CHAPTER III

RESULTS

3.1 Effect of *Z. officinale* extracts on cell viability of A549 cells

The effect of *Z. officinale* extracts and 6-paradol on the viability of A549 cells were studied using the sulforhodamine B (SRB) assay, to use as a guide in choosing the appropriate concentration in subsequent experiments. A549 cells were treated with the indicated concentration of each *Z. officinale* extracts or 6-paradol for 72 h. Dose-response curve between the compound concentration and percent of cell viability are shown in Figures 3.1. The IC_{50} values were derived using the software Curve Expert 1.4 (Table 3.1). The data represent the mean value ± standard deviation of three independent experiments performed in triplicate.

Table 3.1 IC_{50} values of *Z. officinale* extracts and 6-paradol on cell viability of A549 cells.

***Z. officinale* extracts**	**IC_{50} values (µg/ml)**
E1	54.9 $\pm$ 3.5
E2	21.1 $\pm$ 1.6
E3	38.9 $\pm$ 0.7
E4	25.6 $\pm$ 4.3
E2.1	43.2 $\pm$ 4.5
E2.3	5.89 $\pm$ 2.10
E2.2.1	26.5 $\pm$ 1.6
E2.2.2	23.6 $\pm$ 1.2
E2.2.3	17.4 $\pm$ 3.9
	IC_{50} values (µM)
6-paradol	36.2 $\pm$ 0.69

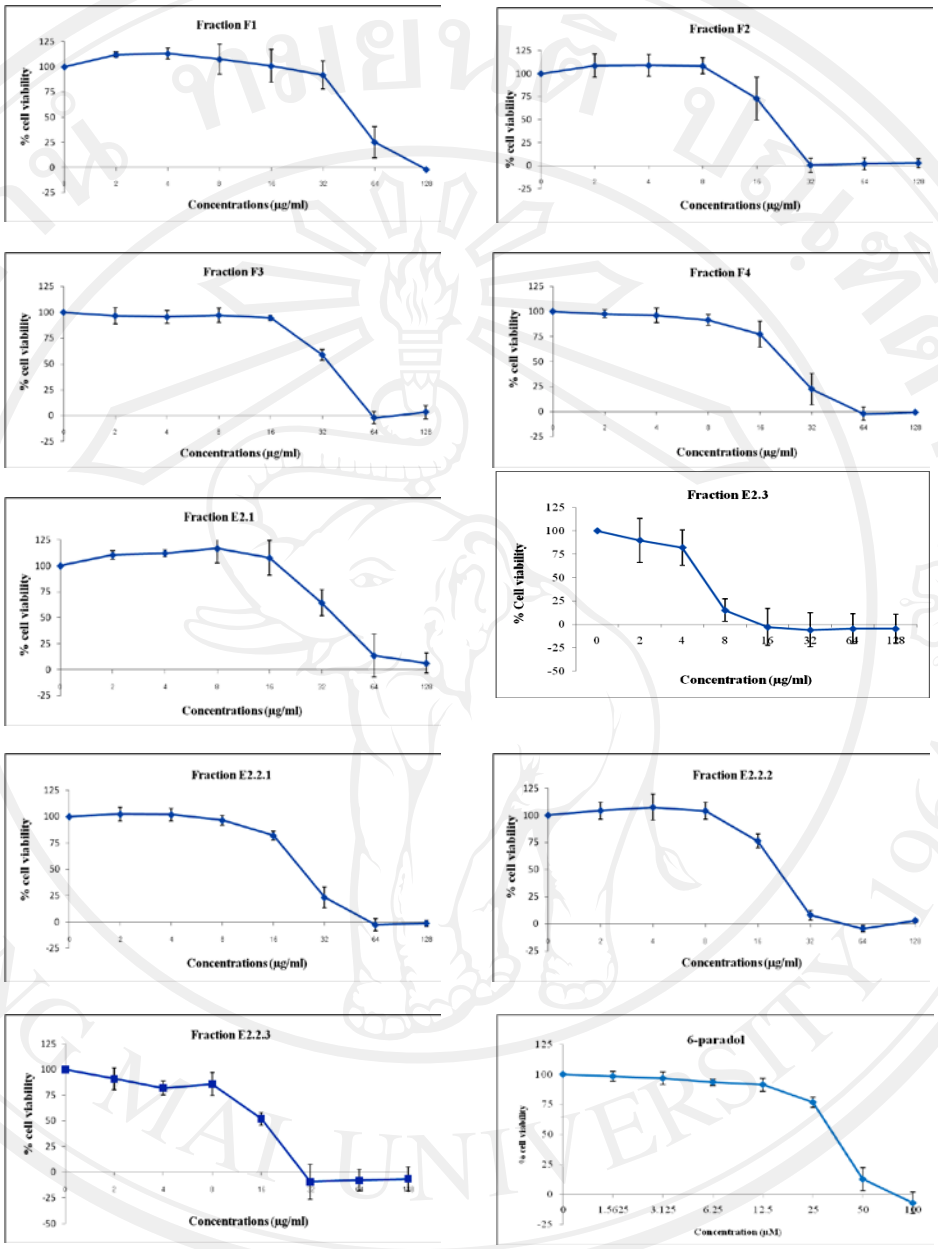


Figure 3.1 Effect of *Z. officinale* extracts and 6-paradol on cell viability of A549 cells. A549 cells (1.0 x 10^4 cell/well), in 200 µl medium were grown in the presence of 0.1% DMSO (vehicle control), or indicated concentration of the *Z. officinale* extracts or 6-paradol for 72 h. The number of viable cells was determined by SRB colorimetric assay. Each point represents the mean values ± standard deviation of three independent experiments performed in triplicate.

3.2 Effect of *Z. officinale* extracts (E1-E4 fractions) on *hTERT* and *c-Myc* mRNAs expression in A549 cells using semi-quantitative RT-PCR.

To investigate whether *Z. officinale* extracts (E1-E4 fractions) down-regulated *hTERT* at the transcriptional level, A549 cells were treated with *Z. officinale* extracts from each fraction for 24 h, and the level of *hTERT* and *c-Myc* genes expression was determined by semi-quantitative RT-PCR analysis. The results are shown in Figures 3.2; panel A shows the gel data, panel B shows graphs representing the relative expression of *c-Myc* mRNA and *hTERT* mRNA and its β-variant (β-splicing of hTERT), after normalization with the expression of *GAPDH* mRNA, the internal control gene. The relative down-regulation of *hTERT* and *c-Myc* mRNAs expression by E1-E4 fractions at 32 µg/ml shows in Table 3.2. Since the E2 fraction was found to be the most effective among the four fractions, we chose this fraction for further studies.

Table 3.2 The relative down-regulation of *hTERT* and *c-Myc* mRNAs expression of A549 cells.

