	47	65 ±8.2
	6	71	53	31	57 ± 9.7
	12	68	39	30	46 ± 9.9
	24	48	34	26	39 ± 11.9
Half-life = 14.4 h					

Data is the mean ± standard error of sample mean of three independent experiments.

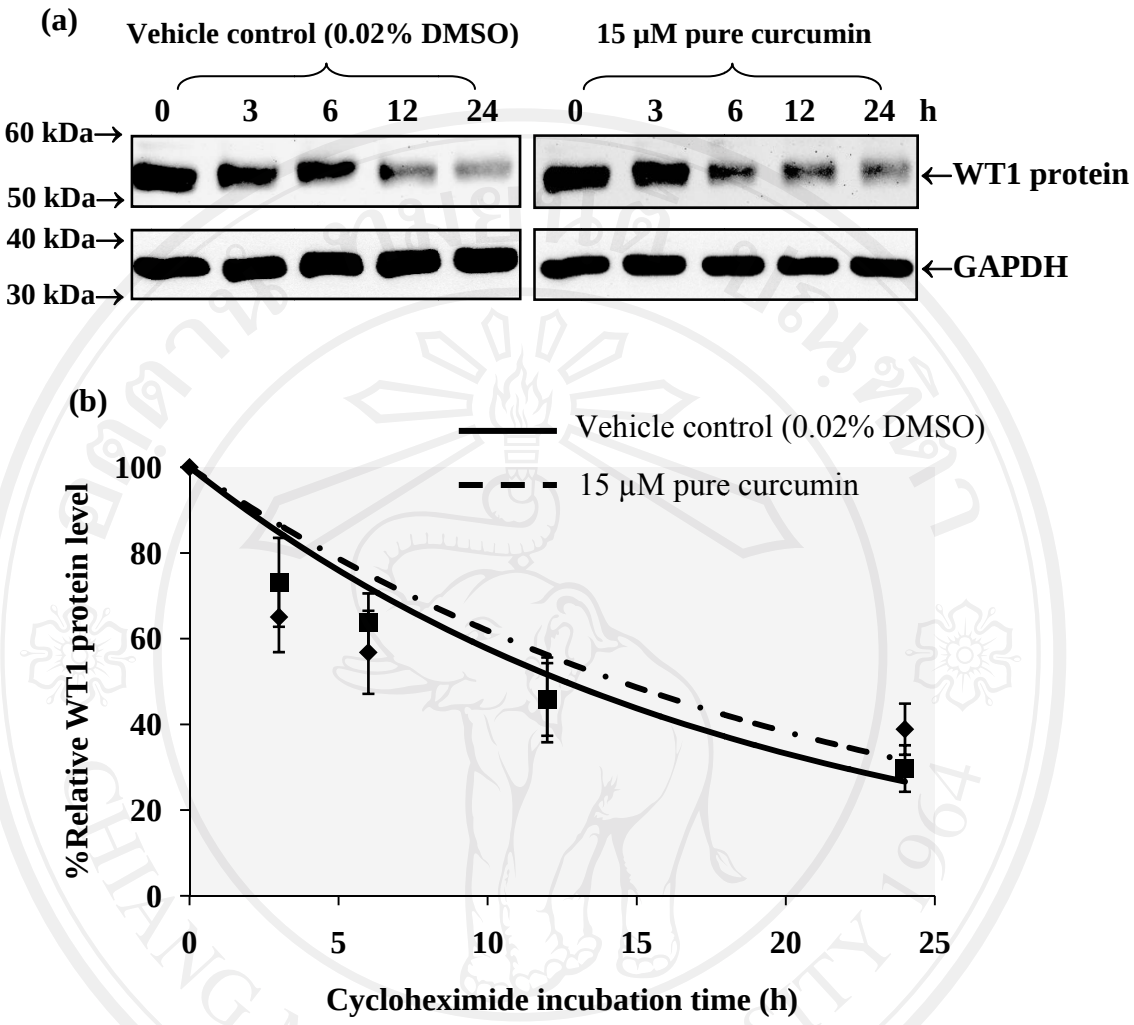


Figure 18 Effect of pure curcumin on WT1 protein stability using cycloheximide.

To investigate the effect of pure curcumin on protein stability, K562 cells were treated with 50 µg/mL cycloheximide in the presence or absence of 15 µM pure curcumin for 0, 3, 6, 12, and 24 h and assessed by (a) Western blotting, (b) protein half-life quantitation graphed as percent relative WT1 protein levels for vehicle control versus 15 µM pure curcumin treatment. Data is the mean value ± SEM of three independent experiments.

3.4.3 Effect of proteasome inhibitor on WT1 protein half-life

To confirm the half-life of the WT1 protein in the presence of cycloheximide by using proteasome inhibitors, this experiment used three proteasome inhibitors, MG132, EGCG, and lactacystin, to inhibitor proteasome activity. K562 cells were treated with pure curcumin in the presence or absence of MG132, EGCG or lactacystin to determine whether proteasome inhibition would interfere with the repressive effect of pure curcumin on WT1. The result showed the pure curcumin still decreased WT1 protein level after MG132 treatment (Table 16 and Figure 19). Moreover, this experiment also indicated that neither lactacystin nor EGCG had any effect on the ability of curcumin to downregulate WT1 (Table 17 and Figure 20), confirming the observations with MG132 experiment. Proteasome inhibitors did not show significant difference between each type.

3.5. Effect of pure curcumin on phosphorylated protein kinase in K562 cells

3.5.1 Effect of 15 μM pure curcumin on phosphorylated protein kinase in K562 cells using human phospho-kinase array

To investigate whether the decrease of *WT1* gene expression by pure curcumin involved in phosphorylated protein kinase signaling cascade, phosphorylated protein kinase array was used. Cells were treated with 15 μM of pure curcumin for 24 h. The lysate protein was added to the phosphorylated protein kinase array (Table 18 and Figure 21). Pure curcumin strongly decreased phospho-c-Jun (S63) and phospho-JNKpan (T183/Y185, T221/Y223), showing low levels compared to control cells.

Table 16 Percentage of relative WT1 protein levels in K562 cells after treatment with or without MG132 in the presence or absence of 15 μM pure curcumin at different time points

Treatment	Pure curcumin treatment (μM)	%Relative level of WT1 protein expression			
		1	2	3	Mean ± SEM
Treatment control (0.05% DMSO)	Vehicle control (0.02% DMSO)	100	100	100	100 ± 0.0
	15 μM pure curcumin	32	22	15	23 ± 5.0*
5 μM MG132	Vehicle control (0.02% DMSO)	100	100	100	100 ± 0.0
	15 μM pure curcumin	24	16	10	17 ± 4.0*

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

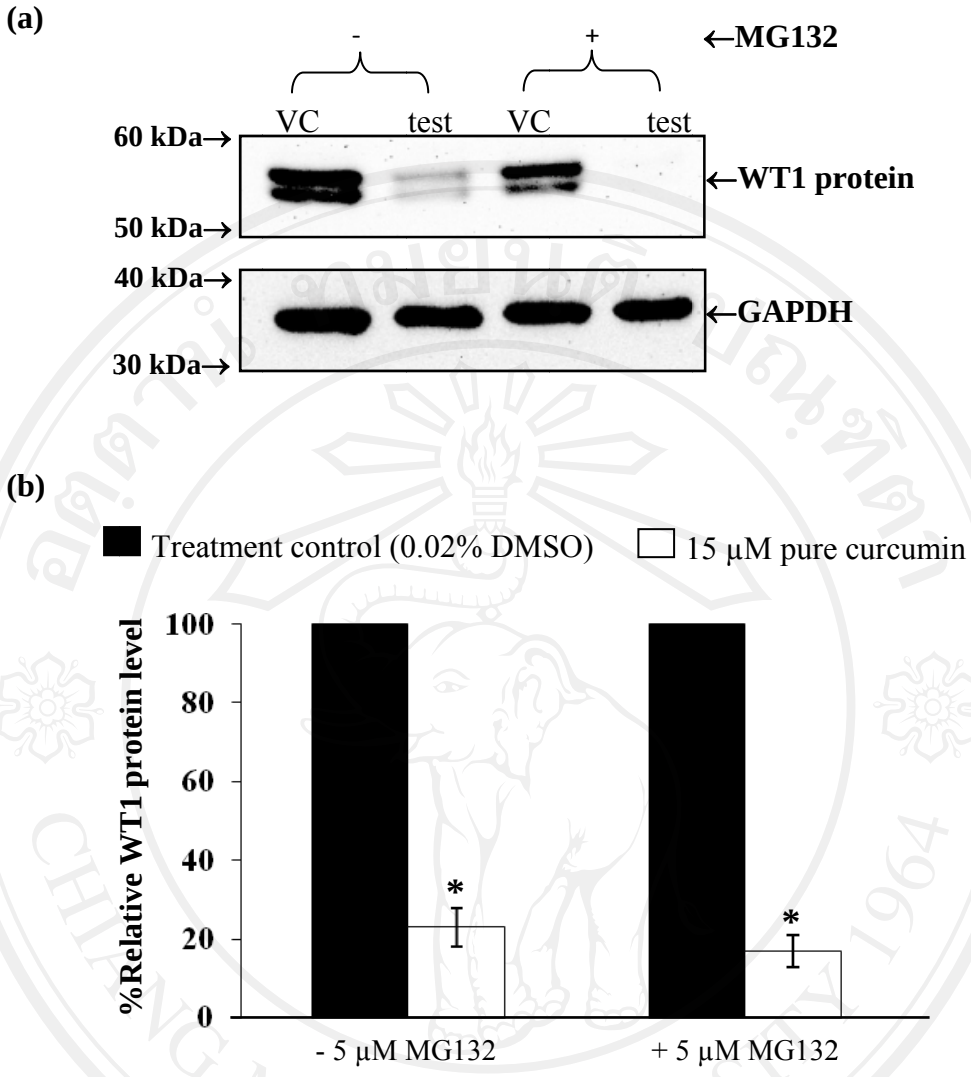


Figure 19 Effect of pure curcumin on WT1 protein stability using MG132. Proteasomal degradation was tested on relative WT1 protein levels for vehicle control versus 15 μM pure curcumin treatment in the presence or absence of 5 μM MG132 and assessed by (a) Western blotting, (b) protein quantitation graphed as percent relative WT1 protein levels for vehicle control versus 15 μM pure curcumin treatment. GAPDH was used as loading control. Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

Table 17 Percentage of relative WT1 protein levels in K562 cells after treatment with or without EGCG or lactacystin in the presence or absence of 15 μM pure curcumin

Treatment	Pure curcumin treatment (μM)	%Relative level of WT1 protein			
		1	2	3	Mean ± SEM
Treatment control (0.05% DMSO)	Vehicle control (0.02% DMSO)	100	100	100	100 ± 0.0
	15 μM pure curcumin	46	43	19	36 ± 8.4*
10 μM EGCG	Vehicle control (0.02% DMSO)	100	100	100	100 ± 0.0
	15 μM pure curcumin	42	38	24	35 ± 5.3*
Treatment control (0.05% DMSO)	Vehicle control (0.02% DMSO)	100	100	100	100 ± 0.0
10 μM Lactacystin	15 μM pure curcumin	23	32	21	26 ± 3.3*

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

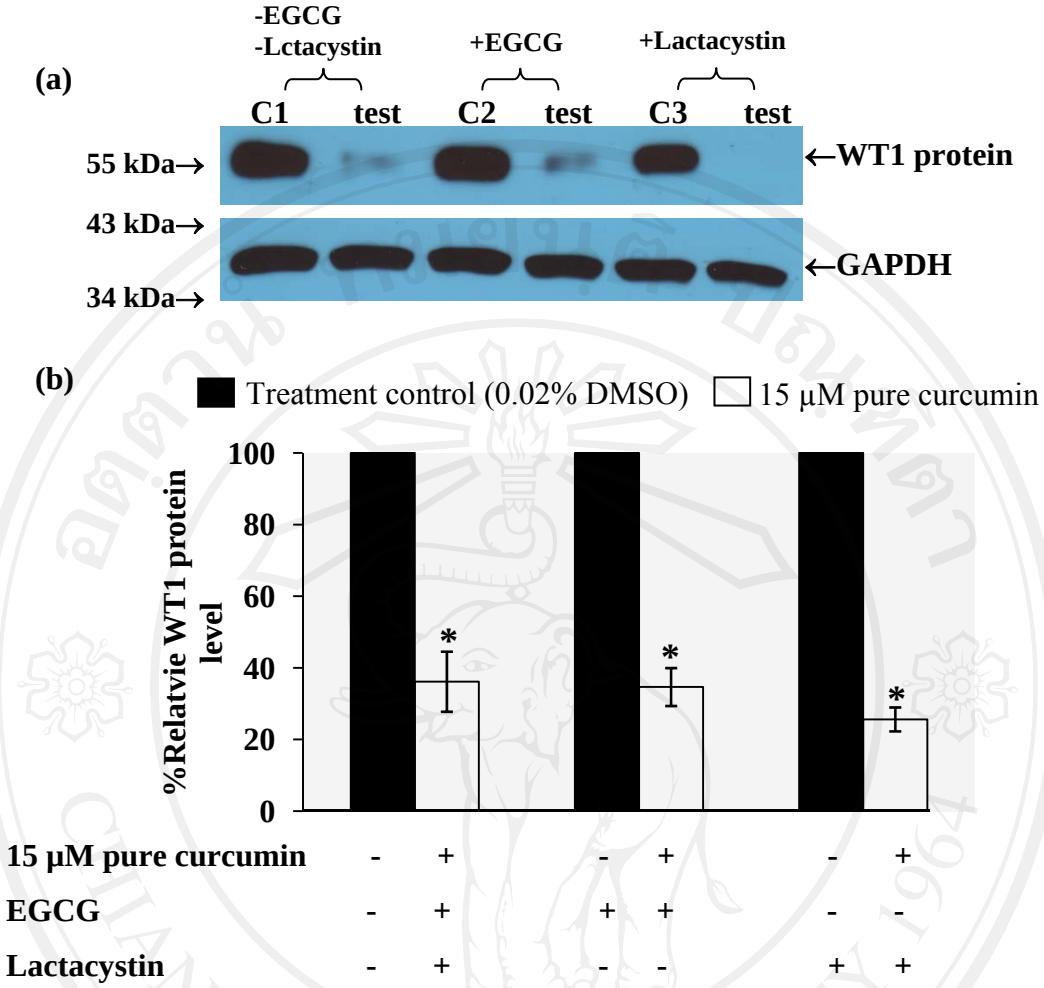


Figure 20 Effect of pure curcumin and proteasome inhibitors on proteasomal degradation. K562 cells were treated with 15 μM pure curcumin in the presence or absence of a proteasome inhibitor (10 μM EGCG or 10 μM lactacystin) and assayed by (a) Western blot analysis, (b) protein quantitation graphed as percent relative WT1 protein levels for vehicle control versus 15 μM pure curcumin treatment. GAPDH was used as loading control. C1 is treatment control of the absent proteasome inhibitor. C2 and C3 are treatment controls of EGCG and lactacystin, respectively. GAPDH was used as loading control. Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control ($P<0.05$).

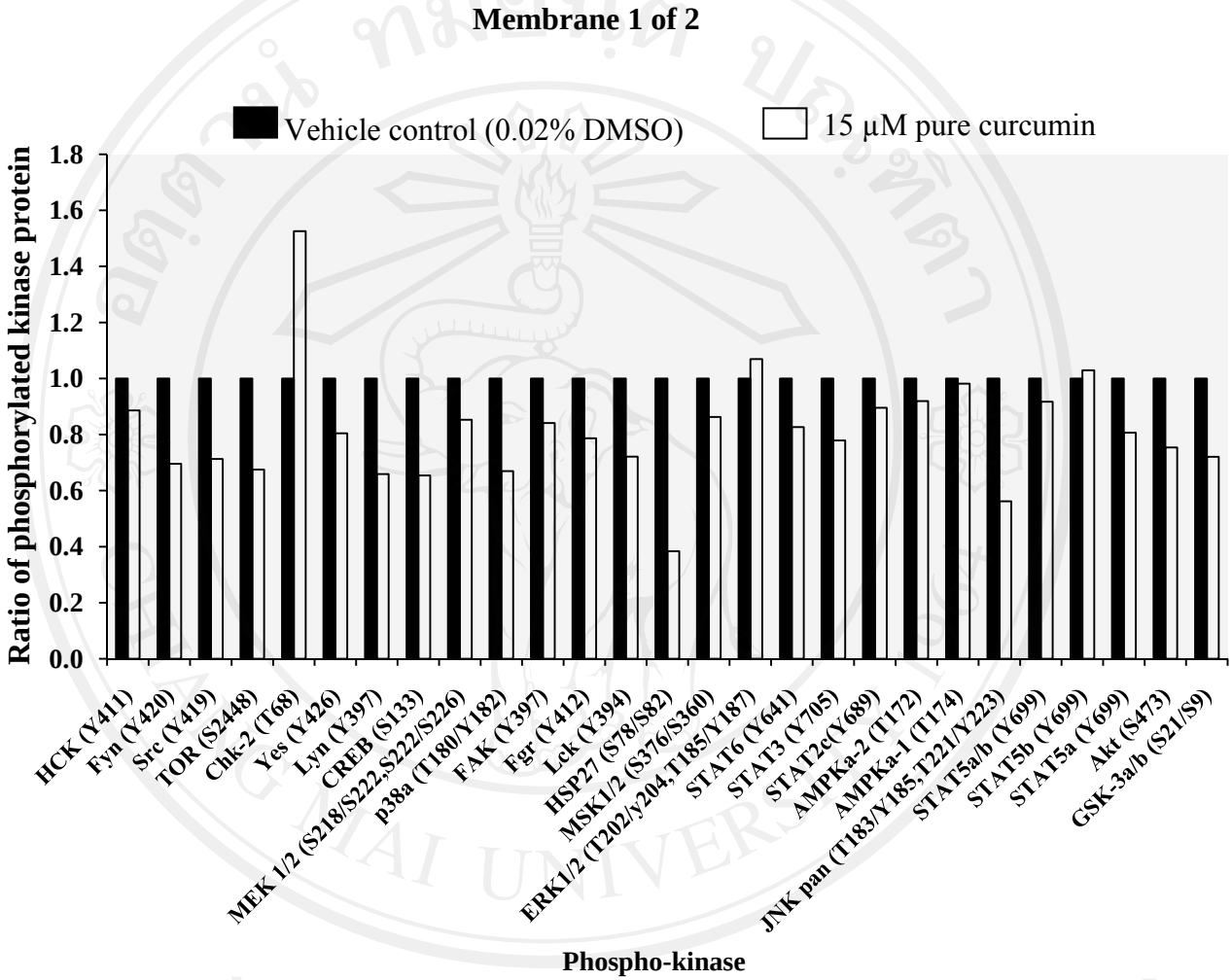
Table 18 Relative ratio of phospho-kinase protein after pure curcumin treatment in K562 cells

Phospho-kinase protein (Relative ratio level*)		
Low (0.3-0.6)	**Medium (0.7-1.0)**	**High (>1.0)**
HSP27 (S78/S82)	STAT1 (Y701)	Chk-2 (T68)
JNK pan	STAT2c(Y689)	ERK1/2
(T183/Y185,T221/Y223)	STAT3 (Y705)	(T202/y204,T185/Y187)
c-Jun (S63)	STAT4 (Y693)	
	STAT5a/b (Y699)	
	STAT5a (Y699)	
	STAT5b (Y699)	
	STAT6 (Y641)	
	AMPKα-1 (T174)	
	AMPKα-2 (T172)	
	MSK1/2 (S376/S360)	
	MEK 1/2	
	(S218/S222,S222/S226)	
	HCK (Y411)	
	FAK (Y397)	
	Fyn (Y420)	
	Yes (Y426)	
	Fgr (Y412)	
	Akt (S473)	
	Akt (T308)	

Table 18 (continued)

Low (0.3-0.6)	Medium (0.7-1.0)	High (>1.0)
	Lck (Y394)	
	Lyn (Y397)	
	GSK-3a/b (S21/S9)	
	Src (Y419)	
	TOR (S2448)	
	p38α (T180/Y182)	
	CREB (S133)	
	RSK1/2/3 (S380)	
	RSK1/2 (S221)	
	eNOS (S1177)	
	p70S6 kinase (T389)	
	p53 (S15)	
	p70S6 kinase (T421/S424)	
	p70S6 kinase (T229)	
	p53 (S46)	
	p53 (S392)	
	p27 (T157)	
	p27 (T198)	
	PLCg-1 (Y783)	
	Paxillin (Y118)	
	Pyk2 (Y402)	

Asterisks (*) denote values that were normalized with untreated cells.