***Z. officinale* extracts**	**% Relative down-regulation**	
	***hTERT* gene**	***c-Myc* gene**
E1	15%	13%
E2	34%	38%
E3	22%	12%
E4	9%	23%

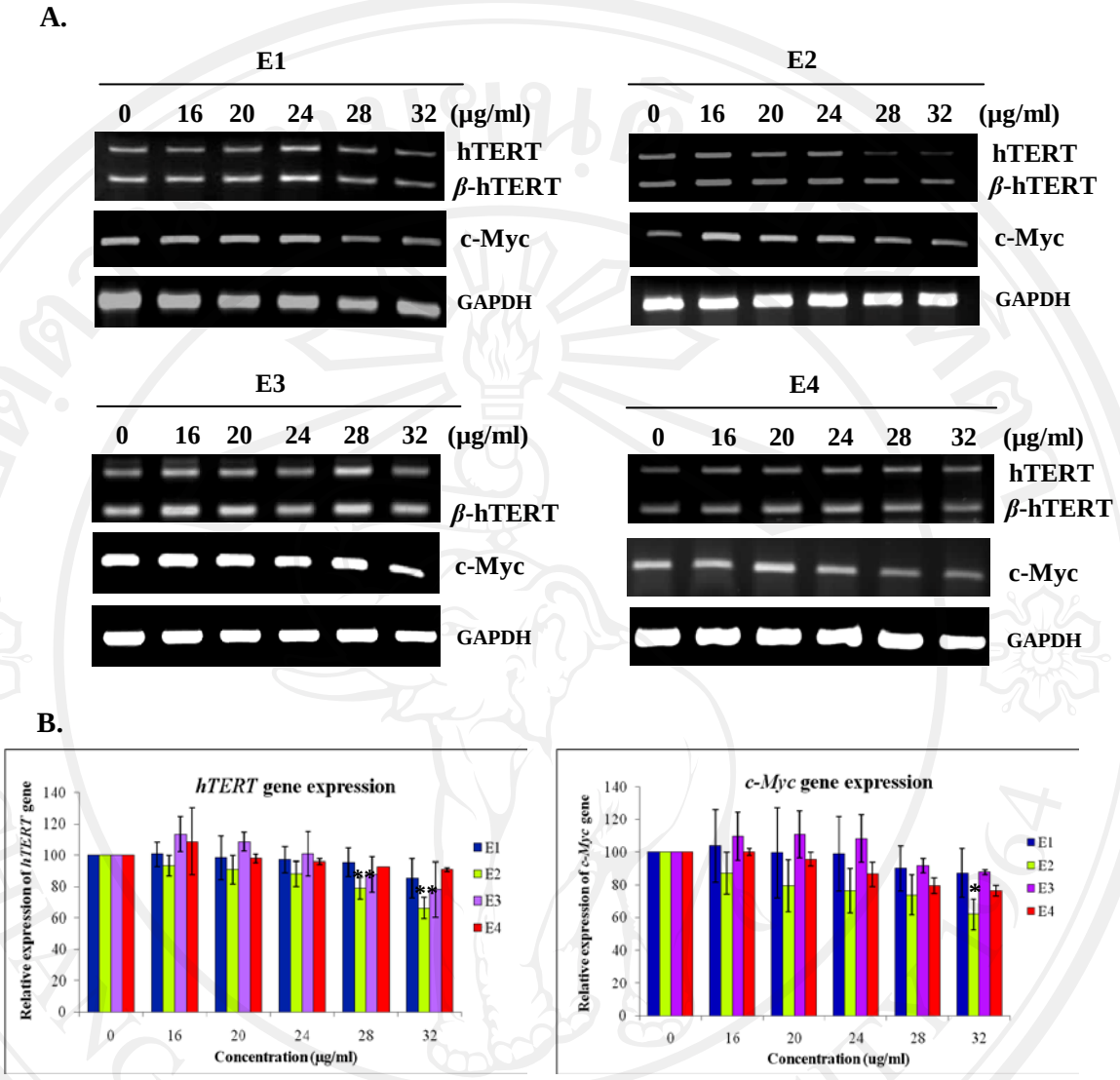


Figure 3.2 Effect of E1-E4 fractions on *hTERT* and *c-Myc* mRNAs expression in A549 cells. A. A549 cells were treated with a various concentration of *Z. officinale* extracts (E1-E4 fractions) for 24 h. At the end of treatment, RNA was extracted, and RT-PCR assays performed to detect *hTERT* (upper panel) or *c-Myc* (middle panel) and *GAPDH* (lower panel) mRNAs. **B.** The density of the various bands was determined by scan densitometer. The *hTERT* and *c-Myc* mRNAs levels were normalized to the levels of *GAPDH* mRNAs. $*P < 0.05$ and $**P < 0.01$ is the statistical significance of the difference between the values for each *Z. officinale* extracts-treated and untreated cells.

3.3 Effect of *Z. officinale* extracts (E2 subfractions) on *hTERT* and *c-Myc* mRNAs expression in A549 cells using semi-quantitative RT-PCR and Real-Time quantitative RT-PCR.

We sub-fractionated the E2 fraction and obtained 5 more fractions: E2.1, E2.3, E2.2.1, E2.2.2, and E2.2.3, as shown in Figure 2.1. We analyzed these subfractions for their ability to inhibit *hTERT* and *c-Myc* expression using semi-quantitative RT-PCR. As shown in Figure 3.3, most of the subfractions inhibit *hTERT* and *c-Myc* expression, especially at higher doses. We also analyzed these subfractions (except E2.3 fraction due to this fraction is highly toxic to cells) for their ability to inhibit *hTERT* and *c-Myc* expression using real-time quantitative RT-PCR. Figure 3.4 shows the relative *hTERT* expression (A) and *c-Myc* expression (B) in the presence of various concentrations of the E2 subfraction (E2.1, E2.2.1, E2.2.2, E2.2.3 subfraction). In each subfraction were down-regulation the level expression of *hTERT* and *c-Myc* mRNAs. The fold change in down regulation of the *hTERT* and *c-Myc* mRNAs expression by E2.1, E2.2.1, E2.2.2, and E2.2.3 at 32 µg/ml was shown in Table 3.3. When control represents the 1X expression of the target gene (hTERT and c-Myc) normalized to GAPDH.

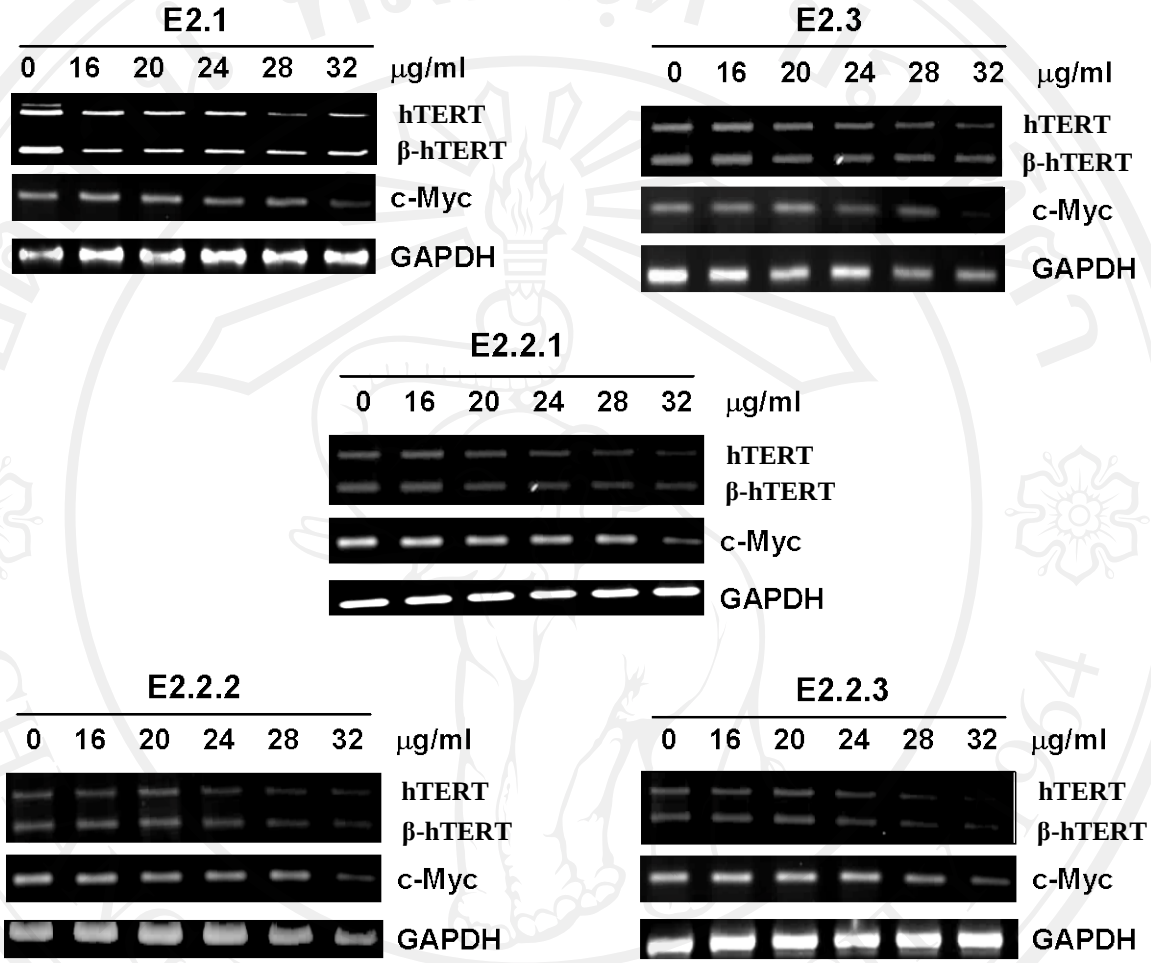
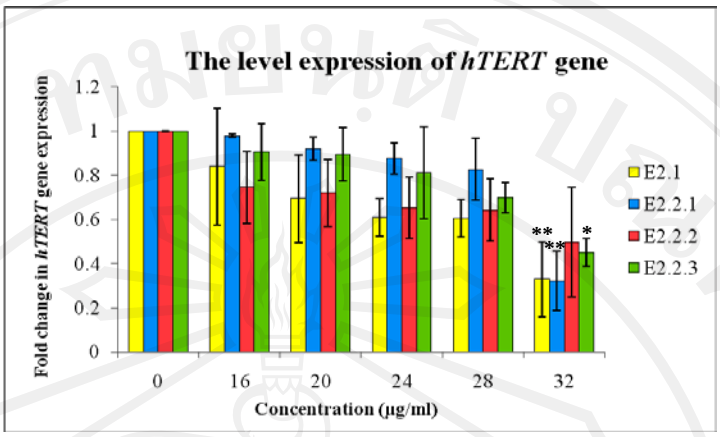


Figure 3.3 Effect of the E2.1, E2.3, E2.2.1, E2.2.2 and E2.2.3 subfraction on *hTERT* and *c-Myc* mRNAs expression in A549 cells by semi-quantitative RT-PCR. A549 cells were treated with each subfraction at the indicated concentrations for 24 h. The cDNA was then amplified by PCR using gene-specific primers. The PCR products were visualized by ethidium bromide staining and UV irradiation. *GAPDH* expression was also analyzed and used as internal control.

A.



B.

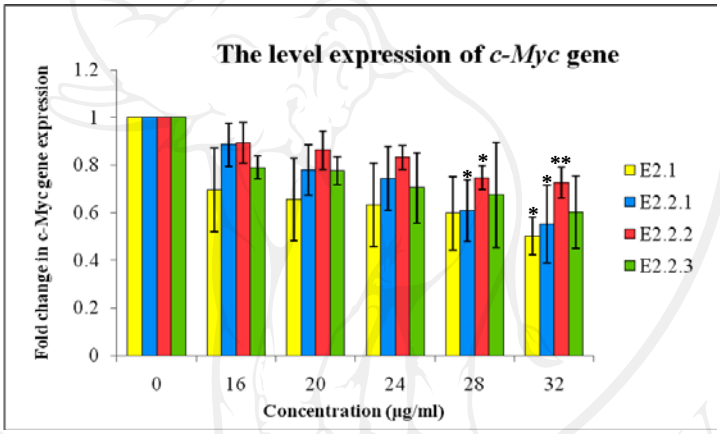


Figure 3.4 Effect of the E2.1, E2.2.1, E2.2.2 and E2.2.3 subfraction in modulating the *hTERT* and *c-Myc* mRNAs expression in A549 cells by Real-Time quantitative RT-PCR . A549 cells were treated with a various concentrations of each subfractions for 24 h. At the end of treatment, RNA was extracted, and subjected to a real time PCR assays to detect *hTERT* and *c-Myc* mRNAs expression. The levels expression of *hTERT* (A) and *c-Myc* (B) were normalized with that of *GAPDH* and presented as fold of the untreated cells. Results are expressed as the mean values ± standard derivation of three experiments. $*P < 0.05$ and $**P < 0.01$ is the statistical significance of the difference between the values for each subfraction-treated and untreated cells.

Table 3.3 The fold of control in down regulation of *hTERT* and *c-Myc* mRNAs expression of A549 cells.

***Z. officinale* extracts**	**Fold of control in down regulation**	
	***hTERT* gene**	***c-Myc* gene**
E2.1	0.70 ± 0.17	0.50 ± 0.08
E2.2.1	0.68 ± 0.13	0.45 ± 0.16
E2.2.2	0.50 ± 0.25	0.30 ± 0.07
E2.2.3	0.50 ± 0.14	0.40 ± 0.3

3.4 Effect of *Z. officinale* extracts (E2 subfractions) on c-Myc protein using Western blot analysis.

In this study, we investigated whether the down-regulation of *c-Myc* mRNAs expression would lead to the reduction of c-Myc protein using western blotting analysis. We did not investigate hTERT protein because hTERT is present in a very low level and could not be detected by the commercially available hTERT antibody (Rockland), as presented in the company's manual. However, we opted to detect telomerase activity instead, as shown in the subsequent experiment. The results in Figure 3.5 show that, when compared with untreated cells, the c-Myc protein (left panel) was reduced in a dose dependent manner after treatment with the subfraction E2.1 or E2.2.1. On the contrary, treatment with the subfraction E2.2.2 or E2.2.3 showed little effect on the c-Myc protein expression in A549 cells. To show equal loading of protein, the blot was stripped and re-probed with anti-β actin antibody, as shown in the right panel.

Figure 3.5 Effect of the E2.1, E2.2.1, E2.2.2 and E2.2.3 subfraction in modulating the c-Myc protein expression in A549 cells. A549 cells were treated with a various concentrations of each subfractions for 48 h. The cell lysate was subjected to Western blotting using monoclonal c-Myc (left panel) at 1:2000, HRP conjugated goat anti-mouse IgG at 1:20000 and detected by Enhanced chemiluminescence (ECL). The β-actin was used as internal loading control (right panel).

3.5 Effect of *Z. officinale* extracts (E2 subfractions) on telomerase activity using modified fluorescent TRAP assay.

We investigated the effect of *Z. officinale* extracts (E2.1, E2.2.1, E2.2.2, E2.2.3 subfraction) on telomerase activity in two aspects: (a) the cellular effect on telomerase activity and (b) the direct effect on telomerase activity in a cell-free system. A549 cells were treated with the indicated extract for 24 h before the cells were lysed, and the crude cellular proteins were used as crude telomerase extract. The results in Figure 3.6 show that telomerase activity in the cells treated with each subfraction was reduced in a concentration-dependent manner. In Figure 3.7, we show that the telomerase inhibition seen in Figure 3.6 was not arisen from the direct inhibition of the enzyme itself. The results show that each subfractions did not affect telomerase activity, even at the highest concentration of 32 μg/ml when it was directly incubated in a cell-free system TRAP assay.