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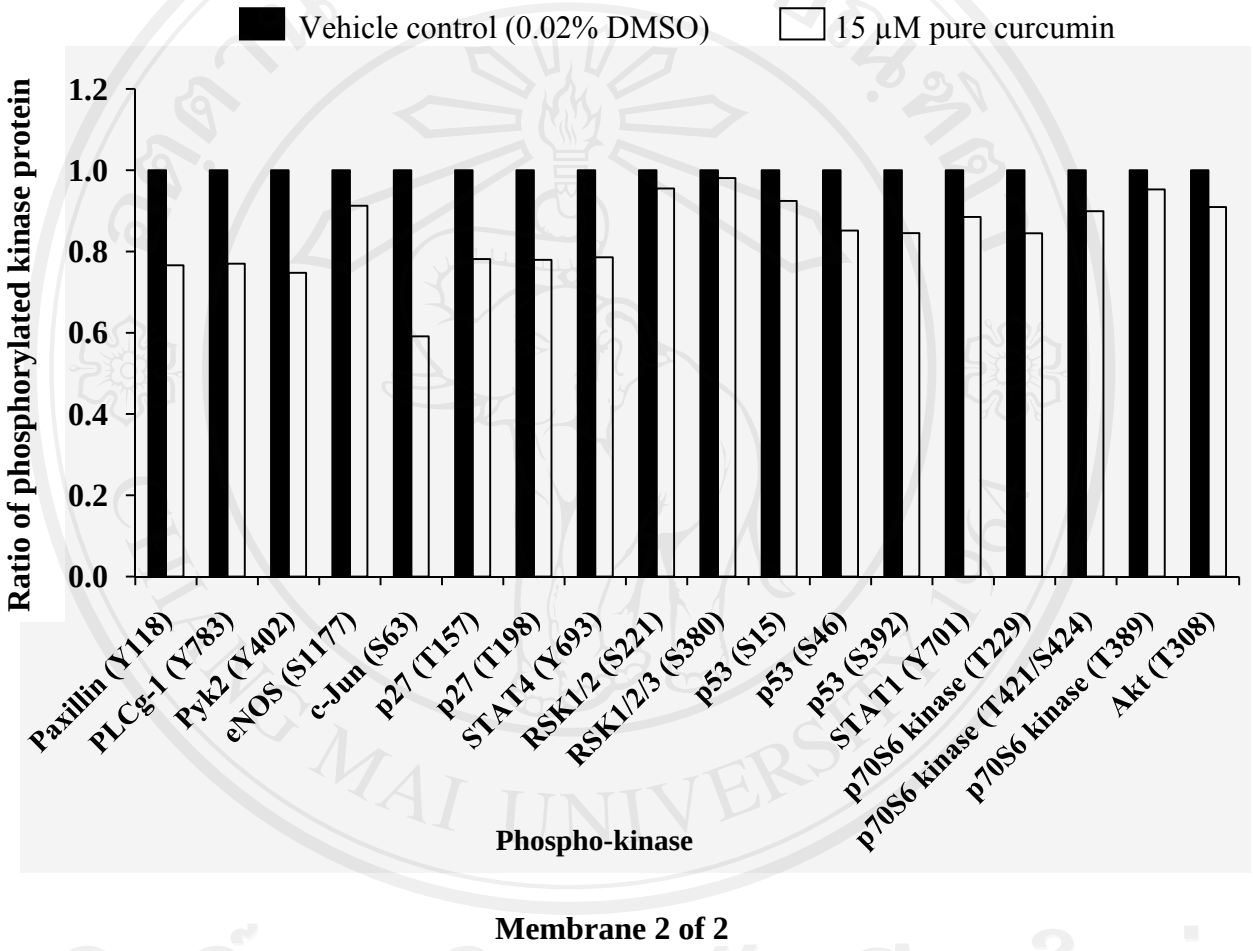


Membrane 2 of 2

Figure 21 Effect of pure curcumin on phosphorylated protein kinase in K562 cells using human phospho-kinase array kit. K562 cells were treated with 15 µM pure curcumin for 24 h. The whole protein lysate was probed into human phospho-kinase array (a) membrane 1 and (b) membrane 2.

3.5.2 Effect of 15 μM pure curcumin on protein kinase C signaling cascade in K562 cells

To examine the effect of pure curcumin on protein kinase C (PKC) isoenzyme in K562 cells, Western blot analysis was used in the study. The PKC isoenzymes utilised were PKCalpha (PKCα), PKCdelta (PKCδ), and PKCzeta (PKCζ). K562 cells were cultured with 15 μM of pure curcumin for 24 h. The membrane lysate was used to determine the level of PKCα by Western blotting. The levels of PKCδ and PKCζ were measured using cytosolic protein fraction. Caviolin 3 was used as a cell membrane protein loading control. Pure curcumin at 15 μM decreased the activated PKCα at cell membrane. The percent relative level of PKCα expression after 15 μM of pure curcumin treatment was 57%. However, it did not affect PKCδ and PKCζ. The results are shown in Table 19 and Figure 22.

3.5.3 Effect of 15 μM pure curcumin on protein kinase A cell signaling in K562 cells

To determine the effect of pure curcumin on protein kinase A (PKA) in K562 cells, cells were treated with 15 μM pure curcumin for 24 h. The PKA levels were measured by Western blotting the cytosolic protein fraction. Pure curcumin at the concentration of 15 μM did not affect PKA expression in K562 cells as compared to vehicle control cells. The percent relative level of PKA expression after 15 μM pure curcumin treatment was 99% (Table 19 and Figure 22).

Table 19 Percentage of relative expression of protein kinase C isoenzymes and protein kinase A levels in K562 cells after 15 μM pure curcumin treatment

Protein kinase antibody	**Pure curcumin treatment (μM)**	**%Relative expression level**			
		1	**2**	**3**	**Mean ± SEM**
PKCα	Vehicle control (0.02% DMSO)	100	100	100	100 ± 0
	15 μM pure curcumin	39	57	75	57 ± 10.4*
PKCδ	Vehicle control (0.02% DMSO)	100	100	100	100 ± 0
	15 μM pure curcumin	89	97	105	97 ± 4.6
PKCζ	Vehicle control (0.02% DMSO)	100	100	100	100 ± 0
	15 μM pure curcumin	77	99	95	90 ± 6.8
PKA	Vehicle control (0.02% DMSO)	100	100	100	100
	15 μM pure curcumin	107	96	95	99 ± 3.8

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

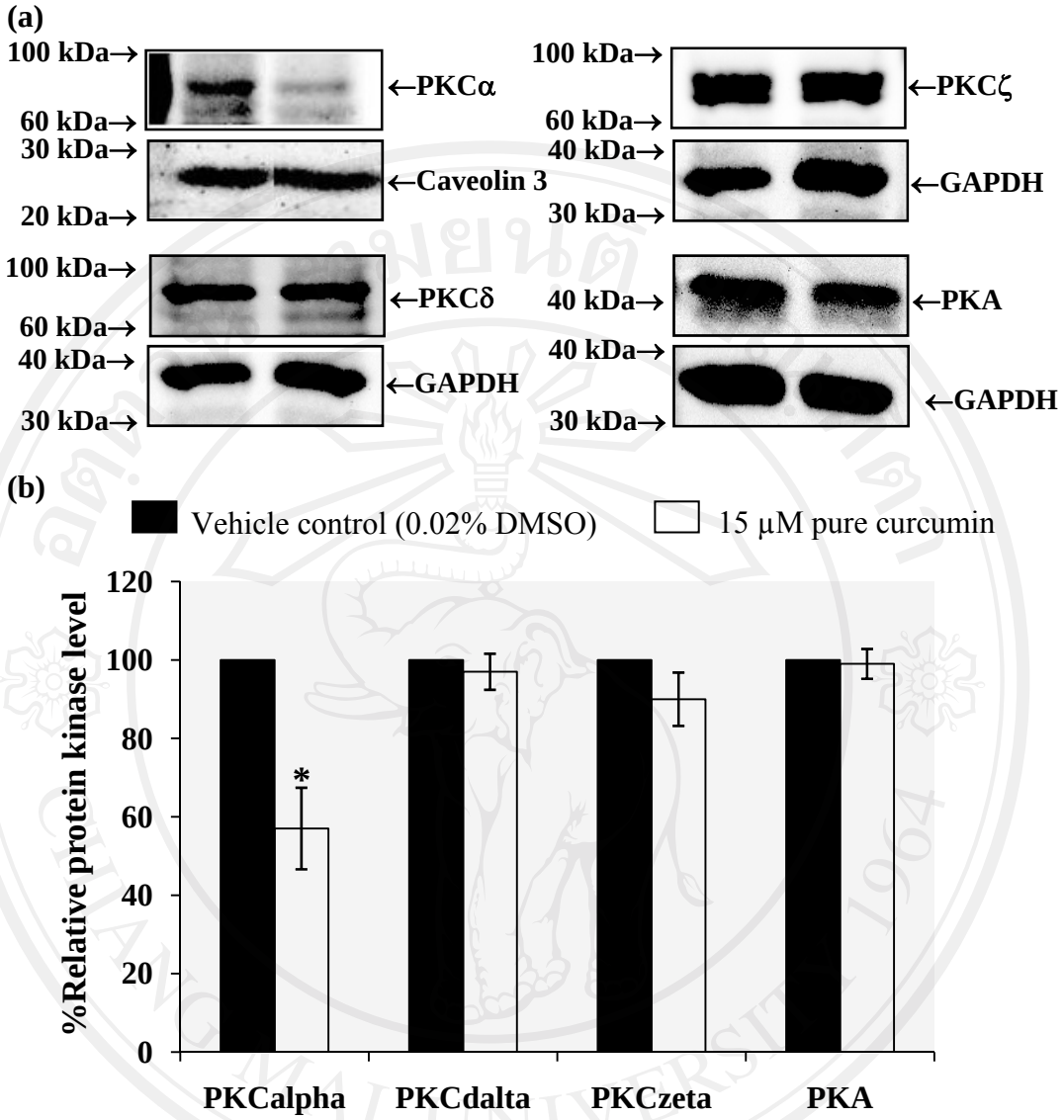


Figure 22 Effect of pure curcumin on the level of protein kinase C isoenzymes and protein kinase A expression in K562 cells. Cells were treated with 15 μM of pure curcumin for 24 h and assessed by (a) Western blotting, (b) protein quantitation graphed as percent relative protein kinase C isoenzymes and protein kinase A levels for vehicle control versus 15 μM pure curcumin treatment. Caveolin 3 and GAPDH were used as loading control. Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

3.5.4 Effect of various concentrations of pure curcumin on PKCα in K562 cells

To examine whether the decrease of activated PKCα protein in K562 cells depends on concentrations of pure curcumin, cells were cultured with 5, 10, and 15 μM of pure curcumin for 24 h. The cell membrane proteins were analysed for levels of activated PKCα by Western blot analysis. The result showed that the level of activated PKCα was decreased by pure curcumin in an independent manner (Table 20 and Figure 23). The levels of activated PKCα expression after treatment with 5, 10, and 15 μM of pure curcumin for 24 h were 80, 61, and 57%, respectively. The concentrations at 10 and 15 μM of pure curcumin significantly decreased activated PKCα expression (P<0.05).

Table 20 Percentage of relative activated PKCα protein levels in treated K562 cells with various concentrations of pure curcumin

Pure curcumin treatment (μM)	%Relative level of activated PKCα protein			
	1	2	3	Mean ± SEM
Vehicle control (0.02% DMSO)	100	100	100	100 ± 0
5	84	74	82	80 ± 3.1
10	28	71	83	61 ± 16.7*
15	39	57	75	57 ± 10.4*

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

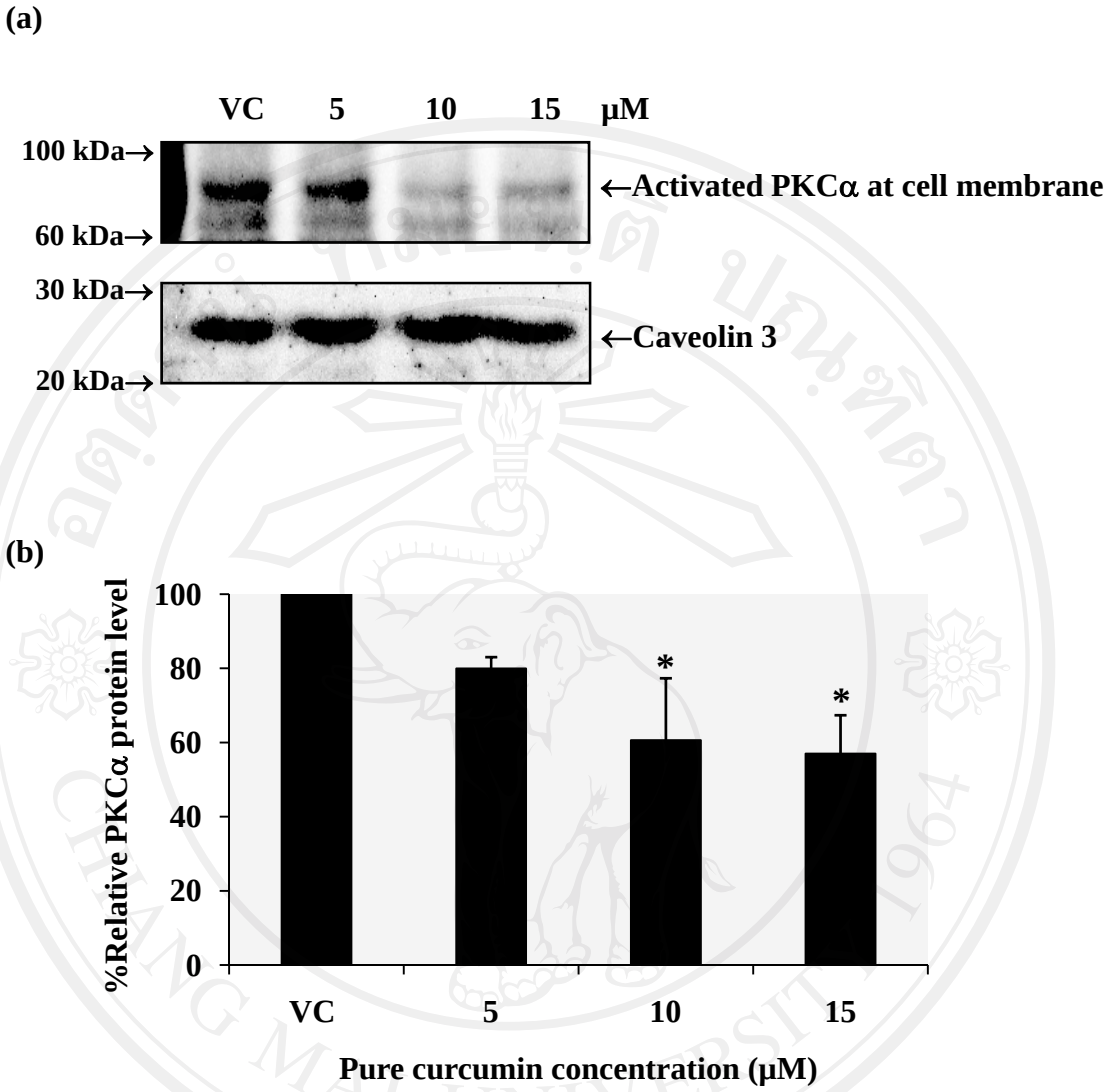


Figure 23 Effect of various concentrations of pure curcumin on the level of activated protein kinase Cα expression in the cell membranes of K562 cells. Cells were treated with 5, 10, and 15 μM of pure curcumin for 24 h. Cell membrane protein fraction was assessed by (a) Western blotting, (b) protein quantitation graphed as percent relative protein kinase Cα level. Caveolin 3 was used as loading control. Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

3.6 Effect of specific protein kinase inhibitor on *WT1* gene expression in K562 cells

3.6.1 Effect of PKC inhibitor (GF109203x) on WT1 mRNA expression in K562 cells

To confirm whether the inhibition of *WT1* gene expression by pure curcumin relates to the PKCα signaling cascade in K562 cells, 0.0084 μM PKCα inhibitor (GF109203x) was used to examine its effect on WT1 mRNA levels for 0, 1, 3, 5, and 7 h. The levels of WT1 mRNA are shown in Table 21 and Figure 24. The results indicated that the inhibition of PKCα activity decreased WT1 mRNA in a time-dependent manner after GF109203x treatment for 3 and 5 h. The level of WT1 mRNA remained decreased at 5 h when compared to control at 0 h. The WT1 mRNA expression at 3 and 5 h after treatment was 84 and 59%, respectively. Therefore, an incubation time of 5 h significantly decreased WT1 mRNA expression (P<0.05).

3.6.2 Effect of PKC inhibitor (GF109203x) on WT1 protein expression in K562 cells

To confirm whether the inhibition of WT1 protein by 15 μM pure curcumin relates to PKCα signaling cascade in K562 cells, GF109203x was used to inhibit PKC activity. Cells were treated with 0.0084 μM GF109203x for 0, 1, 3, 5, and 7 h. The levels of WT1 protein were measured by Western blot analysis. The results indicated that inhibition of PKCα activity reduced WT1 protein levels after GF109203x treatment for 3 h, and the levels of protein expression were decreased at 5 and 7 h. The WT1 protein expression was significantly decreased (P<0.05) after GF109203x treatment for 3, 5, and 7 h by 77, 45, and 84%, respectively (Table 22 and Figure 25).