All of these experiments show convincingly that these *Z. officinale* extract subfractions can down-regulate *hTERT* expression, leading to a reduction in telomerase activity for the treated cells. The reduced telomerase activity is likely due to the diminished protein production rather than the direct inhibition of the enzyme. This down-regulation of *hTERT* expression paralleled the down-regulation of *c-Myc*. Since c-Myc and Max form heterodimers that activate *hTERT* expression, it is likely that the down-regulation of *c-Myc* precedes the down-regulation of *hTERT*.

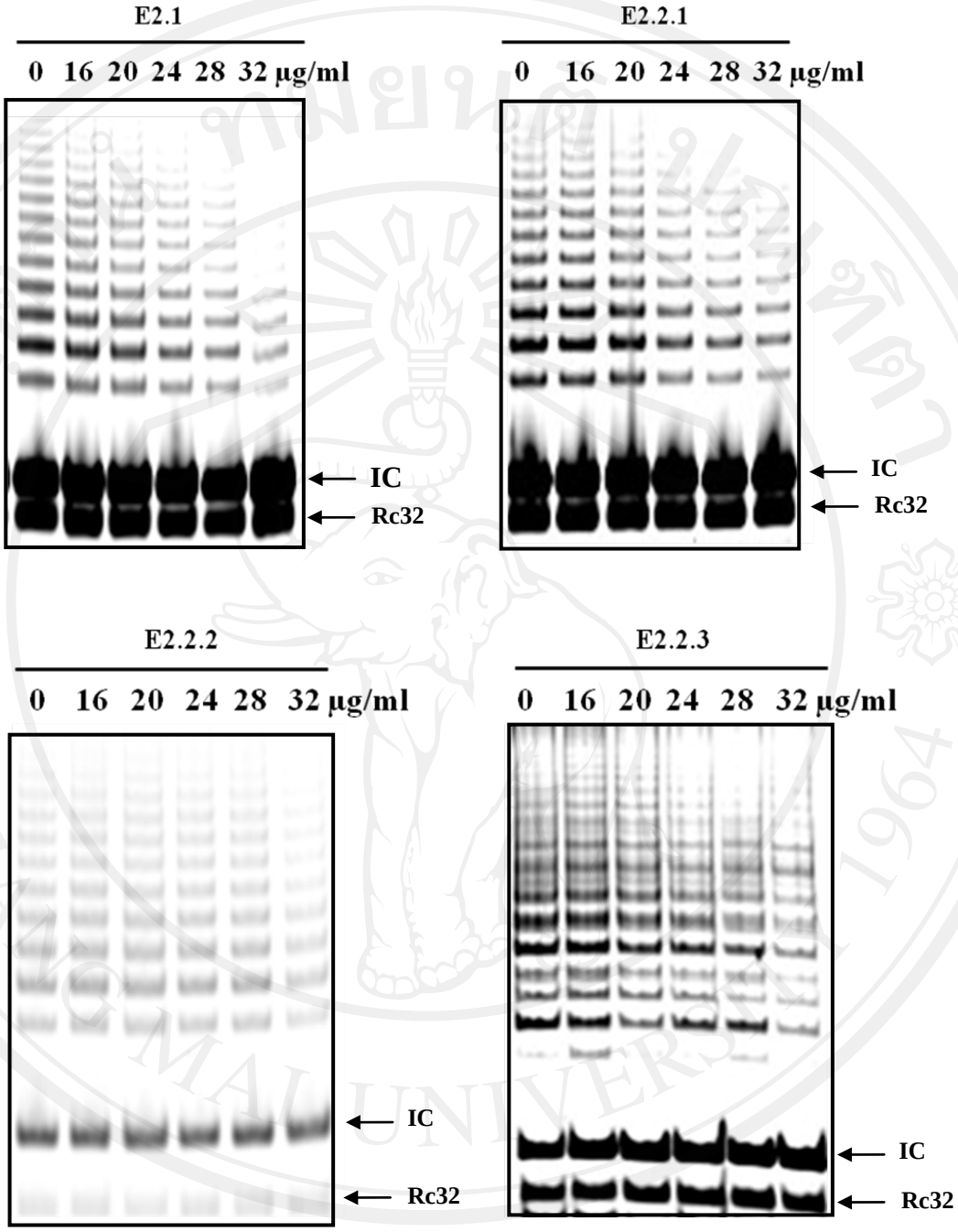


Figure 3.6 The cellular effect of the E2.1, E2.2.1, E2.2.2 and E2.2.3 subfraction on telomerase activity. A549 cells were treated with the indicated concentration of each subfractions for 48 h before 125 µg of the crude cell lysate was used as the source of telomerase in a modified fluorescent TRAP assay. IC represents the internal control and Rc32 represents the recovery control.

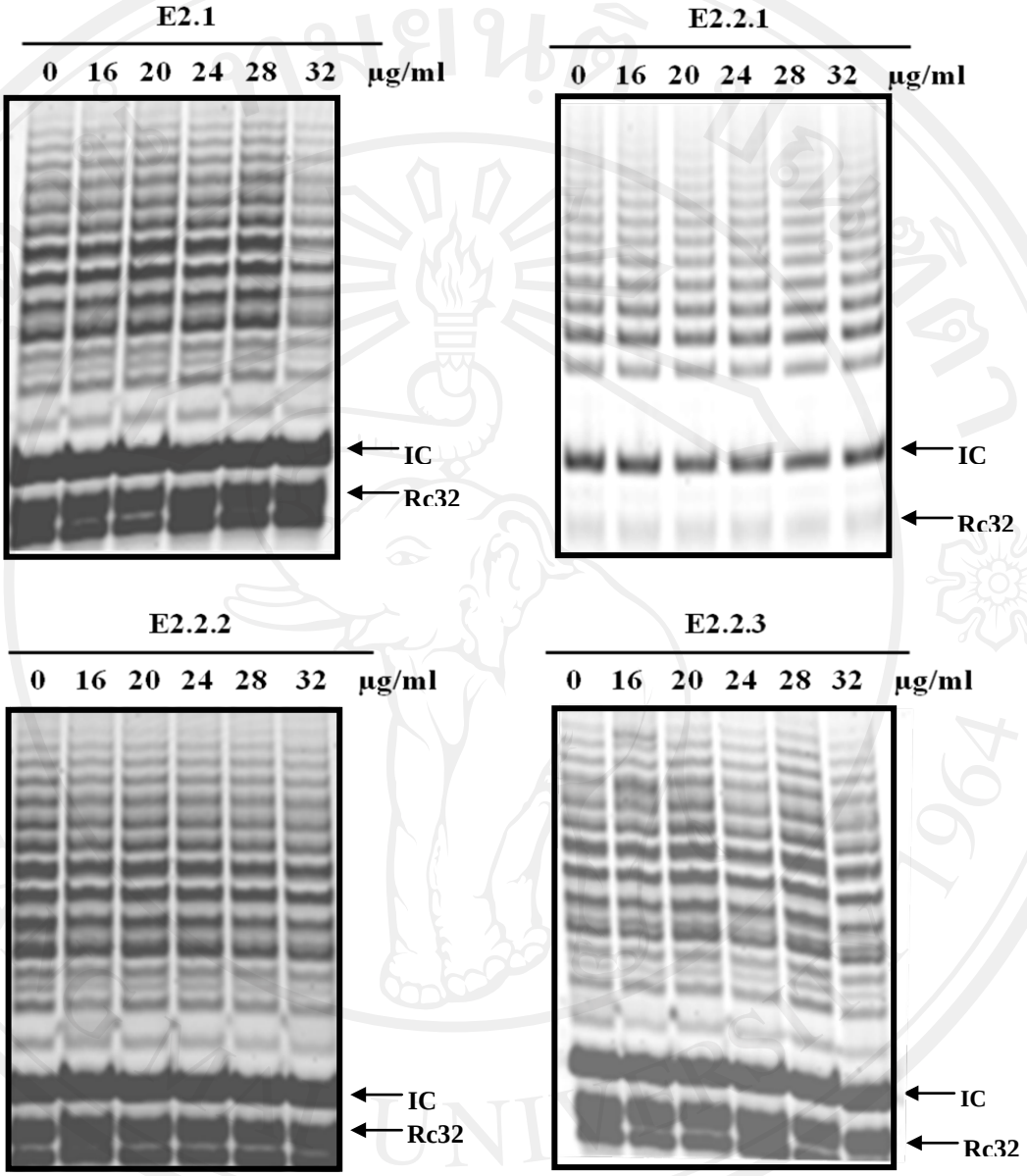


Figure 3.7 The direct effect of the E2.1, E2.2.1, E2.2.2 and E2.2.3 subfraction on telomerase activity in a cell-free system. The indicated concentration of each subfractions was incubated with the crude telomerase (125ng) produced from transfected HEK293T cells in modified fluorescent TRAP assay reaction mixture. IC represents the internal control and Rc32 represents the recovery control.