Table 21 Percentage of relative WT1 mRNA levels in treated K562 cells with 0.0084 μM (IC_{50} value) GF109203x (PKCα inhibitor)

Incubation time (h)	**%Relative level of WT1 mRNA expression**			
	1	**2**	**3**	**Mean ± SEM**
0	100	100	100	100 ± 0
1	110	104	98	104 ± 3.5
3	74	90	89	84 ± 5.2
5	42	66	68	59 ± 8.3*
7	106	116	103	108 ± 3.8

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

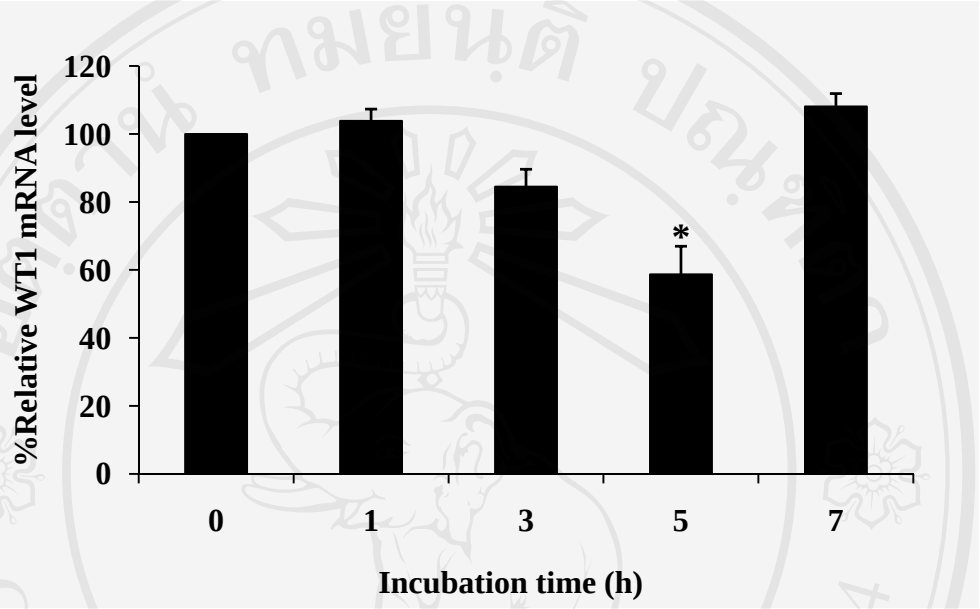


Figure 24 Effect of protein kinase C inhibitor (GF109203x) on the level of WT1 mRNA expression in K562 cells. Cells were treated with 0.0084 μM of GF109203x for 0, 1, 3, 5, and 7 h. The level of WT1 mRNA expression was measured by qRT-PCR. *GAPDH* gene was used as internal control. Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control ($P<0.05$).

Table 22 Percentage of relative WT1 protein level in treated K562 cells with 0.0084 μM (IC_{50} value) GF109203x (PKCα inhibitor)

Incubation time (h)	**%Relative level of WT1 protein expression**			**Mean ± SEM**
	1	**2**	**3**	
0	100	100	100	100 ± 0
1	75	161	139	118 ± 25.8
3	72	82	66	77 ± 4.7*
5	33	57	33	45 ± 8.0*
7	89	80	77	84 ± 3.6

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

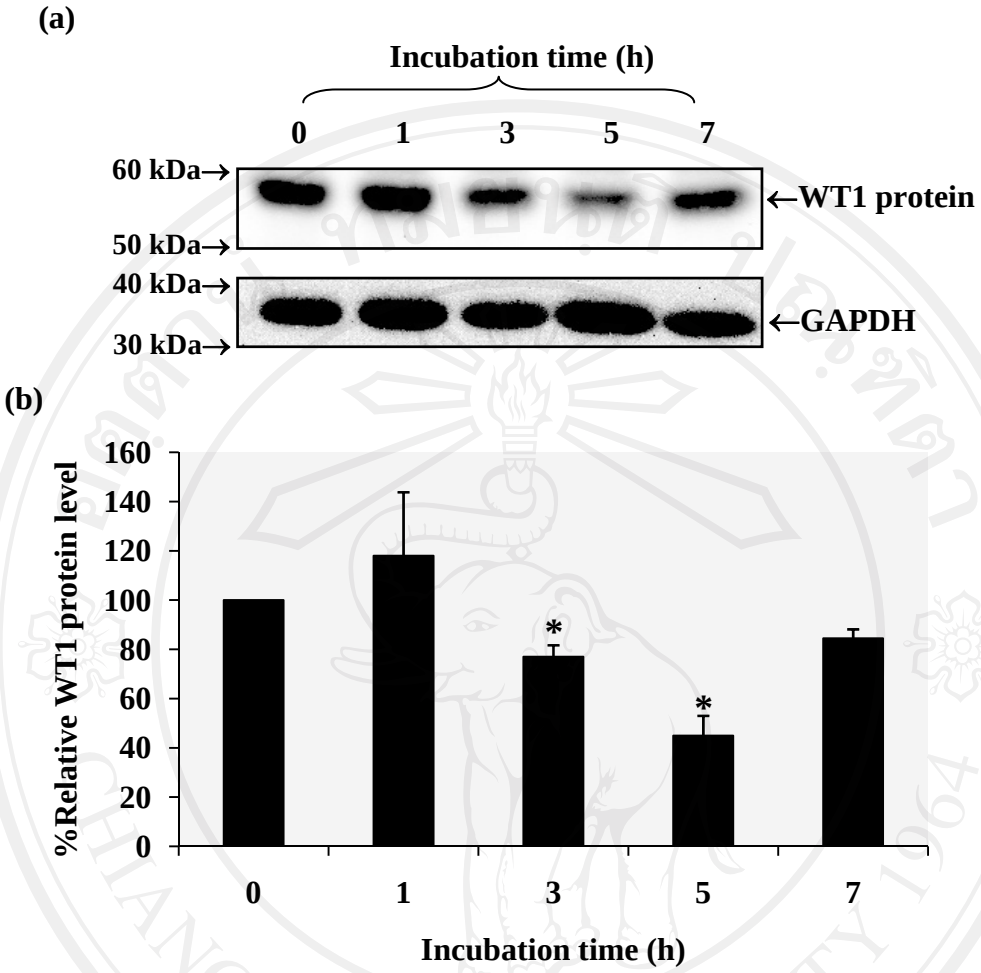


Figure 25 Effect of PKC inhibitor on the level of WT1 protein expression in K562 cells. Cells were treated with 0.0084 μM GF109203x for 0, 1, 3, 5, and 7 h. The whole protein lysate fraction was assessed by (a) Western blotting, (b) protein quantitation graphed as percent relative WT1 protein level. GAPDH was used as loading control. Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

3.7 PKCα activation up-regulates WT1 expression and reverses pure curcumin inhibition of WT1 expression in K562 cells

To investigate whether PKCα activation could induce an increase in endogenous WT1 protein levels in K562 cells, Myr.PKCα vector was used to increase the PKCα activation level in K562 cells. Cells were transfected with Mry.PKCα vector for 48 h and Western blot analysis was used to measure the WT1 protein levels. The high level of activated PKCα increased endogenous WT1 protein expression in K562 cells 1.5 times, compared to that of the pcDNA3.1 transfection condition (Table 23 and Figure 26). To investigate whether the increase of activated PKCα could reverse the pure curcumin activity to downregulate WT1 protein expression in K562 cells, cells were transfected with Myr.PKCα vector for 48 h and then treated with 15 μM pure curcumin for 24 h. The WT1 protein levels were measured by Western blot analysis. The levels of activated PKCα production are shown in Figure 26. Myr.PKCα vector stimulated up-regulation of endogenous WT1 protein expression. The WT1 protein level in transfected cells after pure curcumin treatment increased 1.7 times, compared to that in untransfected cells. The overexpression of endogenous WT1 reversed pure curcumin at 15 μM, compared to pcDNA3.1 transfected K562 cells, as shown in Table 24 and Figure 27.

Table 23 Percentage of relative WT1 protein expression level is higher after transfection with activated PKCα

Transfection	%Relative level of WT1 protein expression			
	1	**2**	**3**	**Mean ± SEM**
pcDNA3.1	100	100	100	100 ± 0
Myr.PKCα	148	164	141	151 ± 6.8*

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

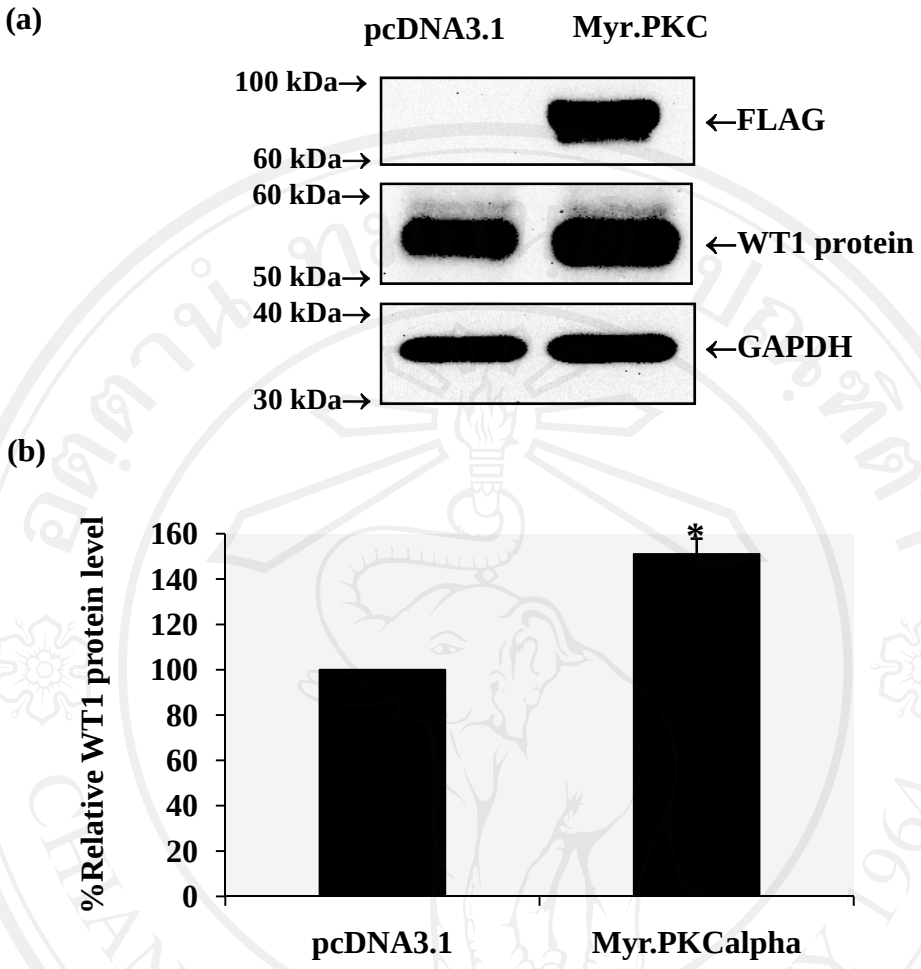


Figure 26 Activated PKCα stimulated endogenous WT1 protein expression in K562 cells. Cells were transfected with Myr.PKCα and pcDNA3.1 vectors for 48 h. The levels of endogenous WT1 protein expression were measured by (a) Western blot analysis. (b) Densitometry, which was used to quantitate the protein levels and graph as the percentage of relative WT1 protein level. GAPDH was used as loading control. Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

Table 24 Activated PKCα stimulated endogenous WT1 protein expression and reversed pure curcumin activity in K562 cells

Transfection	**Pure curcumin treatment (μM)**	**%Relative level of endogenous WT1 protein expression**			
		1	**2**	**3**	**Mean ± SEM**
pcDNA 3.1	Vehicle control (0.02% DMSO)	100	100	100	100 ± 0
	15 μM pure curcumin	75	55	81	70 ± 7.9*
Myr.PKCα	Vehicle control (0.02% DMSO)	120	145	125	130 ± 7.6
	15 μM pure curcumin	109	140	115	121 ± 9.5**

Data is the mean ± standard error of sample mean of three independent experiments.

Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

Double asterisks (**) denote values that were significantly different from pcDNA3.1 transfections (P<0.05).

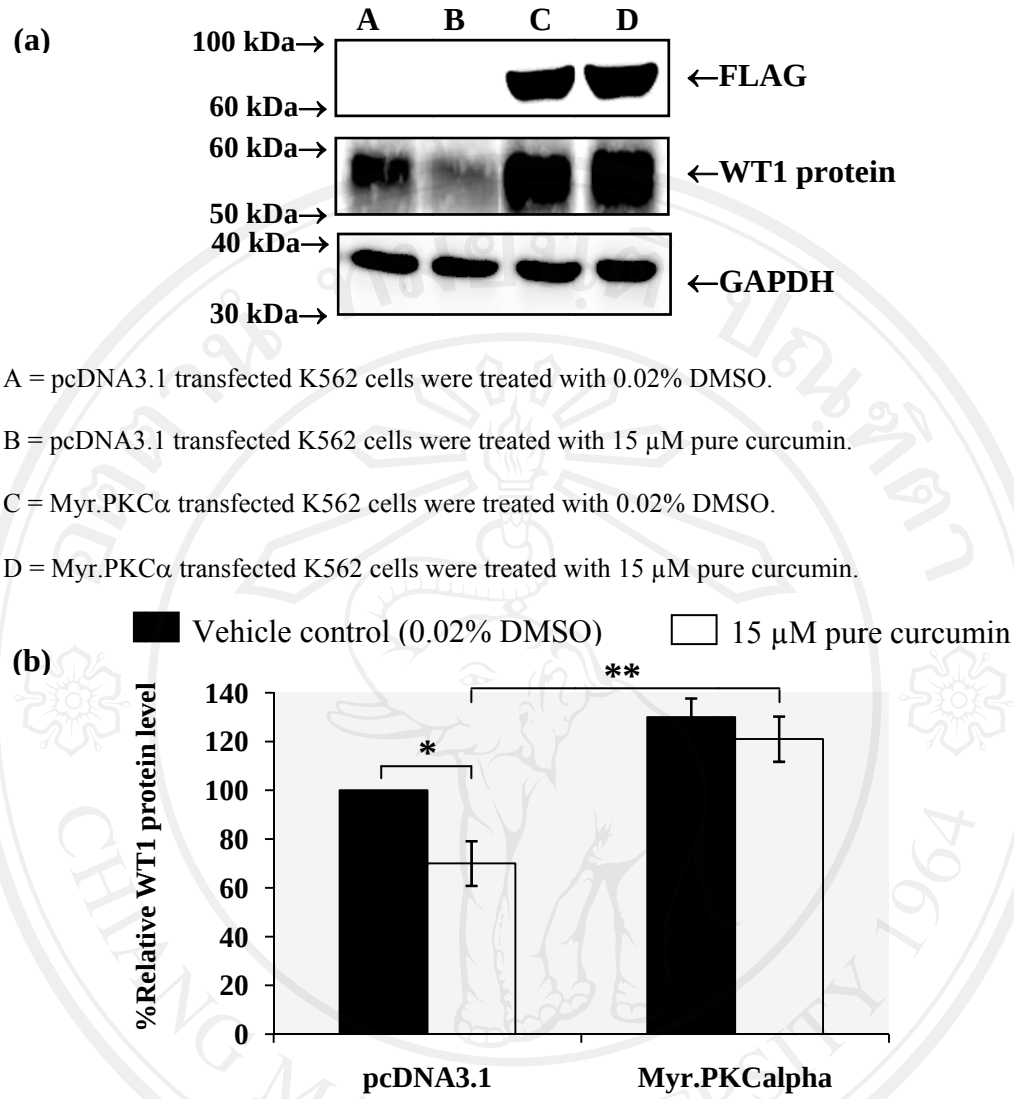


Figure 27 PKCα activation stimulated endogenous WT1 protein expression in K562 cells. Myr.PKCα or pcDNA3.1 vectors were transfected into K562 cells for 48 h then treated with or without 15 μM of pure curcumin. The whole protein lysates were assessed by (a) Western blotting, (b) densitometry, which was used to quantitate the protein levels and graph as the percentage of relative WT1 protein level. GAPDH was used as loading control. Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05). Double asterisks (**) denote values that were significantly different from pcDNA3.1 transfections (P<0.05).

3.8 Investigation upstream and downstream of PKCα signaling cascade in K562 cells

To verify upstream and downstream of PKCα signaling involved in the downregulation of WT1 expression by pure curcumin, this experiment used several protease kinase inhibitors, LY294002, U0126, and SP600125 to inhibit JNK, PI3K, and MEK signaling proteins, respectively. The signaling transduction pathway cited in this experiment is shown in Figure 28.

3.8.1. Investigation upstream of PKCα signaling cascade in K562 cells

To examine whether PI3K might be upstream of PKCα signaling cascade and could lead to the downregulation of WT1 expression by pure curcumin in K562 cells. Cells were cultured with 1.4 μM (IC_{50}) LY294002 for 0, 3, 5, and 7 h. The WT1 mRNA levels were measured by qRT-PCR. The WT1 mRNA level decreased after PI3K was inhibited for 3 and 5 h (Table 25 and Figure 29). The WT1 mRNA expression at 3 and 5 h after treatment was 70 and 37%, respectively. The incubation time of 5 h significantly decreased WT1 mRNA expression ($P<0.05$). However, the activity of LY294002 reached maximum activity to reduce WT1 mRNA expression at 3 h and sustained activity until 5 h. The results indicated that the inhibition of PI3K activity by LY294002 for 3 and 5 h leads to the decrease WT1 mRNA, similar to pure curcumin activity.

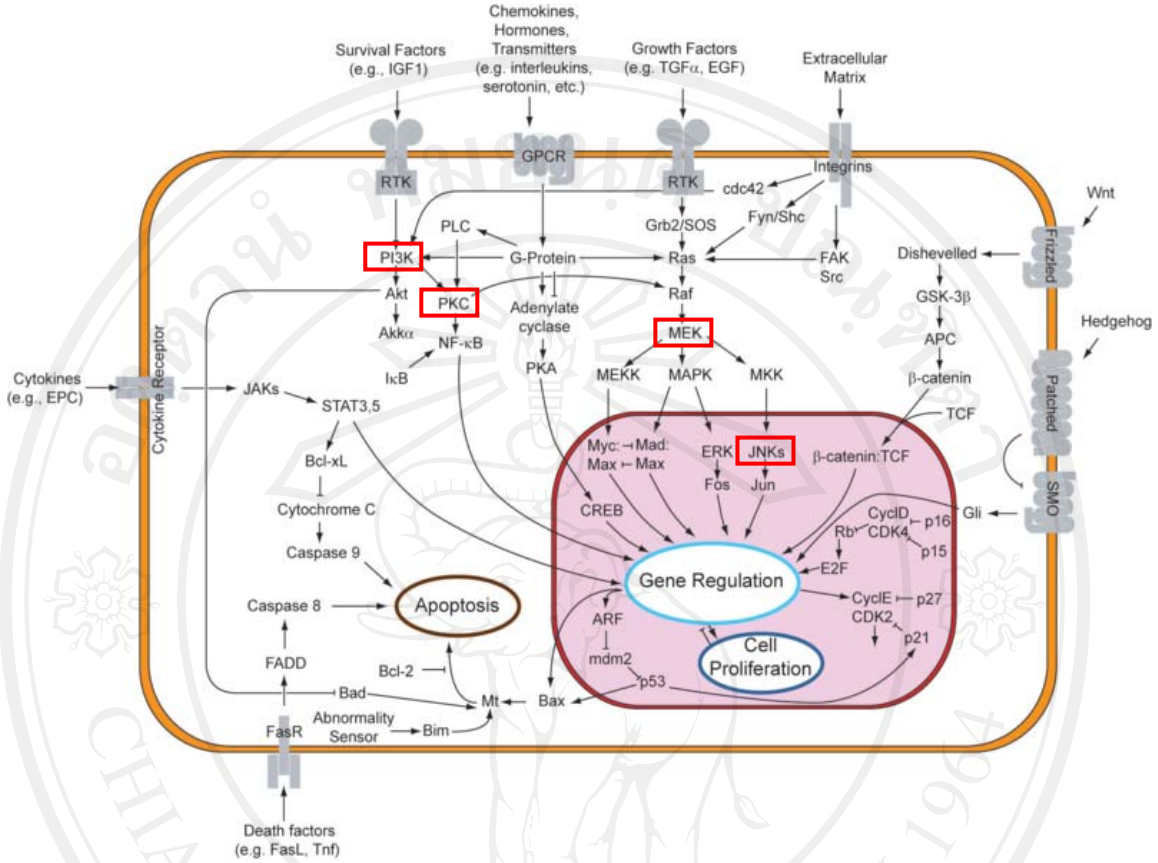


Figure 28 Overview of the signaling transduction pathway (271). The red boxes show signaling molecules that are probably involved upstream and downstream of the PKC signaling cascade to downregulate WT1 expression by pure curcumin.