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3.6 Analyses of *Z. officinale* extracts by thin-layer chromatography (TLC).

In an effort to identify the active compound that is responsible for the down-regulation of *hTERT* and *c-Myc*, we employed TLC, HPLC, and GC/MS to examine the composition of the *Z. officinale* extracts. The TLC fingerprints (Figure 3.8) of each fraction and subfraction were separated and visualized with p-anisaldehyde/ sulfuric acid. The TLC fingerprints show that although each fraction was not pure, it contains major compounds that travel at the same R_f ratio. The results also show that 6-gingerol, appeared as a violet spot (R_f ratio of 0.39), was found as a major compound in E3 and E4 fractions, but not found in E1 and E2 fractions.

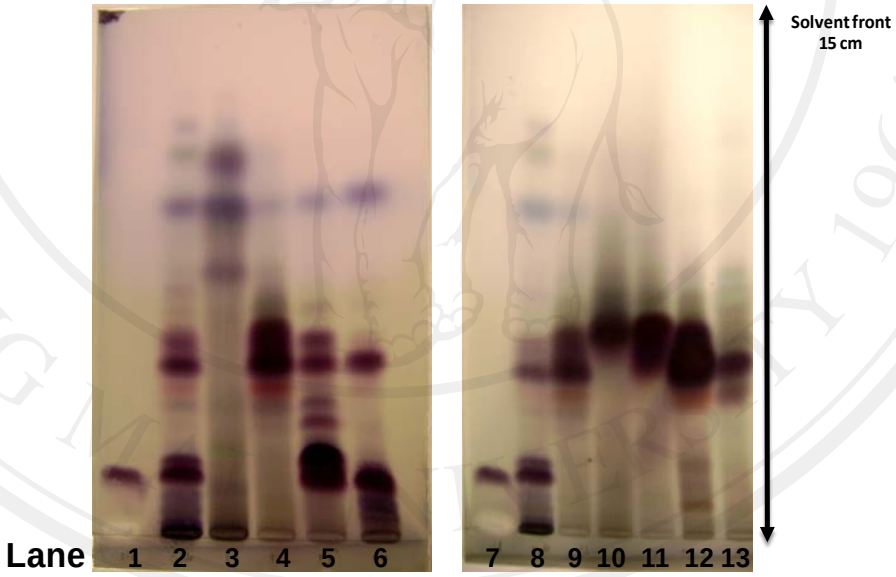


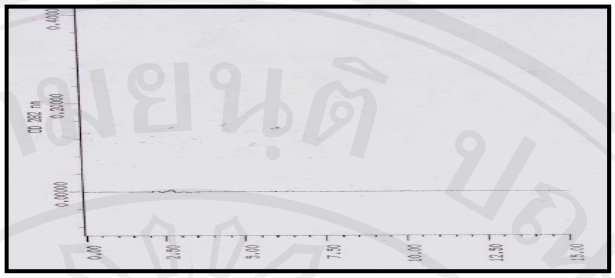
Figure 3.8 TLC fingerprints of *Z. officinale* extracts. TLC separation was run using the mixture of hexane:ethyl acetate (3:1) as mobile phase. The TLC plate was then dipped in p-anisaldehyde/sulfuric acid reagent before color developing by heating at 100°C. Lane1,7: Standard 6-gingerol; Lane 2,8 crude ethyl acetate fraction; Lane 3-6, the E1-E4 fractions, respectively; Lane 10-13, E2.1, E2.2.1, E2.2.2, and E2.2.3 subfraction, respectively.

3.7 Quantification of 6-gingerol content in *Z. officinale* extracts (E1-E4 fractions) by high performance liquid chromatography (HPLC).

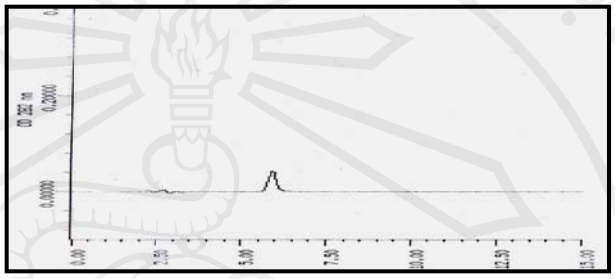
To determine 6-gingerol in the E1-E4 fractions for use as future reference for subsequence extraction, HPLC was exploited to detect the amount of 6-gingerol in each fraction. Figure 3.9 shows the chromatograms from 4 concentrations of standard 6-gingerol (Sigma). Table 3.4 summarizes the retention time (RT) and peak area from the chromatograms in two separate experiments. The average peak area values were then plotted against the amount of 6-gingerol to produce a standard curve (Figure 3.10).

The HPLC chromatograms of each fraction are shown in Figure 3.11-3.14. The retention time and peak area of each chromatogram are summarized in the Tables following the GC chromatogram. The retention time within 5.8-5.9 min was assumed to be 6-gingerol and its amount was quantified by comparing with the standard graph of 6-gingerol in Figure 3.10. In summary, E1 and E2 fractions contain less than 1% of 6-gingerol, while E3 and E4 fractions contain about 30% and 40% of 6-gingerol, respectively. Since E1 and E2 fractions contained less than 1% of 6-gingerol, the E2 subfraction were not subjected to HPLC analysis.

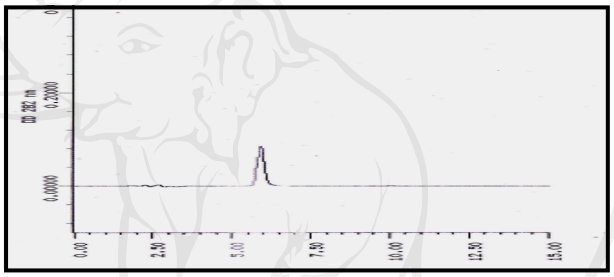
(A) 0 µg/ml



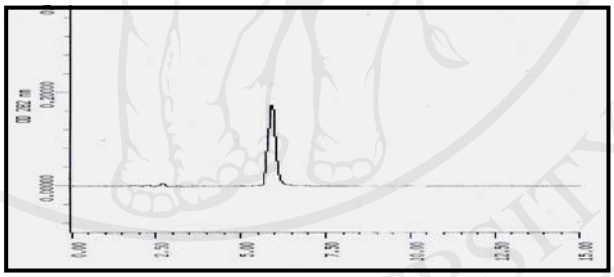
(B) 62.5 µg/ml



(C) 125 µg/ml



(D) 250 µg/ml



(E) 500 µg/ml

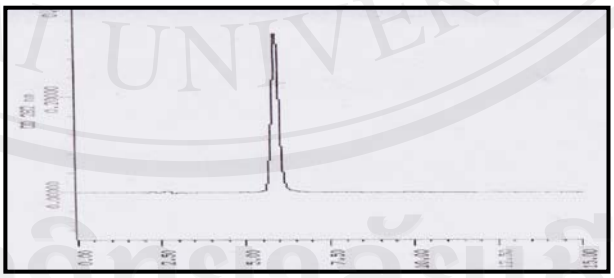


Figure 3.9 HPLC chromatograms of standard 6-gingerol. The 20 µl of standard 6-gingerol at the indicated concentration was separated by HPLC. The compound was detected by UV-visible detector (Spec Monitor® 3200) at 282 nm.