Table 25 Percentage of relative WT1 mRNA level in treated K562 cells with 1.4 μM (IC_{50} value) LY294002 (PI3K inhibitor)

Incubation time (h)	**%Relative level of WT1 mRNA expression**			
	1	**2**	**3**	**Mean ± SEM**
0	100	100	100	100 ± 0.0
1	113	107	105	108 ± 2.0
3	89	54	68	70 ± 9.5*
5	42	28	40	37 ± 4.4*
7	113	106	104	108 ± 2.3

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

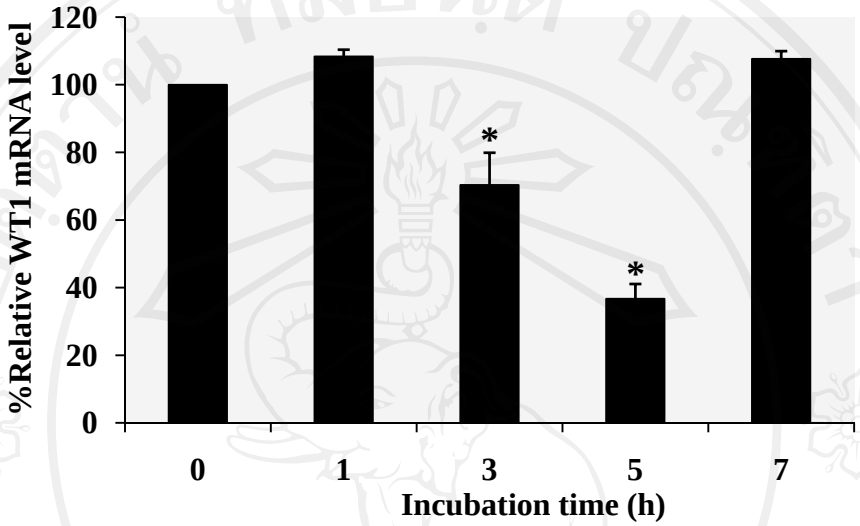


Figure 29 Effect of PI3K inhibitor (LY294002) on the level of WT1 mRNA expression in K562 cells. Cells were treated with 1.4 μM of LY294002 for 0, 1, 3, 5, and 7 h. The level of WT1 mRNA expression was measured by qRT-PCR. *GAPDH* gene was used as internal control. Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control ($P<0.05$).

3.8.2. Investigation downstream of PKCα signaling cascade in K562 cells

To verify whether MEK and JNK signaling cascade might be downstream of PKCα and could lead to decreased WT1 expression by pure curcumin in K562 cells. In this experiment, U0126 was used to inhibit MEK activity signaling cascade and then the level of WT1 mRNA expression in K562 cells was measured. Cells were cultured with 0.53 μM (IC_{50} value) U0126 (MEK inhibitor) for 0, 1, 3, 5, and 7 h. The levels of WT1 mRNA expression were measured by qRT-PCR. The results showed that the inhibition of MEK activity led to decreased WT1 mRNA expression. WT1 mRNA levels significantly decreased after MEK activity was inhibited for 3 and 5 h (Table 26 and Figure 30). However, the activity of U0126 reached maximum activity to reduce WT1 mRNA expression at 3 h and activity was sustained at 5 h.

Furthermore, in this experiment SP600125 was also used to inhibit JNK activity and the levels of WT1 mRNA in K562 cells were assayed. Cells were treated with 40 nM (IC_{50} value) SP600125 (JNK inhibitor) for 0, 1, 3, 5, and 7 h. WT1 mRNA levels were measured by qRT-PCR. The WT1 mRNA level decreased after JNK activity was inhibited (Table 27 and Figure 31). These results indicated that the inhibition of JNK activity by SP600125 for 3 and 5 h leads to decreased WT1 mRNA. The WT1 mRNA expression at 5 h after treatment was 65% - a significant decrease when compared to 0 h of incubation time ($P<0.05$).

These results showed that the decrease of WT1 mRNA levels by three specific kinase inhibitors exhibits the same pattern. The WT1 mRNA was significantly decreased at 3 and 5 h of incubation time. Then, this study also investigated whether the effect of 0.014% DMSO interfered with the reduction of WT1 mRNA expression by a specific kinase inhibitor in K562 cells. Cells were cultured with 0.014% DMSO

for 0, 1, 3, 5, and 7 h. The results indicated that the decrease of WT1 mRNA expression by specific kinase inhibitor was not affected by the 0.014% DMSO (Figure 32).

Table 26 Percentage of relative WT1 mRNA levels in treated K562 cells with 0.53 μM (IC_{50} value) U0126 (MEK inhibitor)

Incubation time (h)	**%Relative level of WT1 mRNA expression**			
	1	**2**	**3**	**Mean ± SEM**
0	100	100	100	100 ± 0.0
1	114	123	99	112 ± 7.0
3	84	60	74	73 ± 7.0*
5	30	23	28	27 ± 2.1*
7	123	118	101	114 ± 6.7

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

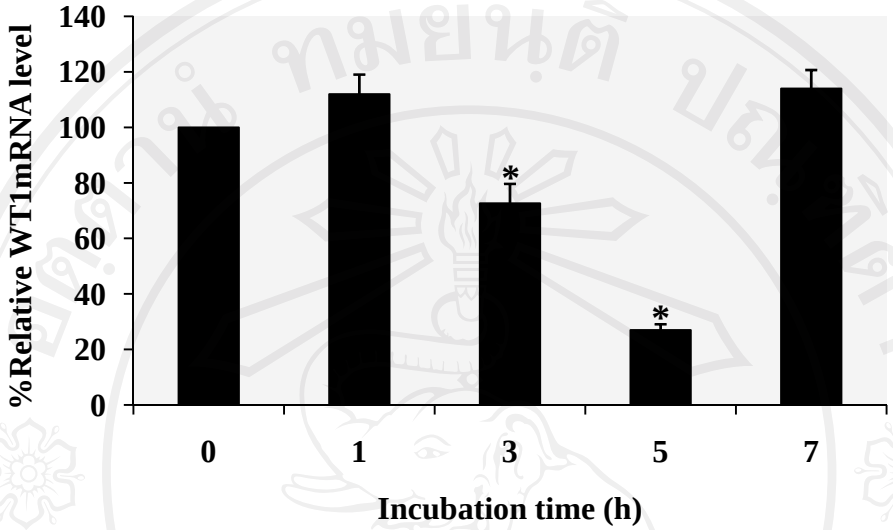


Figure 30 Effect of MEK inhibitor (U0126) on the level of WT1 mRNA expression in K562 cells. Cells were treated with 0.53 μM of U0126 for 0, 1, 3, 5, and 7 h. The level of WT1 mRNA expression was measured by qRT-PCR. *GAPDH* gene was used as internal control. Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control ($P<0.05$).

Table 27 Percentage of relative WT1 mRNA levels in treated K562 cells with 40 nM (IC_{50} value) SP600125 (JNK inhibitor)

Incubation time (h)	**%Relative level of WT1 mRNA expression**			
	1	**2**	**3**	**Mean ± SEM**
0	100	100	100	100 ± 0.0
1	99	108	103	103 ± 3.7
3	101	85	90	92 ± 6.5
5	58	69	67	65 ± 4.5*
7	105	110	104	106 ± 2.0

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

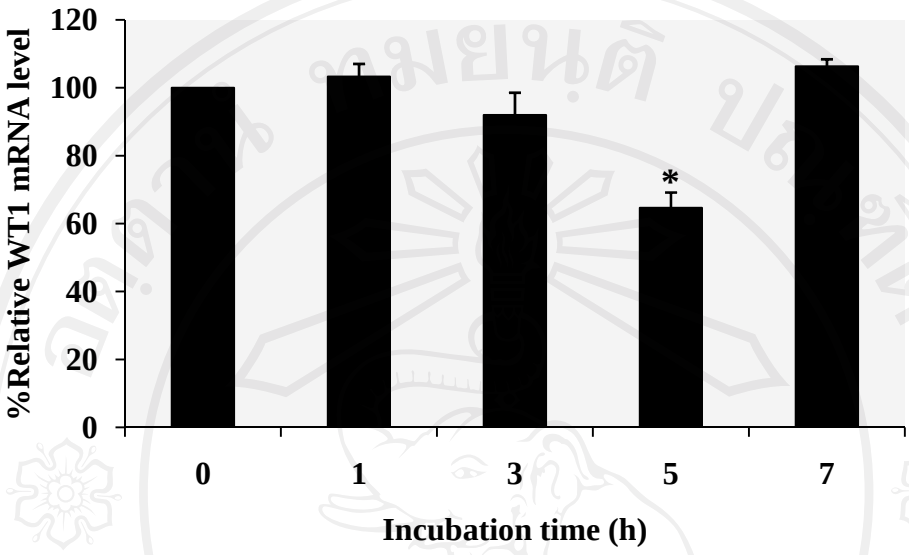


Figure 31 Effect of SP600125 (JNK inhibitor) on the level of WT1 mRNA expression in K562 cells. Cells were treated with 40 nM of SP600125 for 0, 1, 3, 5, and 7 h. The level of WT1 mRNA expression was measured by qRT-PCR. *GAPDH* gene was used as internal control. Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control ($P<0.05$).

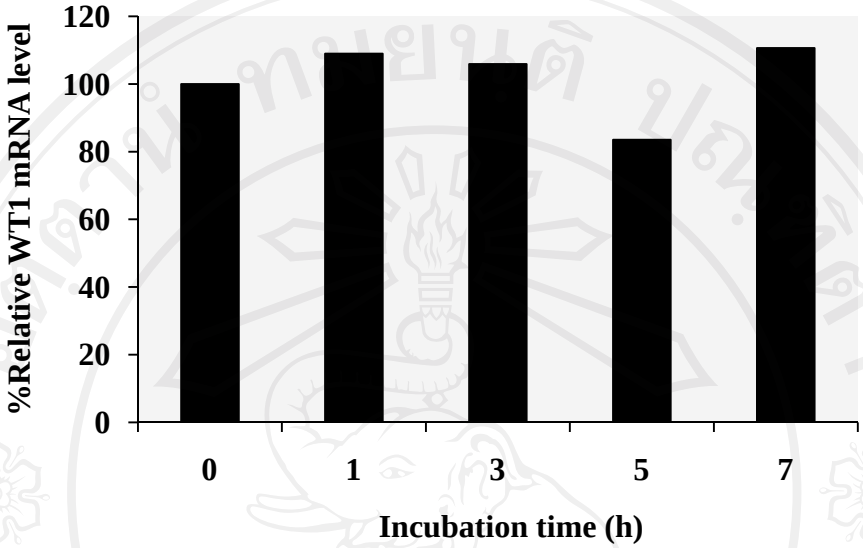


Figure 32 Effect of 0.014% DMSO on the level of WT1 mRNA expression in K562 cells. Cells were treated with 0.014% DMSO for 0, 1, 3, 5, and 7 h. The level of WT1 mRNA expression was measured by qRT-PCR. *GAPDH* gene was used as internal control.

3.9 Effect of pure curcumin on WT1 proximal promoter in K562 cells

Chromatin immunoprecipitation (ChIP) assay was used to verify whether the decrease of *WT1* gene expression by 15 μM of pure curcumin for 24 h involved in attenuation of protein-DNA binding complex and if WT1 transcription factor binds to WT1 proximal promoter. Previous results have shown that 15 μM pure curcumin decreased both WT1 mRNA and protein levels with the independent degradation pathway. However, the downregulation of *WT1* gene expression is involved in the PKCα activation and JNK signaling pathway. Then the effect of pure curcumin on the binding between WT1 transcription and WT1 proximal promoter in K562 cells was further investigated. The WT1 proximal promoter used in this study was obtained from GeneBank (accession No. U77682) and it was based on a DNA sequence published by Fraizer *et al.* (1994) (270). The DNA sequence is shown in Figure 33. K562 cells were treated with 15 μM pure curcumin or 0.02% DMSO for 24 h. The activity of WT1 transcription factor and WT1 proximal promoter binding was studied using ChIP assay. The result showed that 15 μM pure curcumin distinctly inhibited WT1 transcription factor binding activity to WT1 proximal promoter, compared to vehicle control by standard PCR (Figure 34). Furthermore, this experiment also showed the relation between the decrease of WT1 protein levels and the occupancy of the WT1 promoter (Figure 35).

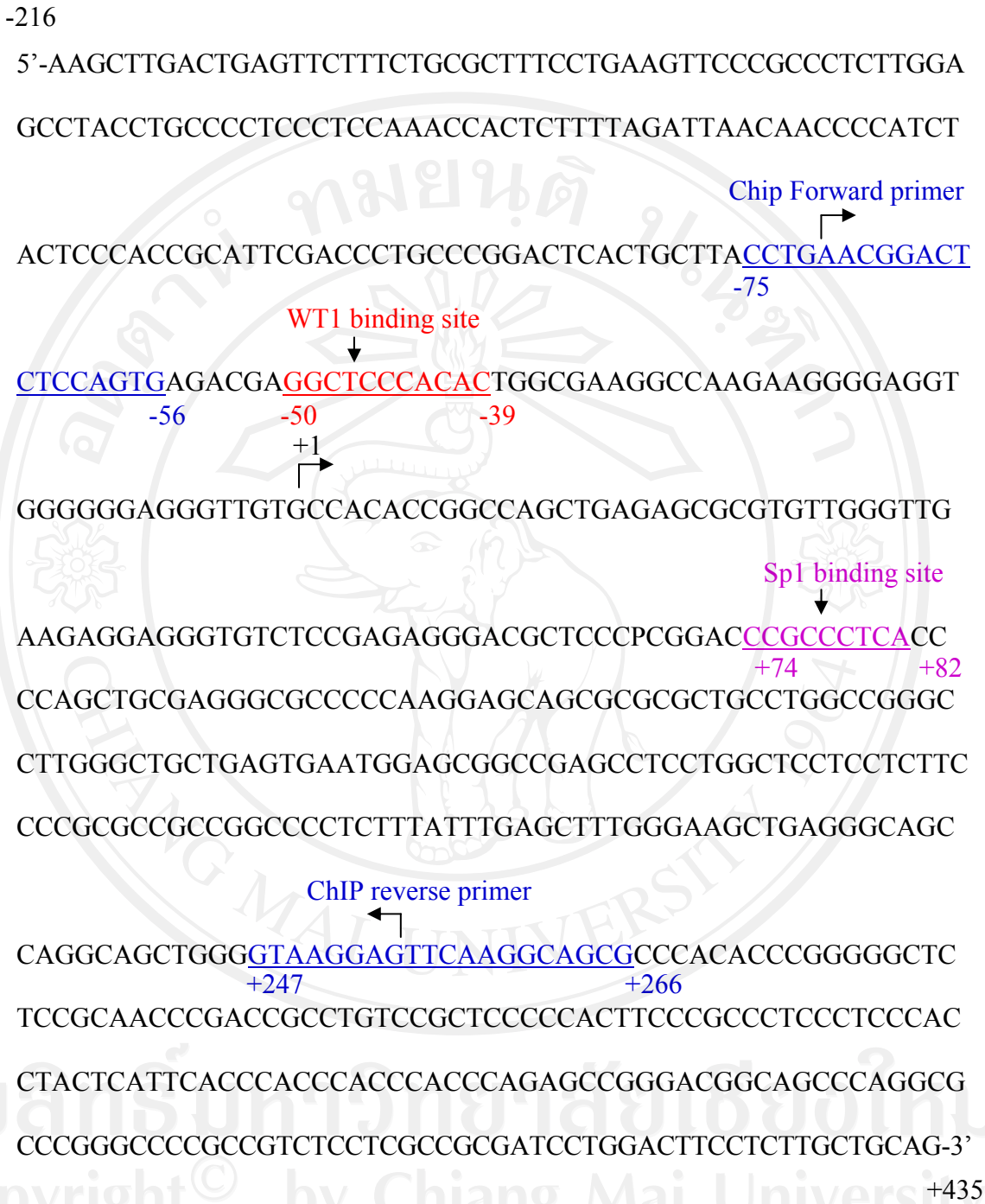


Figure 33 Sequence of the WT1 proximal promoter region. Potential transcription factor-binding sites are underlined. The major start sites are marked as +1. They were used for primer design (270).

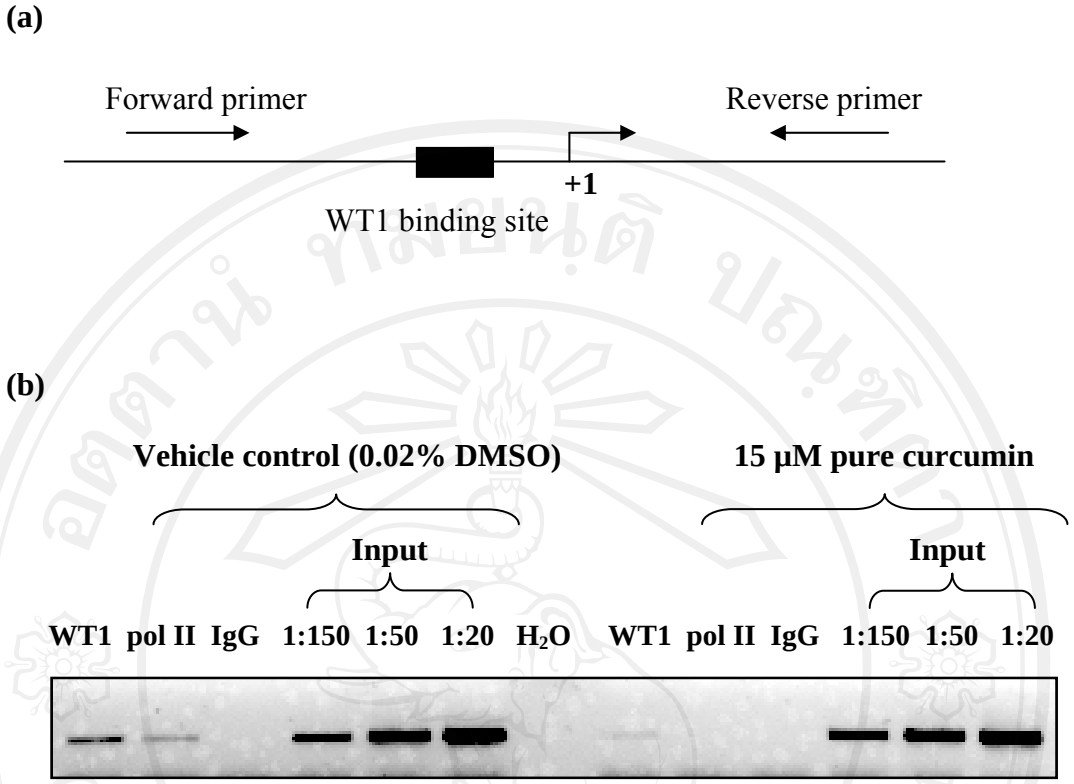


Figure 34 Pure curcumin treatment of attenuated WT1 protein transcription factor binding to the proximal *WT1* gene promoter. (a) Schematic showing the *WT1* gene proximal promoter and location of WT1 binding site and ChIP primers relative to the furthest upstream transcription start site (TSS). K562 cells were treated with 15 µM pure curcumin or 0.02% DMSO for 24 h. (b) Chromatin immunoprecipitated lysates for WT1, pol II (positive control for promoter activation), IgG (negative control), or 1:150, 1:50, and 1:50 dilution of input were assayed by standard PCR for binding to the WT1 proximal promoter.

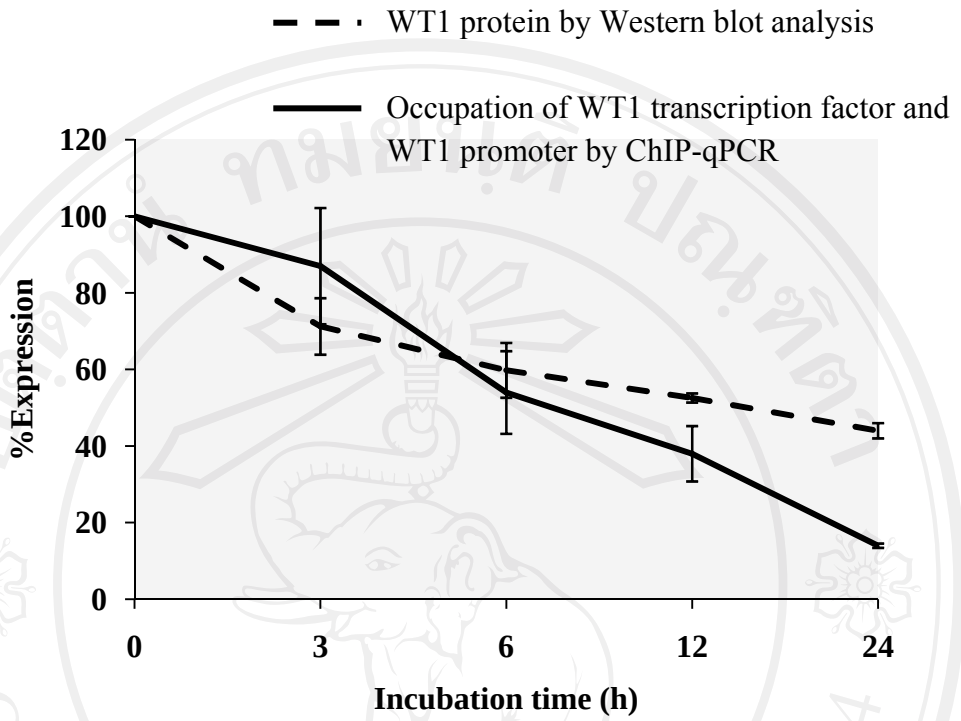


Figure 35 Time-dependent comparison of WT1 protein levels versus promoter occupancy by qPCR. K562 cells were treated with 15 μM pure curcumin for 0, 3, 6, 12, and 24 h. Then WT1 protein levels were measured by Western blot analysis. Promoter occupancy was detected by ChIP assay and qPCR. Data is the mean value ± SEM of three independent experiments.

3.10 Identification of the effect of pure curcumin on Sp1 transcription factor binding *WT1* gene promoter in K562 cells

The aim of this experiment was to identify the activity of pure curcumin specifically affecting WT1 binding protein interaction with WT1 proximal promoter but not other binding proteins.

3.10.1 Effect of various concentrations of pure curcumin on Sp1 protein in K562 cells

To verify the effect of pure curcumin at different concentrations on Sp1 protein levels in K562 cells using Western blot analysis, cells were cultured with 5, 10, and 15 μM of pure curcumin for 24 h and the levels of Sp1 and WT1 protein were determined by Western blotting. The result clearly showed that pure curcumin did not significantly decrease the Sp1 protein expression (P>0.05). However, pure curcumin still down-regulated WT1 protein expression in a dose-dependent manner (Table 28 and Figure 36).

3.10.2 Effect of pure curcumin on binding of Sp1 protein and *WT1* gene promoter in K562 cells

To identify the effect of pure curcumin on DNA protein binding between Sp1 protein and WT1 proximal promoter in K562 cells by ChIP assay, cells were cultured with 15 μM pure curcumin or 0.02% DMSO for 24 h. The chromatin fragments were precipitated with anti-WT1 (C-19) rabbit polyclonal antibody (sc192) or anti-Sp1 rabbit polyclonal antibody. The result showed that 15 μM pure curcumin did not affect the binding of Sp1 and *WT1* gene proximal promoter (Table 29 and Figure 37).

However, pure curcumin still prevented interaction between WT1 transcription factor and WT1 proximal promoter (Table 29 and Figure 37).

Table 28 Percentage of relative WT1 and Sp1 protein levels after treatment with various concentrations of pure curcumin for 24 h

Target protein	Pure curcumin treatment (μM)	%Relative protein level expression			
		1	2	3	Mean ± SEM
WT1	Vehicle control (0.02% DMSO)	100	100	100	100 ± 0
	5	79	81	74	78 ± 2.1
	10	64	69	45	59 ± 7.3*
	15	41	51	45	46 ± 2.9*
Sp1	Vehicle control (0.02% DMSO)	100	100	100	100 ± 0
	5	99	117	112	110 ± 6.7
	10	98	131	81	98± 12
	15	118	80	111	105 ± 9.6

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

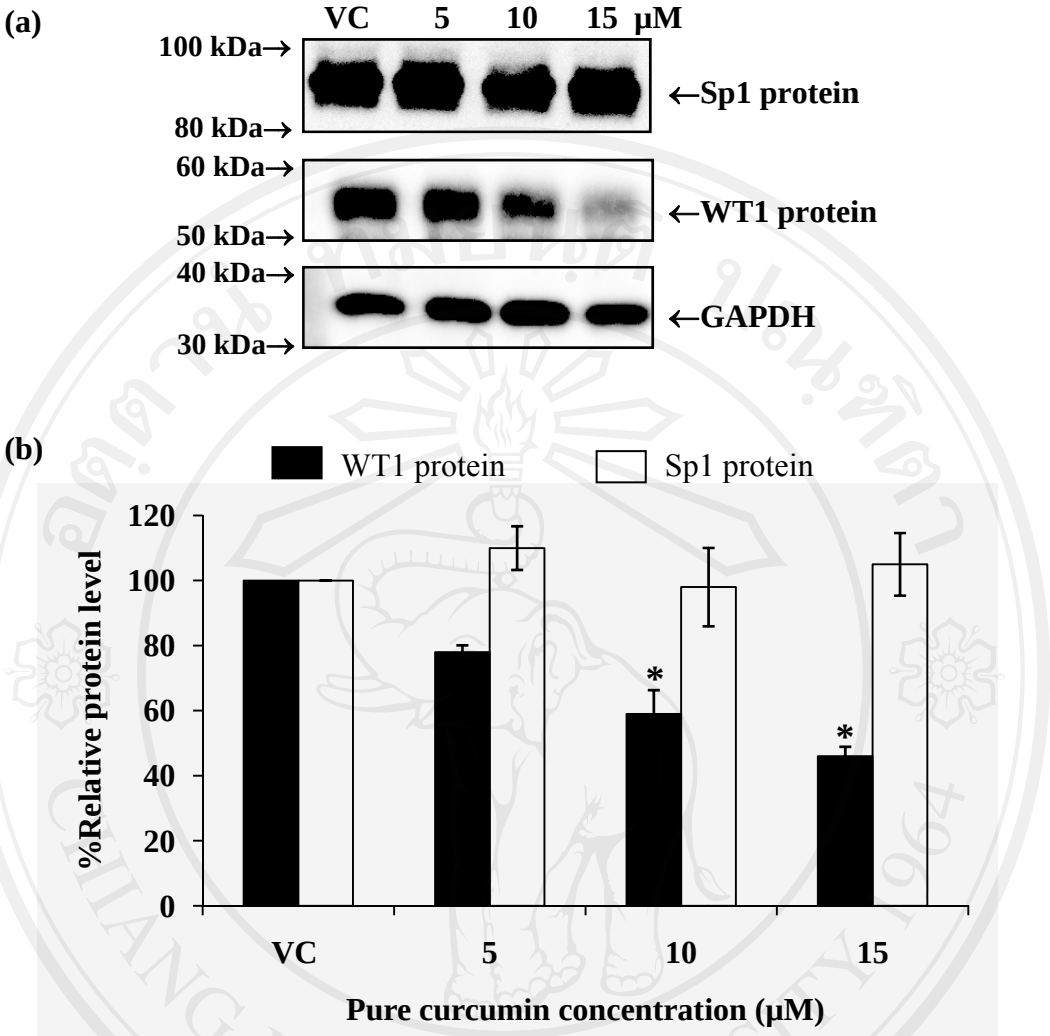


Figure 36 Effect of various concentrations of pure curcumin on Sp1 protein expression in K562 cells. Cells were treated with 5, 10, and 15 µM of pure curcumin for 24 h. The whole protein fraction was assessed by (a) Western blotting. (b) Densitometry, which was used to quantitate the protein levels and graph as the percentage of relative WT1 protein level. GAPDH was used as loading control. Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

Table 29 Relative enrichment ratio of SYBR green qPCR amplification of promoter DNA containing WT1 binding sites in K562 cells treated with 15 μM pure curcumin

Binding site	Pure curcumin treatment	Relative enrichment ratio			
		1	**2**	**3**	**Mean ± SEM**
WT1	Vehicle control (0.02% DMSO)	1	1	1	1 ± 0
	15 μM pure curcumin	0.14	0.45	0.29	0.29 ± 0.15*
Sp1	Vehicle control (0.02% DMSO)	1	1	1	1 ± 0
	15 μM pure curcumin	1.25	0.82	1.03	1.03 ± 0.21

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

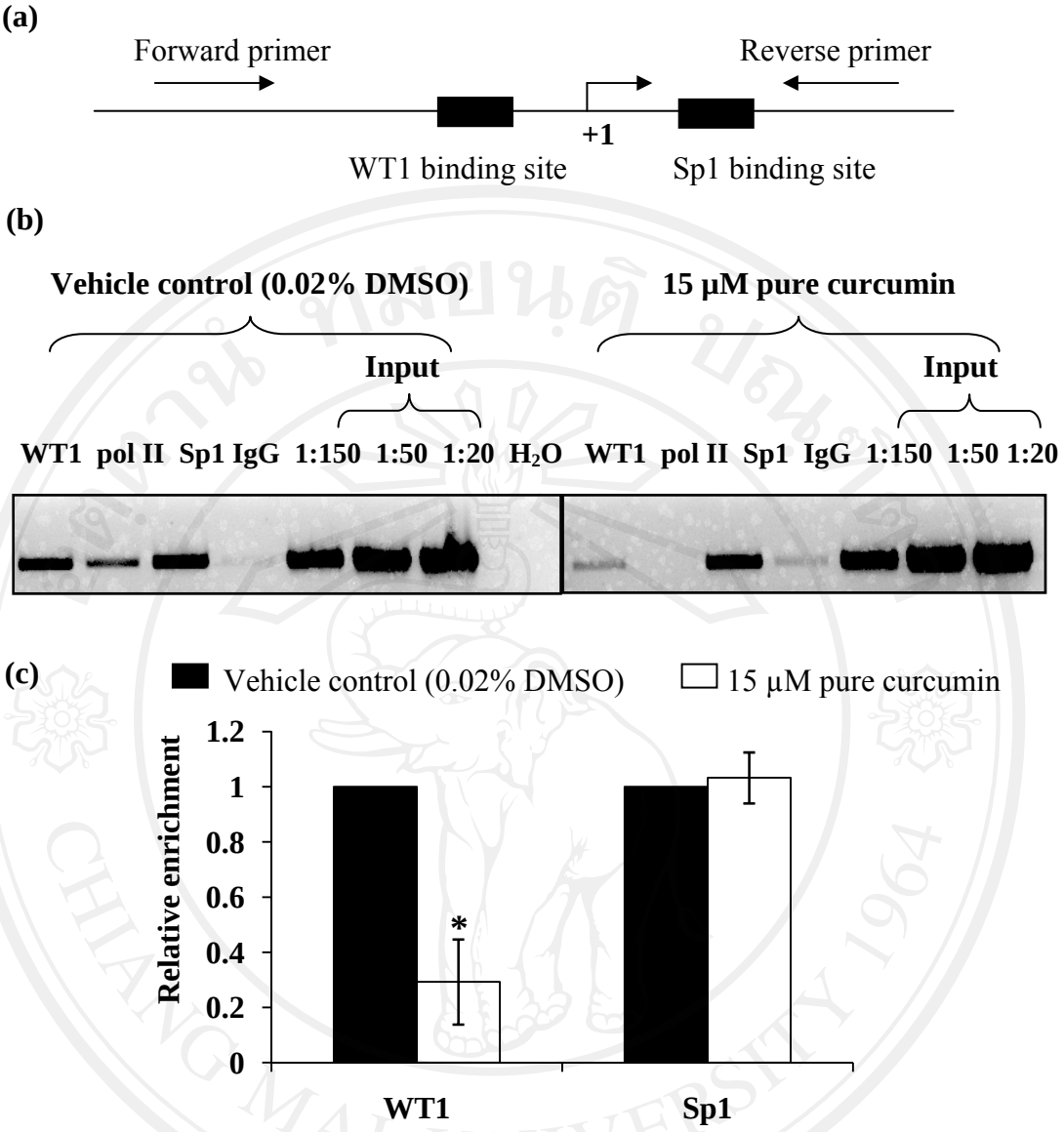


Figure 37 Pure curcumin treatment attenuated WT1 transcription factor protein binding to the WT1 proximal promoter. (a) Schematic showing the WT1 proximal promoter and the locations of the WT1 binding site, Sp1 binding site and ChIP primers relative to the furthest upstream TSS. K562 cells were treated with 15 µM pure curcumin or 0.02% DMSO for 24 h and chromatin immunoprecipitated lysates for WT1, pol II (positive control for promoter activation), IgG (negative control), or input (1:150, 1:50, and 1:20) dilution of input were assayed by (b) standard PCR for binding to the WT1 proximal promoter and (c) SBYR green qPCR. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

3.11 Effect of pure curcumin on WT1 proximal promoter activity

To evaluate the effect of pure curcumin on WT1 proximal promoter activity in K562 cells using the luciferase reporter gene assay, cells were transfected with 301 bp WT1 proximal promoter luciferase vector or pGL3 vector for 24 h and treated with 15 μM pure curcumin for 24 h. The WT1 ChIP experiment 15 μM showed that pure curcumin abrogated WT1 transciption factor binding to the WT1 proximal promoter. Thus the effect of pure curcumin on the WT1 promoter activity was then further investigated using the luciferase reporter gene assay. The reporter construction was used in this experiment to obtain a luciferase reporter construct for the minimal proximal WT1 promoter sequence as required for maximum response in K562 leukaemia cells (272). There are several potential WT1 and Sp1 consensus sites within the WT1 proximal promoter (270). The WT1 (-50 to -39) and Sp1 (-224 to -203) consensus binding sites included within the 301 bp reporter construct are indicated. This experiment used renilla and beta-galactosidase as internal control for co-transfection. The results showed that transfection of the 301 bp construct into K562 cells demonstrated high luciferase activity with vehicle control-treated cells and a diminished response from pure curcumin-treated K562 cells (Table 30 and Figure 38). To determine whether the luciferase activity was driven by WT1 protein binding to the proximal promoter, the WT1 binding site located at -50 to -39 was mutated. Importantly, the luciferase construct containing the mutated WT1 binding site in the proximal WT1 promoter was completely unresponsive when transfected into K562 cells (Figure 39) suggesting that WT1 binding to the proximal promoter was driving firefly luciferase expression. The WT1 binding site included within the luciferase reporter construct and the ChIP-qPCR amplified region suggested that the inhibitory

effect of pure curcumin on WT1 auto-regulation was mediated through the upstream WT1 binding site located at position -50 to -39 in the WT1 proximal promoter.

Table 30 Firefly luciferase activity of 301 bp consensus WT1 promoter constructed pGL3 vector was treated with 15 μM pure curcumin for 24 h

Reporter construct	**Pure curcumin treatment (μM)**	**Mean ± SEM**	
		Firefly luciferase activity	**Firefly luciferase /beta-gal**
pGL3 vector	Vehicle control (0.02%DMSO)	1 ± 0	1 ± 0.1
	15 μM pure curcumin	1 ± 0	1 ± 0.2
-301 bp reporter construct	Vehicle control (0.02%DMSO)	12 ± 2.1	7.2 ± 0.8
	15 μM pure curcumin	6 ± 1.3*	0.2 ± 0.1*

Data is the mean ± standard error of sample mean of seven independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

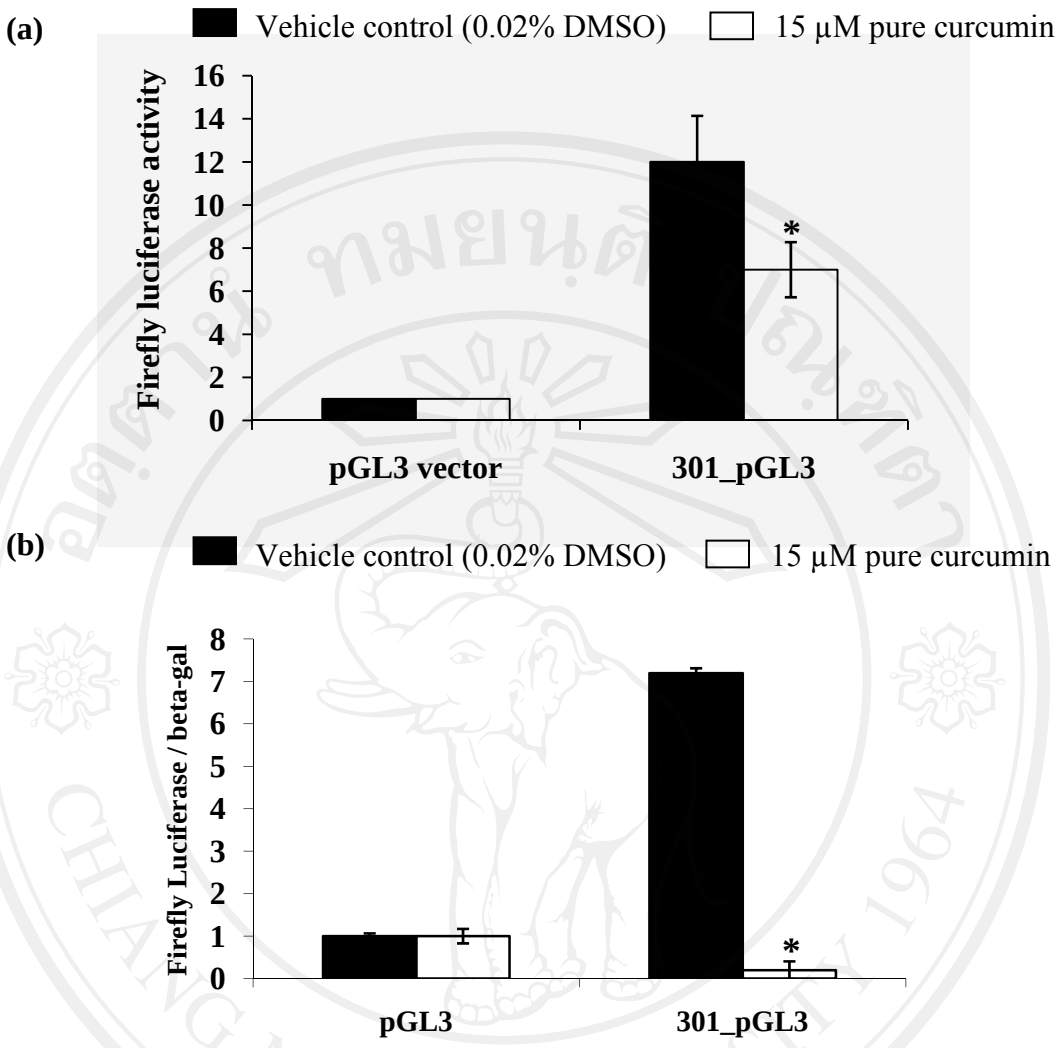


Figure 38 Effect of pure curcumin on WT1 gene activity *via* firefly luciferase reporter. K562 cells were transfected with the pGL3_basic luciferase reporter vector containing 301 bp of the WT1 proximal promoter followed by 15 µM pure curcumin or vehicle treatment for 24 h. (a) The firefly luciferase activity was assayed and relative activity graphed compared to the pGL3 basic vector (n=7). (b) Relative firefly luciferase and beta-galactosidase activity after pure curcumin treatment (n=3). Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