Table 3.4 Retention time and peak area from Standard 6-Gingerol Chromatograms.

6-Gingerol (μg)	Retention Time (min)		Peak Area		
	I	II	I	II	Average
0	0	0	0	0	0
1.25	5.85	5.88	71203	63642	67422.5
2.5	5.87	5.87	135806	127160	131483
5.0	5.87	5.85	274329	235798	255063.5
10.0	5.85	5.87	526047	440065	483056

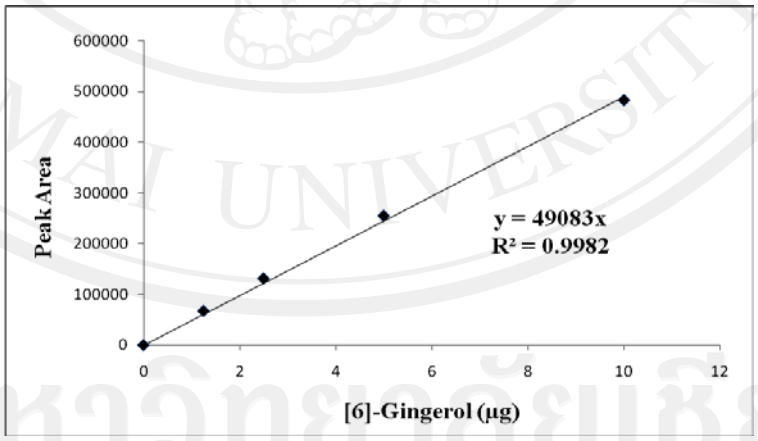
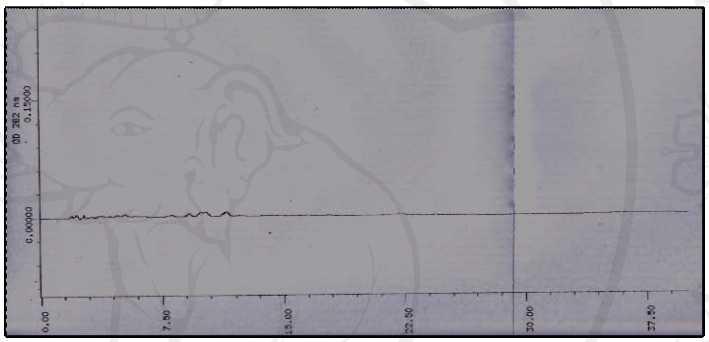


Figure 3.10 The standard curves of 6-gingerol.

(A) 1 mg/ml

(B) 2 mg/ml



(C) 4 mg/ml

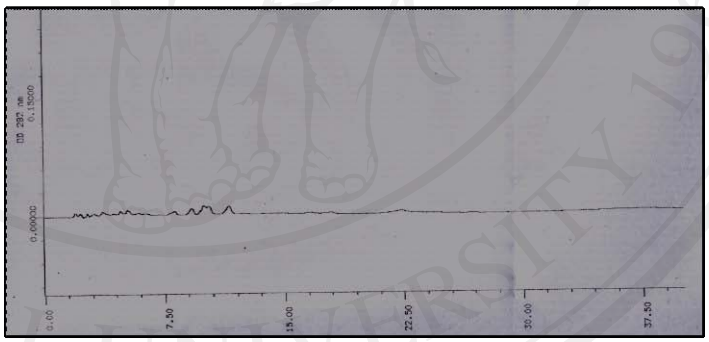


Figure 3.11 HPLC chromatograms of E1 fraction. The 20 μl of E1 fraction at the designated concentration was separated by HPLC using C18 reverse phase column as stationary phase and the mixture of acetonitrile and water (70:30 v/v) as the mobile phase at the flow rate of 1.0 ml/min. The compound was detected by UV-visible detector (Spec Monitor® 3200) at 282 nm.

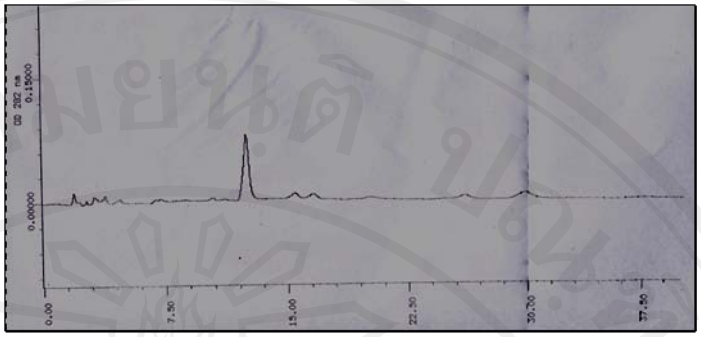
Table 3.5 Retention time and peak area from HPLC chromatograms of E1 fraction at 4 μg/ml

Retention Time (min)	Peak Area	Retention Time (min)	Peak Area
3.62	10539	9.17	14564
4.7	7245	9.9	22414
5.15	9941	10.2	17652
5.85 (6-gingerol)	**2218**	11.45	22323
8.1	10233		

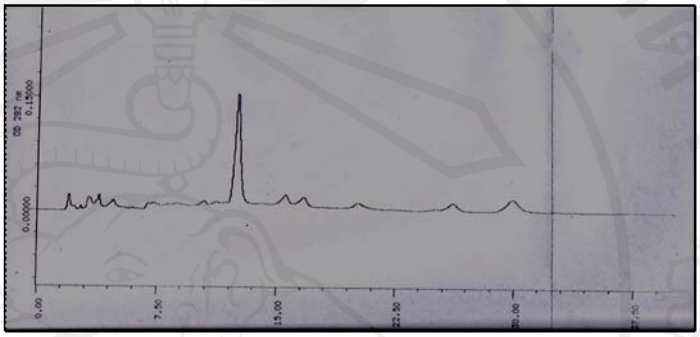
Table 3.6 Quantification of 6-gingerol from E1 fraction

E1 fraction (μg)	Retention Time (min)	Peak Area	Amount of 6-Gingerol (μg)	% of 6-Gingerol
20	-	-	-	-
40	-	-	-	-
80	5.85	2218	0.04	0.05

(A) 1 mg/ml



(B) 2 mg/ml



(C) 4 mg/ml

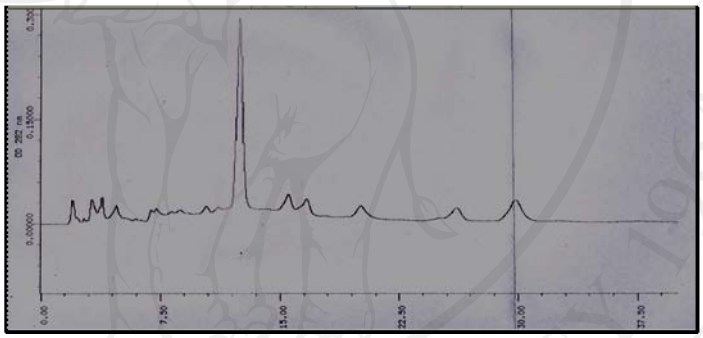


Figure 3.12 HPLC chromatograms of E2 fraction . The 20 µl of E2 fraction at the designated concentration was separated by HPLC using C18 reverse phase column as stationary phase and the mixture of acetonitrile and water (70:30 v/v) as the mobile phase at the flow rate of 1.0 ml/min. The compound was detected by UV-visible detector (Spec Monitor® 3200) at 282 nm.