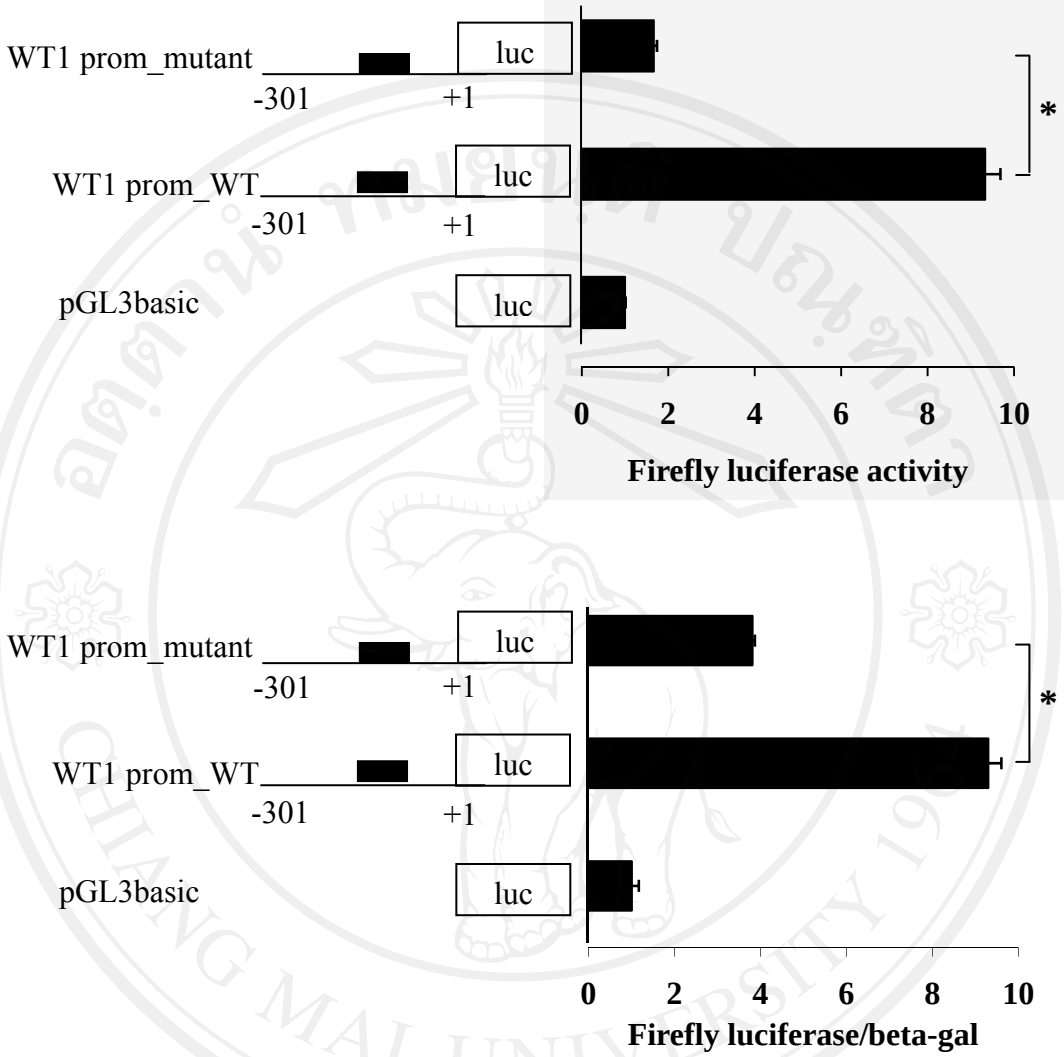


Figure 39 Site-directed mutagenesis of the WT1 consensus sequence (-50 to -39) abolished the WT1 promoter activity compared to the wild type WT1 promoter construct (301 bp WT1). (a) Mutant or WT1 constructs were transfected for 24 h and then assayed for firefly luciferase activity (n=7). (b) Relative firefly luciferase and beta-galactosidase activity after mutant or WT1 constructs were transfected for 24 h (n=3). Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control ($P<0.05$).

3.12 Establishment and characterization of U937 clones constitutively expressing WT1

To investigate the role of WT1 isoforms on cell proliferation in leukemic cells, stable clones of WT1 expressing U937 cells were established. The WT1 (+17AA/+KTS or +/+), WT1 (+17AA/-KTS or +/-), WT1 (-17AA/+KTS or -/+), and WT1 (-17AA/-KTS or -/-) constructs were transfected into U937 cells by lipofection. The overexpression of WT1 mRNA in different clones was detected using RT-PCR and qRT-PCR. Transfected U937 cells displayed high levels of WT1 isoform mRNA. WT1 +/+ clones were designated as WT1 +/+ /1C7, WT1 +/+ /1G3, WT1 +/+ /2E10, and WT1 +/+ /1E3, whereas WT1 +/- clones were designated as WT1 +/- /1C8, and WT1 +/- /1G9, and WT1 -/+ clones were designated as WT1 -/+ /1D3 and WT1 -/+ /1D6. The WT1 -/- clone was designated as WT1 -/- /1E9. Mock control cells were transfected with pcDNA 3.1 empty vectors. The control clones showed low levels of endogenous WT1 mRNA (Figure. 40 and 41). Moreover, when expression of the four WT1 isoforms was determined in WT1-tranfected U937, control cells (wild type U937), and mock control using the immunoprecipitation and Western blot analysis, all four isoforms were clearly observed at higher levels in transfected cells than those of the cell control and mock control (Figure 42).

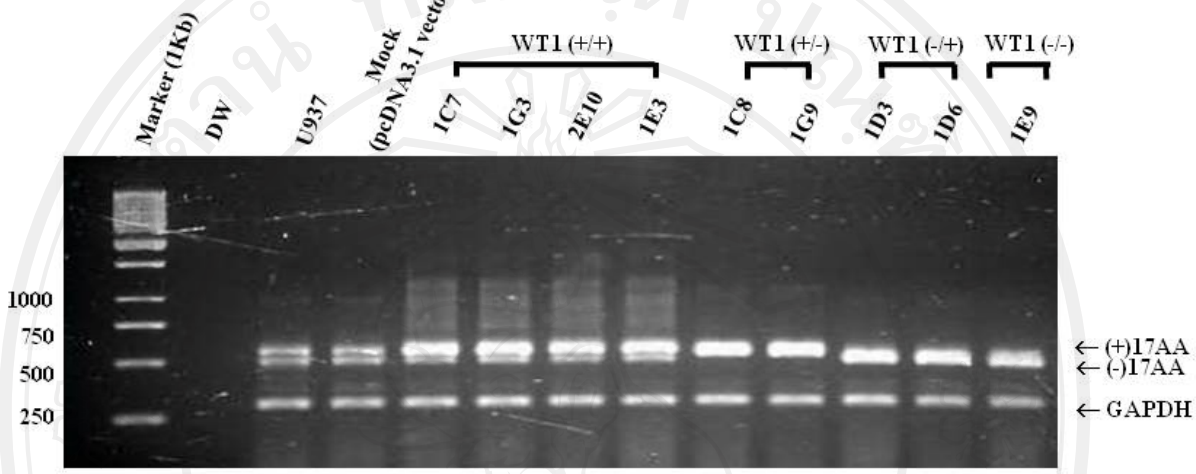


Figure 40 Expression of *WT1* gene in transfected cells by RT-PCR. The expression of WT1 mRNA in WT1 isoform-transfected U937 cells was determined by RT-PCR. The position of WT1 mRNA is 474-540 bp in the transfected clones 1C7, 2E10 (WT1 +/+), 1C8, 1G9 (WT1 +/-), 1D6 (WT1 -/+), and 1E9 (WT1 -/-).

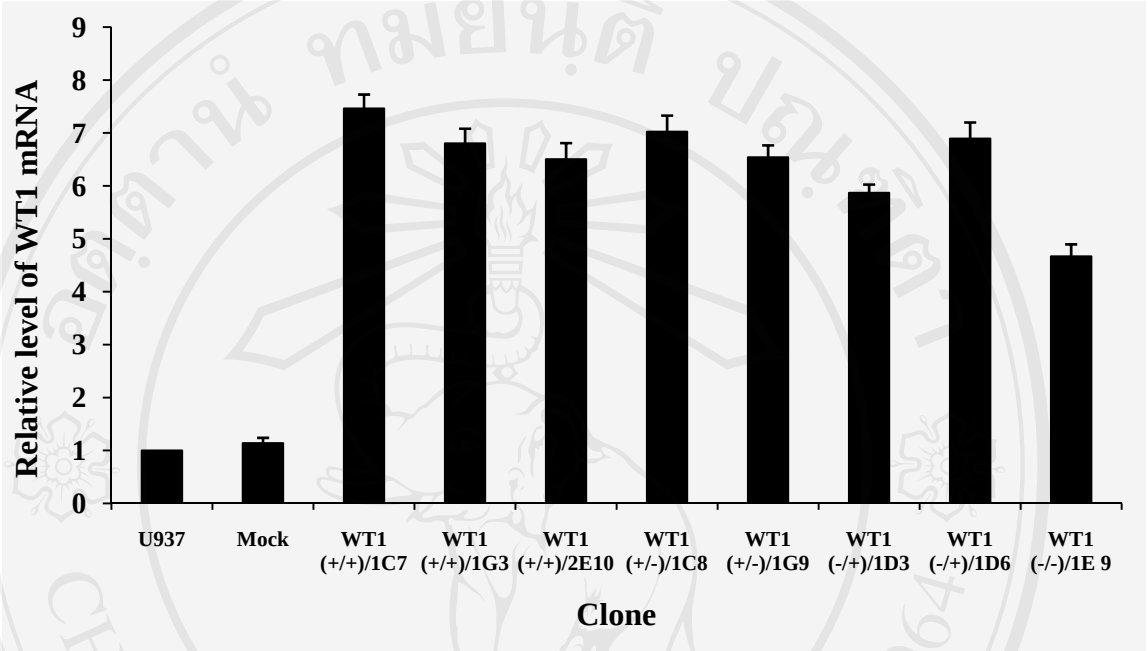


Figure 41 Expression of *WT1* gene in transfected cells by qRT-PCR. The expression of WT1 mRNA in WT1 isoform-transfected U937 cells was determined by real-time PCR. The *β-actin* gene was used as an internal control.

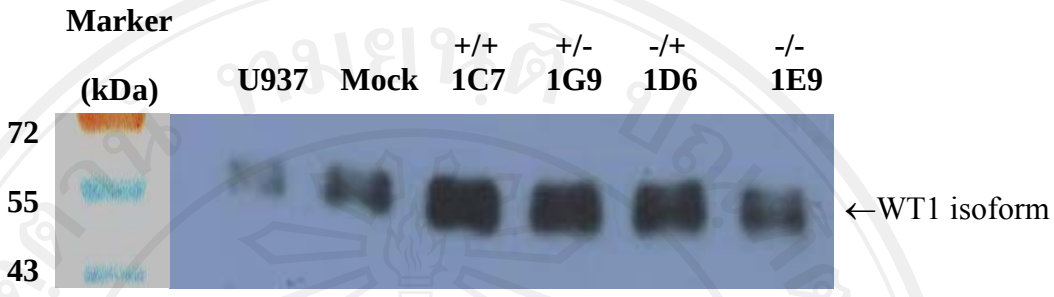


Figure 42 Expression of WT1 protein in transfected U937 cells by immunoprecipitation and Western blot analysis. The expression of WT1 protein in WT1 isoform-transfected U937 cells was determined by immunoprecipitation and detected with the specific anti-WT1 (C-19) rabbit polyclonal antibody using SDS-PAGE (12% gradient gel) and ECL analysis. The position of WT1 protein is 48-57 kDa in the transfected clones 1C7, 2E10 (WT1 +/+), 1C8, 1G9 (WT1 +/-), 1D6 (WT1 -/+), and 1E9 (WT1 -/-).

3.13 Cytotoxic effects of pure curcumin on four WT1 isoforms stably transfected U937 cells

Pure curcumin exhibited a cytotoxic effect on the U937 clones with inhibitory concentrations at 50% growth (IC_{50} value) of approximately 21 ± 1.0 μM (WT1 +/+ /1C7), 19 ± 0.2 μM (WT1 +/- /1G9), 24 ± 0.6 μM (WT1 -/+ /1D6), and 23 ± 0.4 μM (WT1 -/- /1E9), respectively by MTT assay. The IC_{50} values of U937 control cells and mock control cells were 15 ± 1.0 μM and 17 ± 2.2 μM, respectively. Moreover, the vehicle control of 0.02% DMSO did not affect leukemic cell viability (Table 31 and Figure 43). The IC_{20} values of pure curcumin were used for investigations on the inhibitory effect and mechanism of pure curcumin on the *WT1* gene expression.

3.14 WT1 overexpression reverses the effect of pure curcumin activity

To investigate whether high expression of WT1 could reduce pure curcumin activity, four different WT1 isoforms: WT1 +/+, WT1 +/-, WT1 -/+, and WT1 -/- were stably cloned into U937 cells. Stably transfected U937 cells were treated with 15 μM pure curcumin (IC_{50} value of wild type U937 cells) for 48 h and assayed by measuring cell viability and proliferation using the MTT assay. The overexpression of all four WT1 isoforms in stably transfected U937 cells showed resistance to the anti-proliferative effects of pure curcumin activity, compared to transfected vector control (Figure 44).

Table 31 IC_{20} and IC_{50} values of pure curcumin on cytotoxicity of transfected U937 cells

Cells	Pure curcumin concentration (μM)	
	IC_{20} (mean ± SEM)	**IC_{50} (mean ± SEM)**
U937	9 ± 0.6	15.0 ± 1.0
pcDNA3.1	8 ± 1.7	17.0 ± 2.2
WT1 +/+ 1C7	15 ± 0.8	21.0 ± 1.0*
WT1 +/- 1G9	11 ± 1.2	19.0 ± 0.2*
WT1 -/+ 1D6	13 ± 0.3	24.0 ± 0.6*
WT1 -/- 1E9	13 ± 0.5	23.0 ± 0.4*

Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

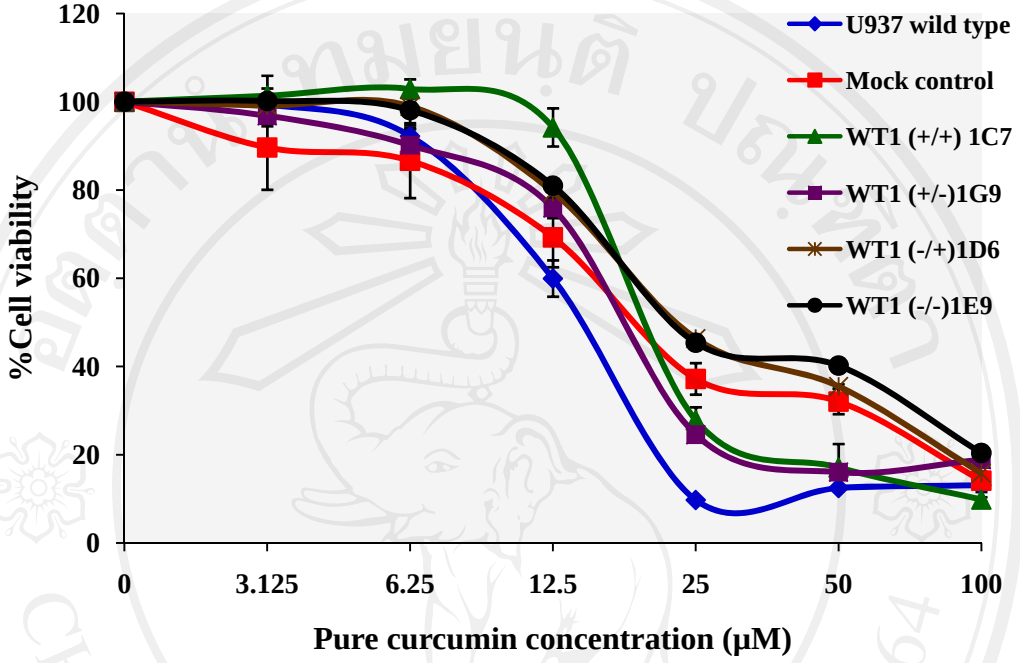


Figure 43 Cytotoxicity of pure curcumin on transfected U937 cells. Transfected U937 cells (1 x 10^5 cells/mL) in 200 µL were grown in the presence of various concentrations of pure curcumin for 48 h. Cell viability was measured by MTT assay. Each point represents the mean value ± SEM of three independent experiments performed in triplicate.

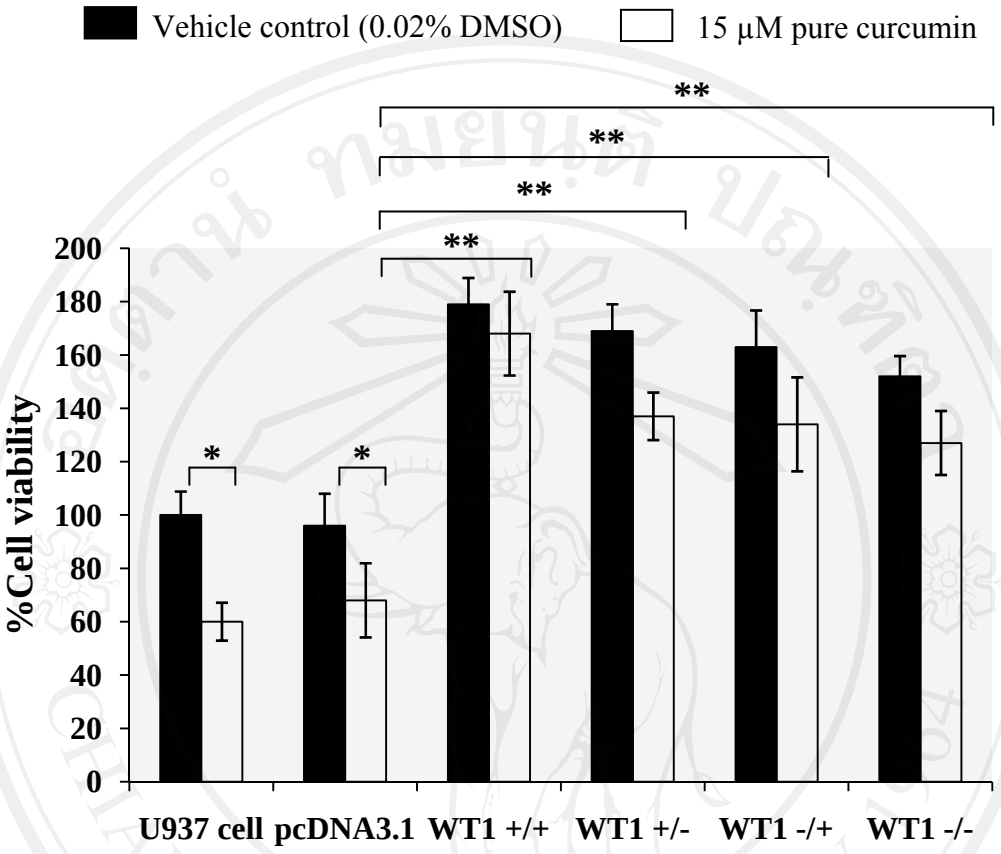


Figure 44 Cytotoxic effect of pure curcumin on WT1 isoform-transfected U937 cells. The WT1 +/+, WT1 +/-, WT1 -/+, WT1 -/-, stably transfected U937 or mock control cells were treated with 15 μM pure curcumin for 48 h. Percent cell viability of WT1-overexpressed U937 cells was assessed by MTT assay. Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05). Double asterisks (**) denote values that were significantly different from those in the pcDNA3.1 transfection cell experiment (P<0.05).

3.15 Effect of pure curcumin on mRNA levels in four WT1 isoform stably transfected cells

The previous results in this study have shown that pure curcumin was able to inhibit the endogenous *WT1* gene and WT1 protein in K562 cells. Thus, the aim of this study was to investigate which WT1 isoform would be inhibited by pure curcumin. The WT1 isoform-transfected cells, control cells, and mock control cells were treated with non-cytotoxic doses (IC_{20} value) of the pure curcumin for 24 h. The concentrations at IC_{20} of WT1 +/+, +/-, -/+, -/-, cell control, and pcDNA3.1 control vector were 15, 11, 13, 13, 9, and 9 μM, respectively. The expression of the exogenous WT1 +/+ isoform was significantly decreased by pure curcumin when compared to total endogenous WT1 mRNA levels in cell control and pcDNA3.1 control vectors. While the WT1 +/- isoform showed a similar trend, pure curcumin did not inhibit WT1 expression in WT1 -/+ and WT1 -/- isoform-transfected cells (Table 32 and Figure 45). Each treatment was compared to the vehicle control (0.02% DMSO alone without the pure curcumin in the culture medium).

3.16 Effect of pure curcumin on exogenous WT1 protein in WT1 +/+ isoform-transfected U937 cells

3.16.1 Effect of various concentrations of pure curcumin on exogenous WT1 +/+ protein in stably transfected U937 cells

To examine whether pure curcumin could inhibit WT1 +/+ protein expression, WT1 protein levels were determined by Western blot analysis. WT1 +/+ isoform-transfected U937 cells were treated with 10, 15, and 17 μM of pure curcumin for 24 h. The levels of WT1 +/+ protein expression after pure curcumin treatment were 37, 13,

and 11%, respectively. Thus the exogenous WT1 +/+ protein levels were significantly decreased by 63, 87, and 89%, respectively (P<0.05), compared to vehicle control (Table 33 and Figure 46).

Table 32 Percentage of relative levels of WT1 isoform-mRNA in transfected U937 cells after treatment with pure curcumin or DMSO

Isoforms	Treatment	%Relative WT1 +/+ mRNA level			
		1	2	3	Mean ± SEM
U937 cells	Vehicle control (0.013% DMSO)	1	1	1	1 ± 0.00
	9 μM pure curcumin	0.81	0.9	0.83	0.85 ± 0.05
pcDNA3.1	Vehicle control (0.013% DMSO)	1.12	1.15	1.1	1.12 ± 0.03
	9 μM pure curcumin	0.79	0.83	0.8	0.81 ± 0.02
WT1 +/+	Vehicle control (0.02% DMSO)	7.48	7.59	7.3	7.46 ± 0.15
	15 μM pure curcumin	4.5	4	4.3	4.27 ± 0.25*
WT1 +/-	Vehicle control (0.016% DMSO)	6.53	6.76	6.34	6.54 ± 0.21
	11 μM pure curcumin	4.8	5.7	5.6	5.37 ± 0.49
WT1 -/+	Vehicle control (0.019% DMSO)	6.5	7.2	7	6.90 ± 0.36
	13 μM pure curcumin	7.6	7.2	8	7.60 ± 0.40
WT1 -/-	Vehicle control (0.019% DMSO)	4.45	4.88	4.69	4.67 ± 0.22
	13 μM pure curcumin	6.3	5.8	5.1	5.73 ± 0.60

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

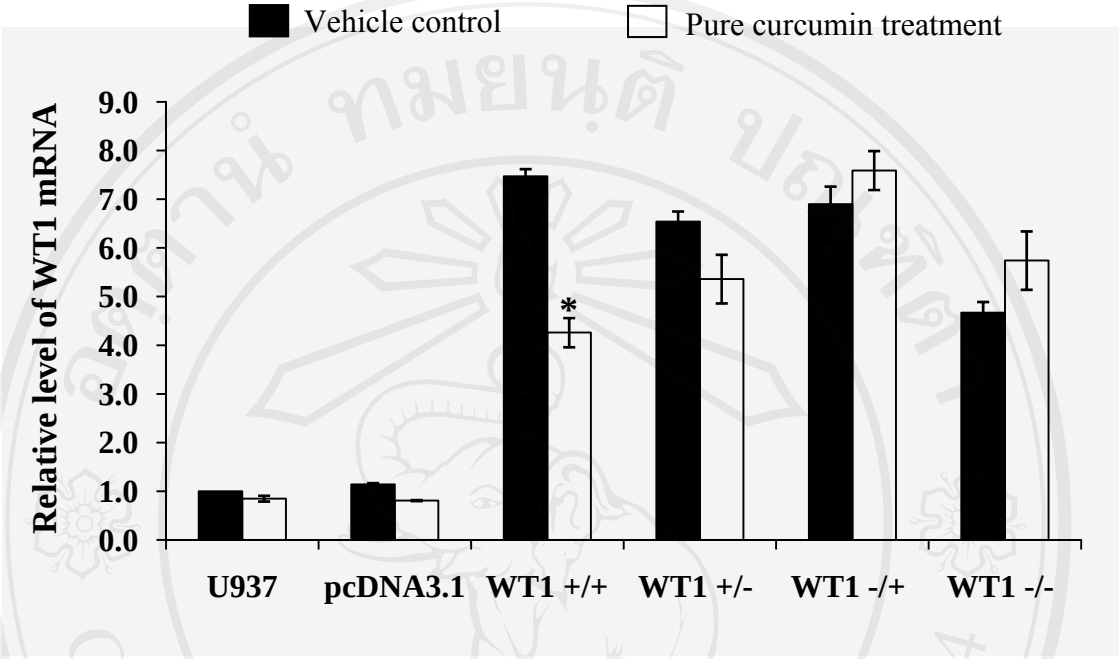


Figure 45 Effect of pure curcumin on WT1 isoform-mRNA level in transfected U937 cells. The WT1 isoform-transfected U937 cells, WT1 +/+, +/-, -/+, and -/- were cultured with non-cytotoxic doses (15, 11, 13, and 13 μM) of pure curcumin. Vehicle control contained 0.02% DMSO. WT1 mRNA levels were measured by qRT-PCR. *β-actin* gene was used as housekeeping gene. Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control ($P<0.05$).

Table 33 Percentage of relative exogenous WT1 +/+ protein levels in transfected U937 cells after treatment with 10, 15, and 17 μM pure curcumin or 0.03% DMSO for 24 h

Pure curcumin concentration (μM)	**%Relative WT1 +/+ protein level expression**			
	1	**2**	**3**	**Mean ± SEM**
Vehicle control (0.03% DMSO)	100	100	100	100 ± 0
10	30	40	42	37 ± 3.7*
15	11	15	14	13 ± 1.2*
17	8	11	13	11 ± 1.5*

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

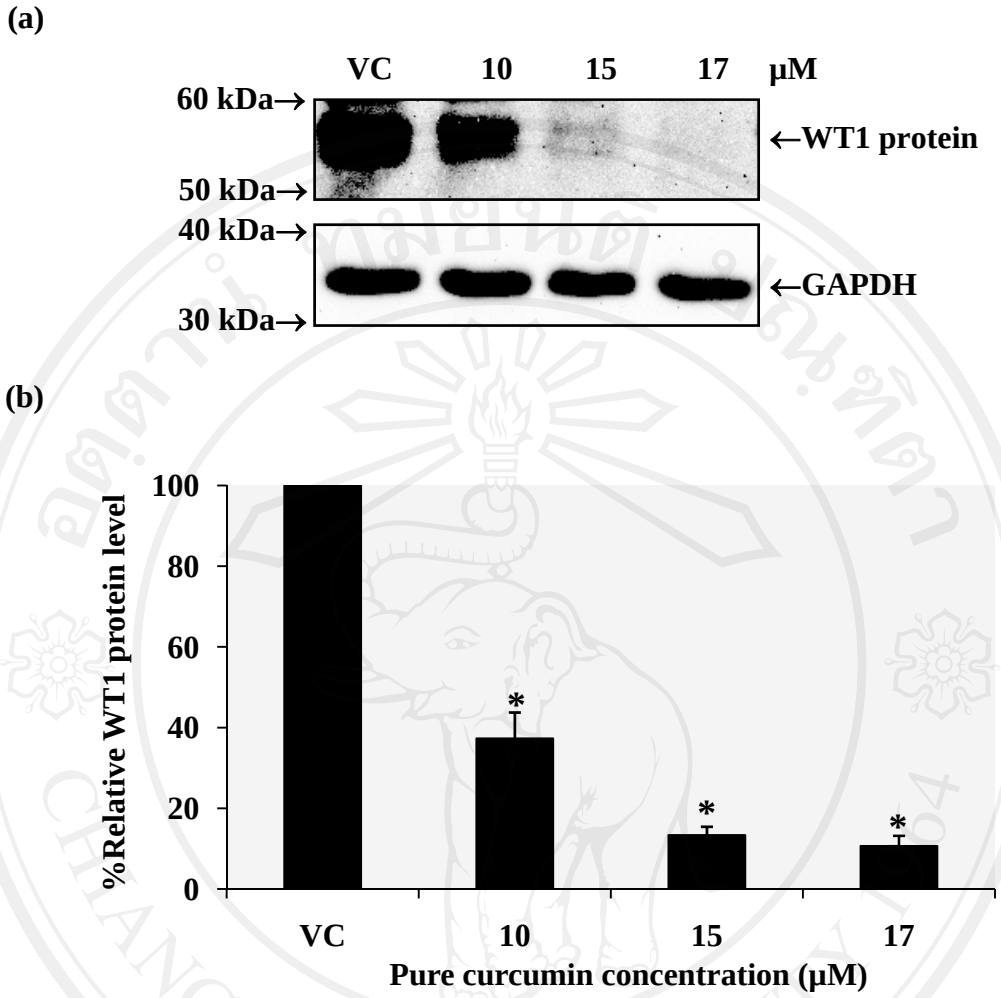


Figure 46 Effect of different concentrations of pure curcumin on exogenous WT1 +/+ proteins in stably transfected U937 cells. WT1 +/+ transfected U937 cells were cultured with 10, 15, and 17 µM pure curcumin or 0.03% DMSO for 24 h. The levels of exogenous WT1 +/+ protein was measured by (a) Western blot analysis and (b) densitometry, which was used to quantitate the protein levels and graphed as the percentage of relative WT1 protein level. GAPDH was used as loading control. Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

3.16.2 Effect of 15 μM pure curcumin on exogenous WT1 protein in stably transfected U937 cells at various time points

To verify whether the effect of 15 μM pure curcumin could down-regulate exogenous WT1 +/+ protein in a time-dependent manner in WT1 +/+ transfected U937 cells. Cells were cultured with 15 μM of pure curcumin for 0, 3, 12, and 24 h and assayed by Western blot analysis. The levels of exogenous WT1 +/+ protein after 15 μM pure curcumin treatment for 3, 6, 12, and 24 h were 52, 31, 19, and 12 %, respectively. Thus the WT1 +/+ protein levels were significantly decreased by 48, 69, 81, and 88%, respectively ($P<0.05$), compared to vehicle control (Table 34 and Figure 47).

3.17 Effect of pure curcumin on exogenous WT1 protein expression via pCMV promoter

To determine whether the effect of pure curcumin on exogenous WT1 expression was specific, this experiment examined the impact of pure curcumin on the expression of four exogenous proteins (GFP, p95ErbB2, ErbB3 and myc-tagged Lrig1) driven by the pCMV promoter in the same pcDNA3.1 vector as WT1. This experiment used *GFP, p95 ErbB2, ErbB3, and myc-tagged Lrig1* genes for pCMV promoter determination after a 15 μM pure curcumin treatment. The protein levels of GFP, p95 EbrB2, ErbB3, and myc-tagged Lrig1 were not decreased compared to the vehicle control in transfected K562 cells, while the 15 μM pure curcumin decreased endogenous WT1 expression (Figure 48).

Table 34 Percentage of relative levels of exogenous WT1 +/+ protein in transfected U937 cells after treatment with 15 μM pure curcumin at different time points

Incubation time (h)	**%WT1 +/+ protein level**			
	1	**2**	**3**	**Mean ± SEM**
0	100	100	100	100 ± 0.0
3	65	43	49	52 ± 6.7*
6	36	30	27	31 ± 2.7*
12	26	13	17	19 ± 4.0*
24	10	10	16	12 ± 1.8*

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

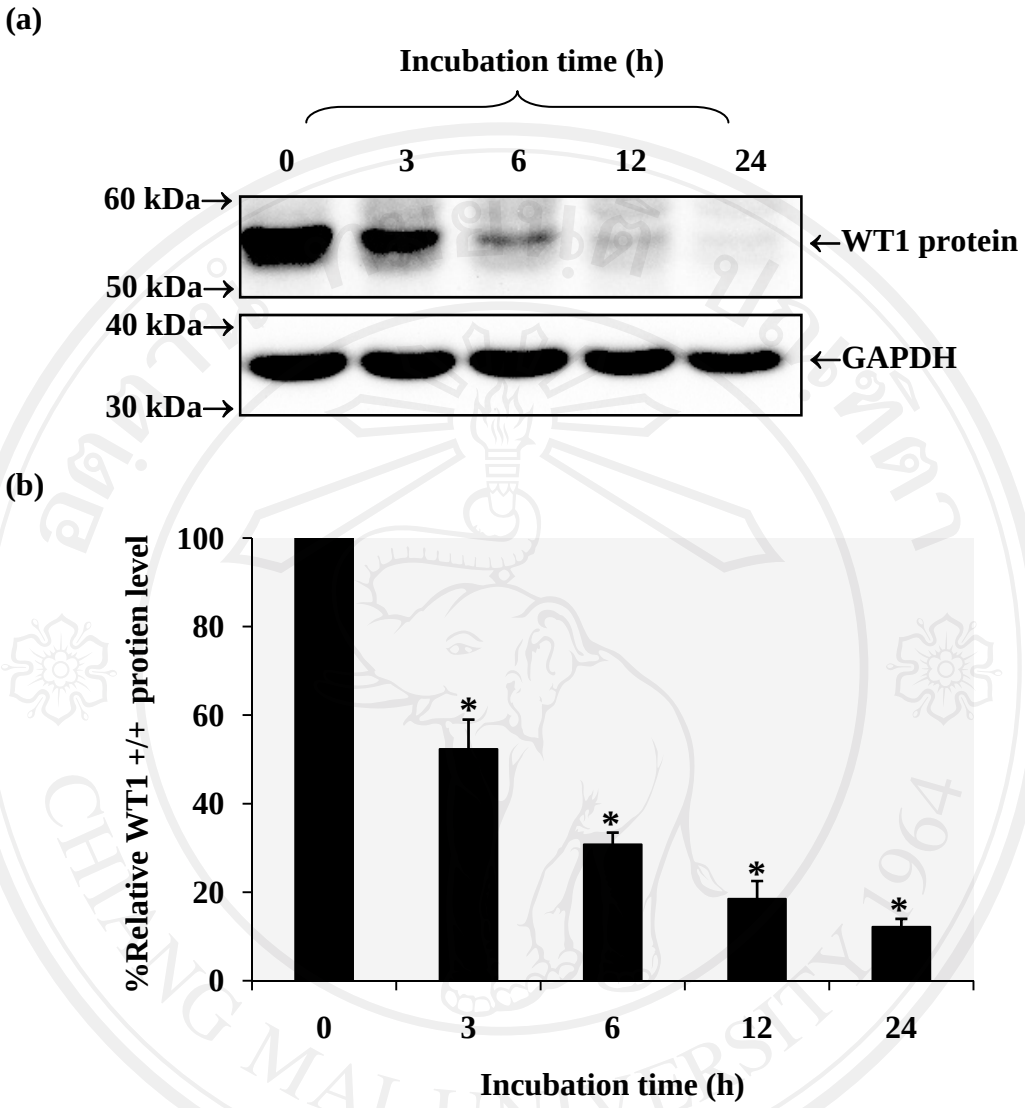
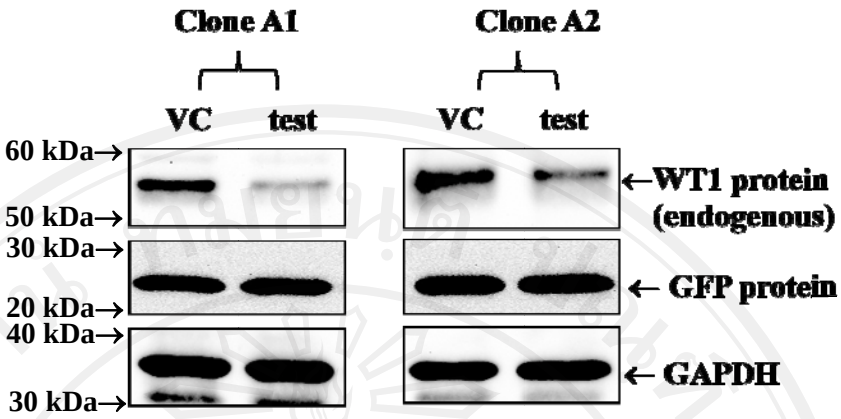
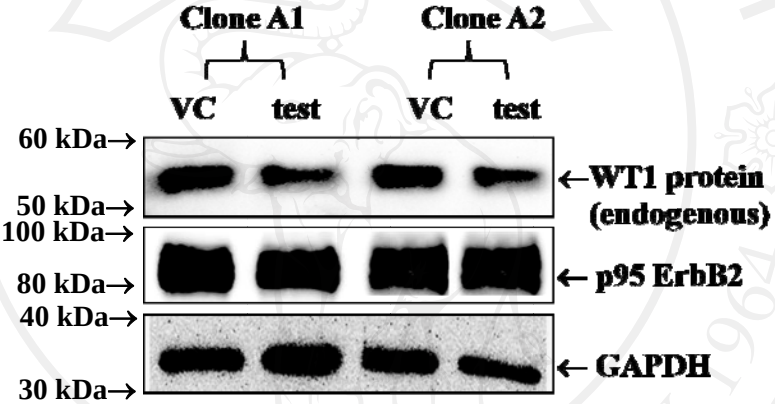


Figure 47 Effect of pure curcumin at 15 µM on exogenous WT1 +/+ protein expression in WT1 +/+ transfected U937 cells. Cells were cultured with 15 µM of pure curcumin for 0, 3, 6, 12 and 24 h. The levels of WT1 +/+ protein was measured by (a) Western blot analysis and (b) protein quantitation graphed as percent relative WT1 protein level. GAPDH was used as loading control. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

(a)



(b)



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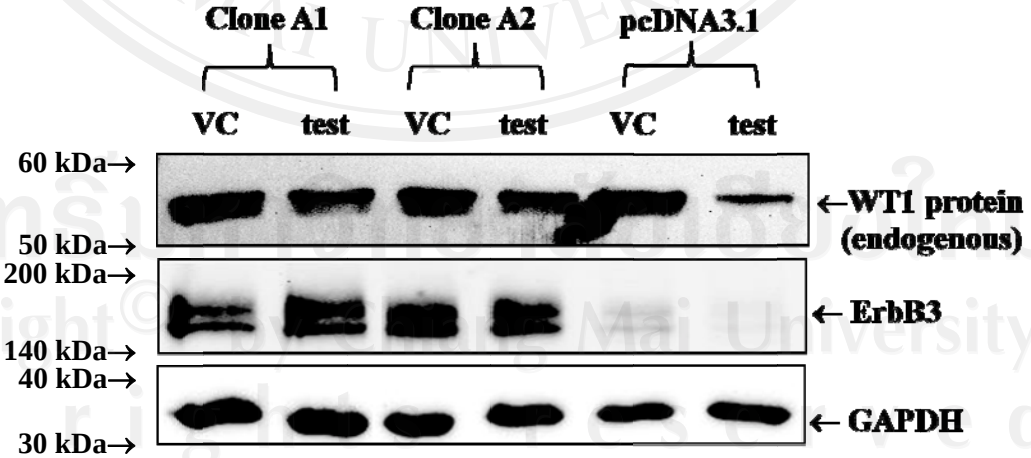

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(d)

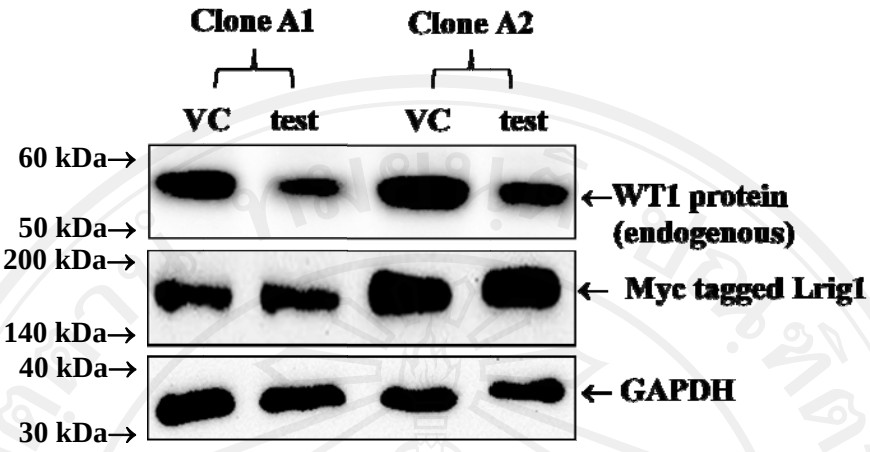

Figure 48 Effect of pure curcumin on other proteins driven by the pCMV promoter. The inserted pcDNA 3.1 vector was transfected into K562 cells and then treated with 15 μM pure curcumin for 24 h. The proteins were detected by Western blot analysis. The membranes were probed with primary antibody against to (a) GFP, (b) p95 ErbB2 protein, (c) ErbB3 and (d) Myc tagged Lrig1. GAPDH proteins were used as a loading control.