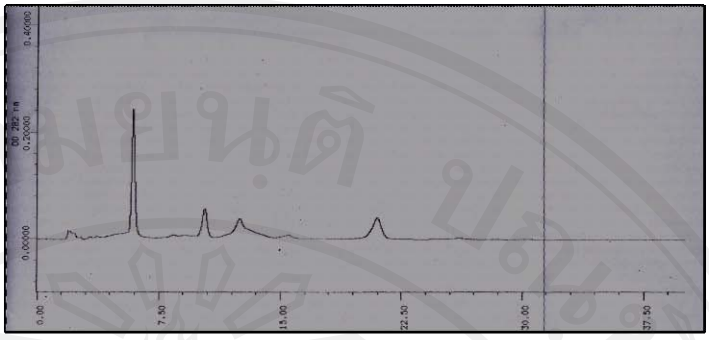
Table 3.7 Retention time and peak area from HPLC chromatograms of E2 fraction at 4 µg/ml

Retention Time (min)	Peak Area	Retention Time (min)	Peak Area
1.95	58443	8.68	14464
3.13	79790	10.33	18532
3.78	70573	12.42	654407
4.67	82563	15.45	37919
5.88 (6-gingerol)	**5096**	16.58	39027
6.87	23494	20	66593
7.18	33337	26.05	52060
8.13	16602	29.78	115122

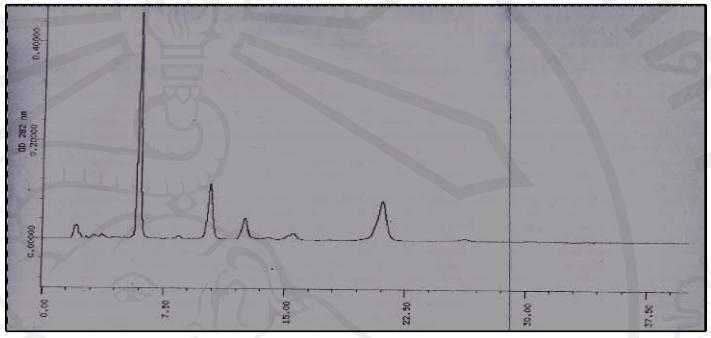
Table 3.8 Quantification of 6-gingerol from E2 fraction

E2 fraction (µg)	Retention Time (min)	Peak Area	Amount of 6-Gingerol (µg)	% of 6-Gingerol
20	5.88	735	0.01	0.07
40	5.88	1456	0.03	0.07
80	5.88	5096	0.10	0.12

(A) 1 mg/ml



(B) 2 mg/ml



(C) 4 mg/ml

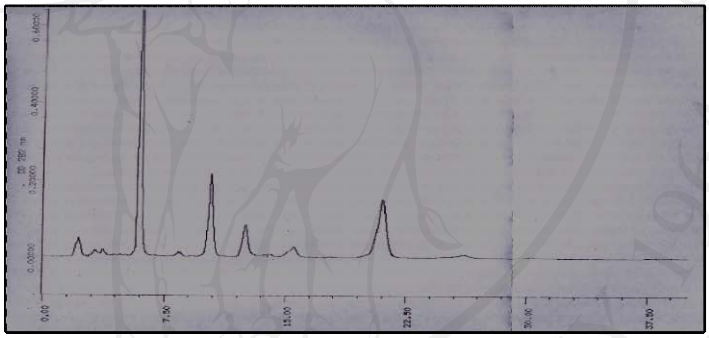


Figure 3.13 HPLC chromatograms of E3 fraction. The 20 µl of E3 fraction at the designated concentration was separated by HPLC using C18 reverse phase column as stationary phase and the mixture of acetonitrile and water (70:30 v/v) as the mobile phase at the flow rate of 1.0 ml/min. The compound was detected by UV-visible detector (Spec Monitor® 3200) at 282 nm.

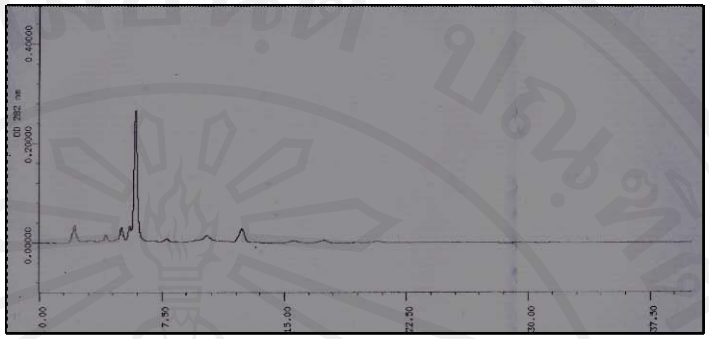
Table 3.9 Retention time and peak area from HPLC chromatograms of E3 fraction at 4 µg/ml

Retention Time (min)	Peak Area	Retention Time (min)	Peak Area
2.1	113134	10.3	448714
3.12	39509	12.43	200064
3.6	23463	15.45	91624
5.87(6-gingerol)	**1153637**	20.95	545834
8.33	26498		

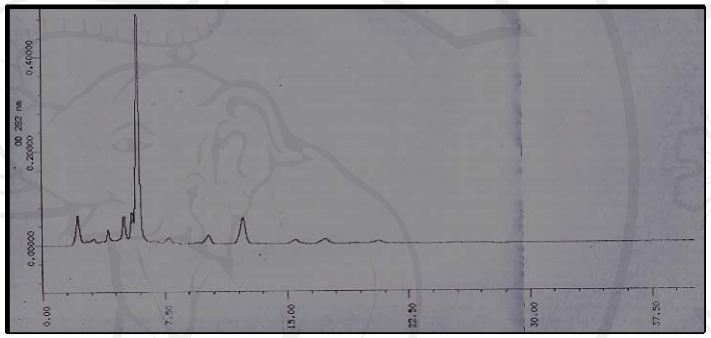
Table 3.10 Quantification of 6-gingerol from E3 fraction

E3 fraction (µg)	Retention Time (min)	Peak Area	Amount of 6-Gingerol (µg)	% of 6-Gingerol
20	5.85	320904	6.29	31.5
40	5.87	632678	12.40	31.0
80	5.87	1153637	22.61	28.3

(A) 1 mg/ml



(B) 2 mg/ml



(C) 4 mg/ml

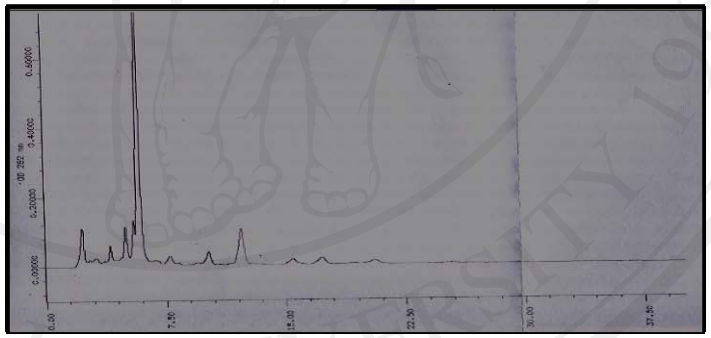


Figure 3.14 HPLC chromatograms of E4 fraction. The 20 µl of E4 fraction at the designated concentration was separated by HPLC using C18 reverse phase column as stationary phase and the mixture of acetonitrile and water (70:30 v/v) as the mobile phase at the flow rate of 1.0 ml/min. The compound was detected by UV-visible detector (Spec Monitor® 3200) at 282 nm.