3.18 Effect of pure curcumin on exogenous WT1 protein half-life in WT1 +/+ isoform-transfected U937 cells

To investigate whether the decrease in the WT1 +/+ protein isoform is involved in a degradation mechanism, WT1 +/+ isoform-transfected U937 cells were treated with cycloheximide to inhibit new protein synthesis, in the presence or absence of 15 μM pure curcumin. WT1 protein expression was then followed over time to measure a half-life. Exogenous WT1 +/+ protein stability after pure curcumin treatment was significantly decreased, compared to various time points of control conditions. the half-life of WT1 protein in the control treatment was 15 h, whereas in the pure curcumin treatment it was 3.6 h (Table 35 and Figure 49). To examine whether pure curcumin promotes the proteasomal degradation of WT1, this experiment determined whether the MG132 and EGCG (proteasome inhibitor) could prevent the effect of pure curcumin on WT1 protein expression. Indeed, the suppressive effect of pure curcumin in the presence of MG132 on WT1 expression was largely prevented (Table 36 and Figure 50).

Table 35 Percentage of relative exogeous WT1 protein level and half-life of WT1 +/+ transfected U937 cells after treatment with cycloheximide in the presence or absence of 15 μM pure curcumin at different time points

Experiment	Incubation time (h)	%Relative level of WT1 protein			
		1	2	3	Mean ± SEM
Vehicle control (0.02% DMSO)	0	100	100	100	100 ± 0
	3	44	88	85	72 ± 14
	6	32	68	63	54 ±11.2
	12	30	73	54	52 ± 12.4
	24	21	47	48	39 ± 8.6
Half-life = 15 h					
15 μM pure curcumin	0	100	100	100	100 ± 0
	3	21	39	53	38 ± 9.2
	6	9	43	45	32 ± 11.6
	12	3	1	6	3 ± 1.3
	24	1	1	3	2 ± 0.5
Half-life = 3.6 h					

Data is the mean ± standard error of sample mean of three independent experiments.

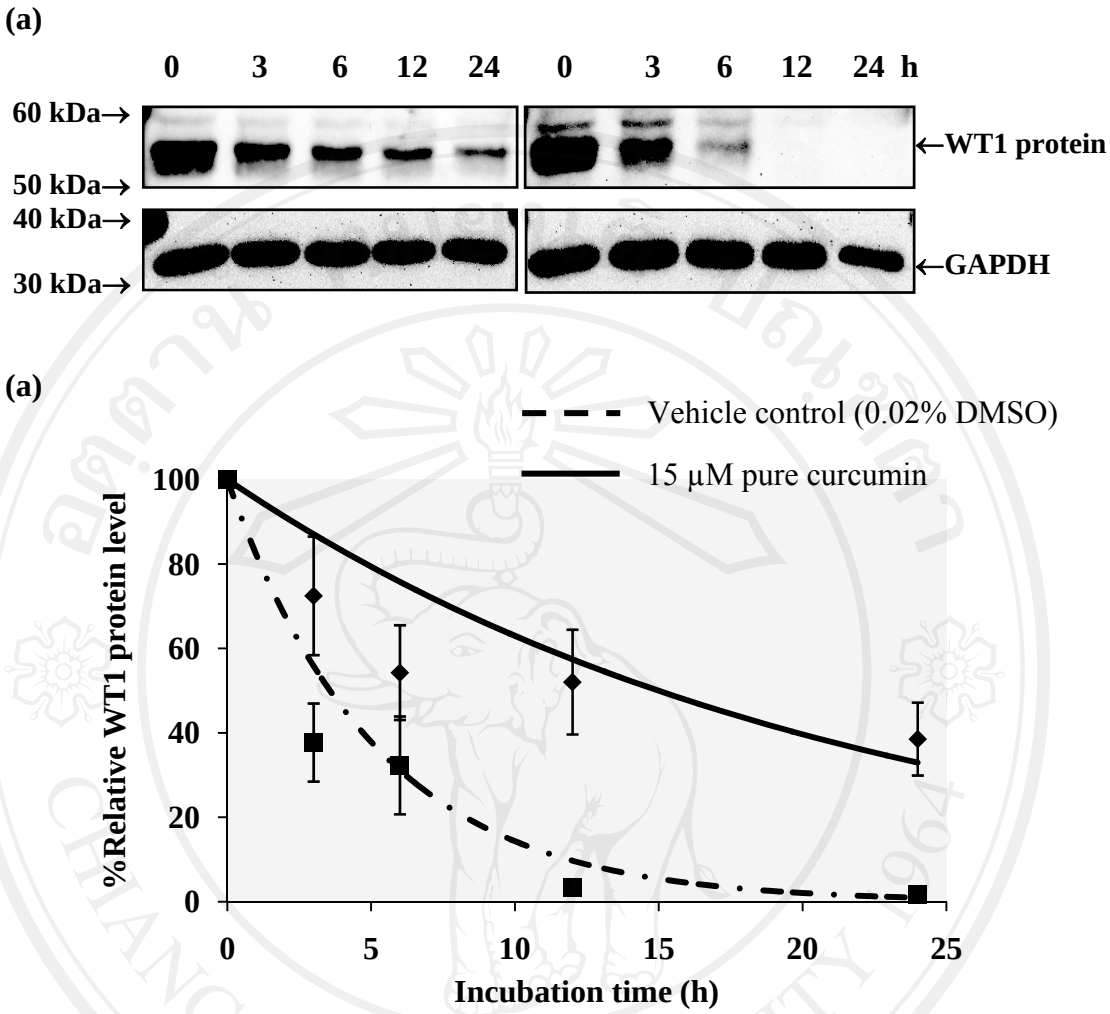

Figure 49 Effect of pure curcumin on exogenous WT1 +/+ protein stability using cycloheximide. To investigate the effect of pure curcumin on exogenous protein stability, WT1 +/+ transfected U937 cells were treated with 50 μg/mL cycloheximide in the presence or absence of 15 μM pure curcumin for 0, 3, 6, 12, and 24 h and assessed by (a) Western blotting, (b) protein half-life quantitation graphed as percent relative exogenous WT1 +/+ protein levels for vehicle control versus 15 μM pure curcumin treatment. Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

Table 36 Percentage of relative exogenous WT1 +/+ protein level in stably transfected U937 cells after treatment with or without MG132 in the presence or absence of 15 μM pure curcumin

Treatment	Pure curcumin treatment (μM)	%Relative level of exogenous WT1 +/+ protein expression			
		1	2	3	Mean ± SEM
Vehicle control (0.05% DMSO)	Vehicle control (0.02% DMSO)	100	100	100	100 ± 0
	15 μM pure curcumin	59	62	60	61 ± 1.5*
5 μM MG132	Vehicle control (0.02% DMSO)	100	100	100	100 ± 0
	15 μM pure curcumin	92	102	98	97 ± 5**

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05). Double asterisks (**) denote values that were significantly different from MG132-untreated WT1 +/+ isoform-transfected U937 cells (P<0.05).

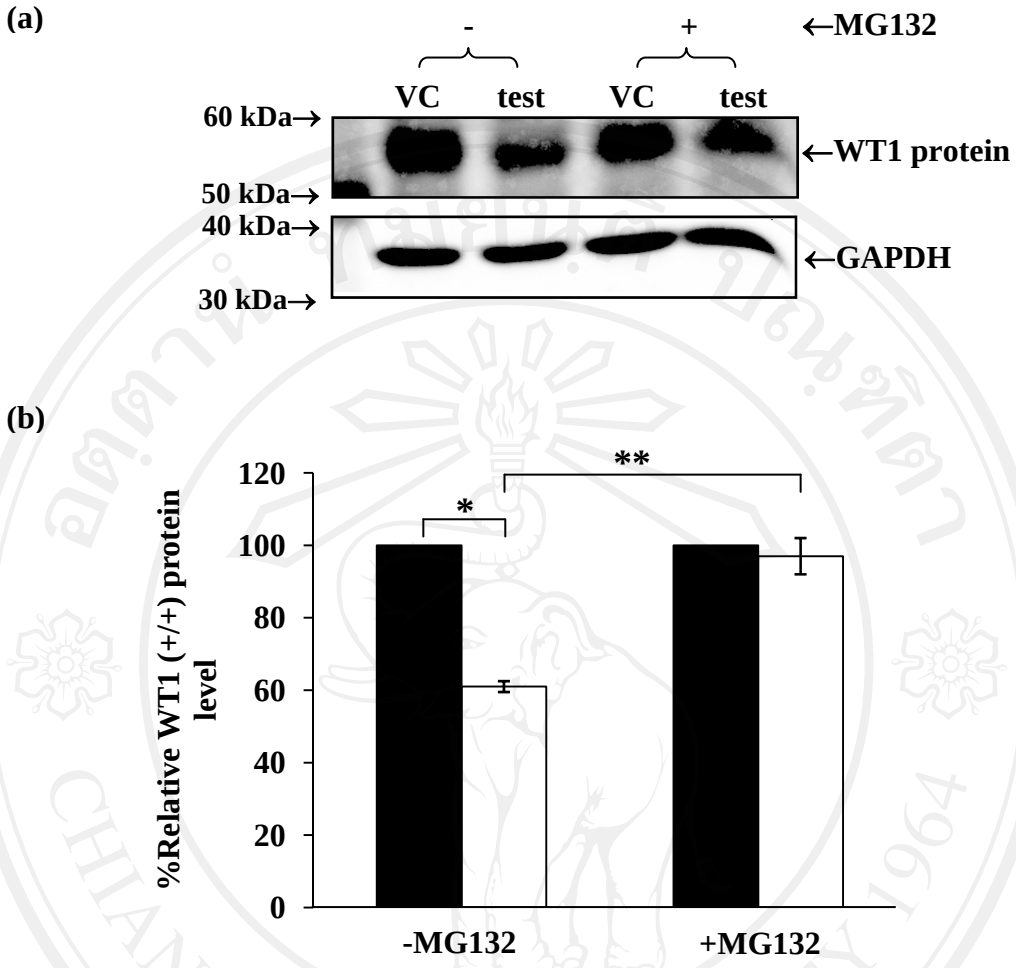

Figure 50 Effect of pure curcumin on exogenous WT1 +/+ protein stability by using MG132. Proteasomal degradation was tested on relative exogenous WT1 +/+ protein levels for vehicle control versus 15 μM pure curcumin treatment in the presence or absence of 5 μM MG132 and assessed by (a) Western blotting, (b) protein quantitation graphed as percent relative exogenous WT1 +/+ protein levels for vehicle control versus 15 μM pure curcumin treatment. GAPDH was used as loading control. Data is the mean value ± SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05). Double asterisks (**) denote values that were significantly different from MG132-untreated WT1 +/+ isoform-transfected U937 cells (P<0.05).

3.19 Effect of PKC inhibitor on exogenous WT1 +/+ protein expression in transfected U937 cells

According to the previous results, pure curcumin clearly and specifically decreased the exogenous WT1 +/+ protein in transfected U937 cells in a protein degradation dependent. Interestingly, phosphorylation of WT1 has been reported in previous studies, indicating that pure curcumin could decrease WT1 phosphorylation by PKC at threonine 638, a site which controls WT1 activation (273). Thus, this experiment aimed to investigate the effect of a PKC inhibitor (GF109203x) on the exogenous WT1 +/+ protein expression in transfected U937 cells. Cells were incubated with 2.5 μM GF109203x at different time points (1, 3, 5, 7, and 24 h) as indicated. The levels of WT1 protein expression were measured by Western blot analysis. The levels of exogenous WT1 +/+ protein significantly decreased after 2.5 μM GF109203x (PKCα, β1, δ, and ε inhibitor) treatment for 3 and 5 h, compared to individual vehicle control. Moreover, 5 h of treatment significantly decreased the level of exogenous WT1 +/+ protein when compared to that of 7 and 24 h (Table 37 and Figure 51).

Table 37 Percentage of relative exogenous WT1 protein level in transfected U937 cells treated with 2.5 μM GF109203x (PKCα, β1, δ, and ε inhibitor)

Incubation time (h)	**%Relative level of WT1 protein expression**			**Mean ± SEM**
	1	**2**	**3**	
0	100	100	100	100 ± 0
1	79	90	91	87 ± 3.7
3	72	94	76	81 ± 6.9*
5	50	79	64	64 ± 8.4*
7	93	101	77	90 ± 7.2
24	104	102	105	104 ± 0.9

Data is the mean ± standard error of sample mean of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).

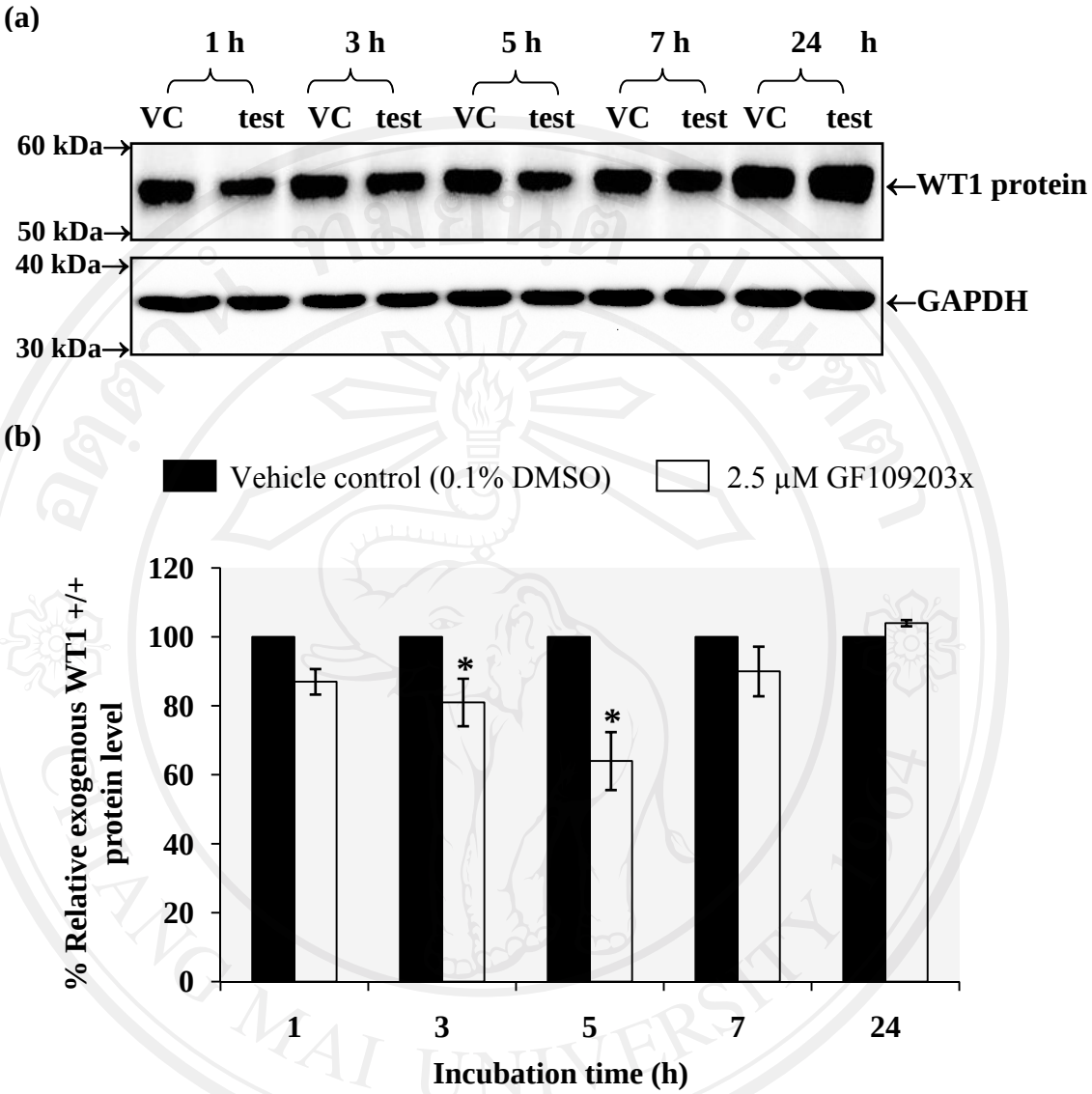

Figure 51 Effect of GF109203x on exogenous WT1 +/+ protein expression in transfected U937 cells. The cells were treated with 2.5 µM GF109203x (PKCα, β1, δ, and ε inhibitor) in 1, 3, 5, 7, and 24 h. The exogenous WT1 +/+ proteins were detected by (a) Western blot analysis and (b) densitometry, which was used to quantitate the protein levels, and graphed as the percentage of relative WT1 +/+ protein level.. GAPDH proteins were used as a loading control. Asterisks (*) denote values that were significantly different from the vehicle control (P<0.05).