Table 3.11 Retention time and peak area from HPLC chromatograms of E4 fraction at 4 µg/ml

Retention Time (min)	Peak Area	Retention Time (min)	Peak Area
2.25	194570	6.78	11833
2.67	23477	7.7	70041
3.12	66410	10.12	80638
3.98	44192	12.17	247646
4.93	113918	15.4	51962
5.45	52015	17.23	65068
5.82 (6-gingerol)	**1659756**	20.48	22920

Table 3.12 Quantification of 6-gingerol from E4 fraction

E4 fraction (µg)	Retention Time (min)	Peak Area	Amount of 6-Gingerol (µg)	% of 6-Gingerol
20	5.83	435927	8.55	42.7
40	5.82	932041	18.27	45.7
80	5.82	1659756	32.5	40.7

3.8 Identify of active compounds in *Z. officinale* extracts by Gas Chromatography/Mass Spectrometry (GC/MS)

In order to identify compounds in each subfractions, we analyzed each subfraction by GC/MS. Figure 3.15-3.17 shows the GC chromatogram, the GC chromatogram data, and selected MS spectra of major peaks were identified for the E2.1 subfraction, respectively. In the same manner, Figure 3.18-3.20 are for E2.2.1, Figure 3.21-3.23 are for E2.2.2, and Figure 3.24-3.26 are for E2.2.3, respectively. Based on the fragment analysis of compounds in ginger reported by Jolad et al.[77], we identified the major compounds in each fraction and summarized in Table 3.13.

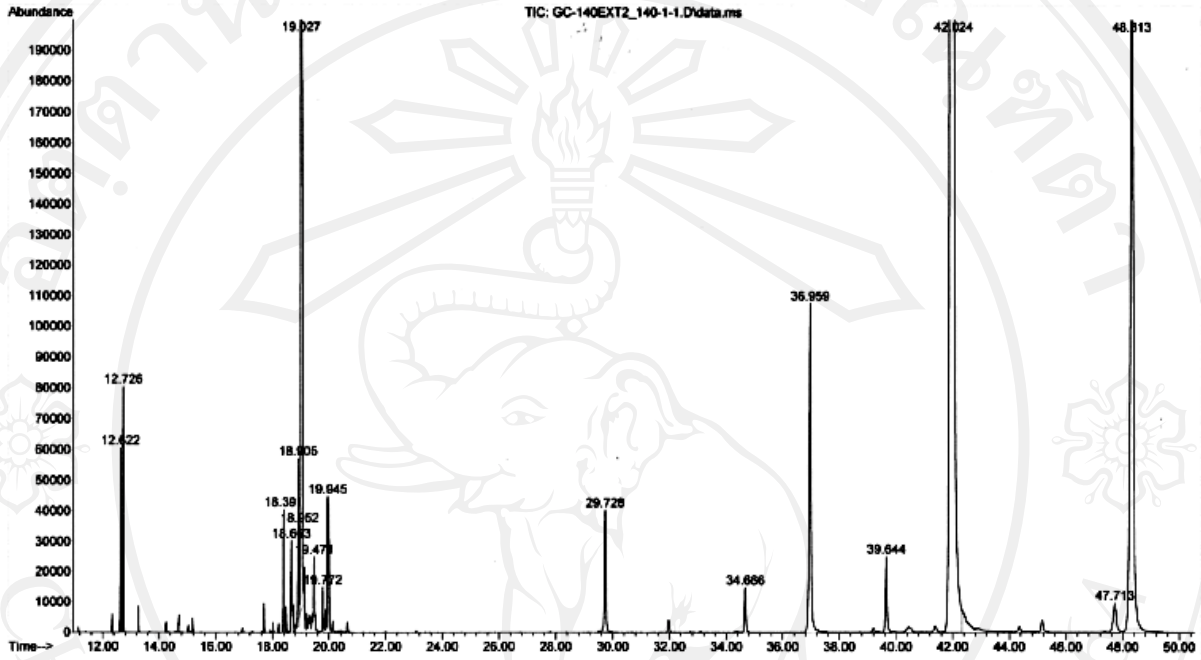


Figure 3.15 GC/MS chromatogram of E2.1 subfraction. GC-MS data were recorded with a GC 7890A from Agilent Technologies and MSD 5975 (EI). The gas chromatograph was fitted with a HP5-MS column (30 m × 0.25 mm ID × 0.25 μm film thickness) and used the following temperature programming (50 °C, 5 min; to 180 °C at 10 °C/min; to 250 °C at 3 °C/min; and 250 °C, 10 min), ionizing voltage 70 eV, and 1 μl split injection (split ratio 25:1). Helium was used as the carrier gas at a flow rate of 1.5 ml/min.

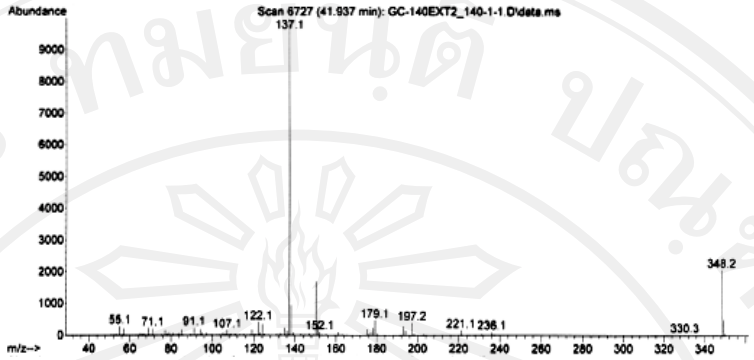
```
Integration Parameters: rteint.p
Integrator: RTE
Smoothing : ON                                  Filtering: 5
Sampling  : 1                                    Min Area: 7000 Area counts
Start Thrs: 0.5                                 Max Peaks: 17
Stop Thrs : 0                                Peak Location: TOP

If leading or trailing edge < 100 prefer < Baseline drop else tangent >
Peak separation: 5

Method    : C:\msdchem\1\METHODS\GC-140EXT2.M
Title     :

Signal     : TIC: GC-140EXT2_140-1-1.D\data.ms
```

peak #	R.T. min	first scan	max scan	last scan	PK TY	peak height	corr. area	corr. % max.	% of total
1	12.622	1645	1651	1658	rBB	60610	77913	0.57%	0.430%
2	12.726	1662	1669	1679	rBB	80543	124995	0.92%	0.690%
3	18.391	2643	2650	2659	rBV	40587	61016	0.45%	0.337%
4	18.663	2691	2697	2707	rBV4	30315	65470	0.48%	0.361%
5	18.905	2734	2739	2744	rBV	55393	94425	0.69%	0.521%
6	18.952	2744	2747	2751	rVV2	30042	44328	0.32%	0.245%
7	19.027	2751	2760	2772	rVV5	421725	1286824	9.42%	7.105%
8	19.471	2833	2837	2843	rVB2	20554	35974	0.26%	0.199%
9	19.772	2877	2889	2893	rBV3	14959	28399	0.21%	0.157%
10	19.945	2912	2919	2922	rBV3	43551	92403	0.68%	0.510%
11	29.728	4602	4613	4639	rVB	40114	134049	0.98%	0.740%
12	34.666	5454	5468	5485	rBV2	14851	54743	0.40%	0.302%
13	36.959	5833	5865	5905	rBV2	107608	524641	3.84%	2.897%
14	39.644	6314	6330	6361	rBV3	25032	110005	0.81%	0.607%
15	42.024	6689	6742	6793	rBV	2063746	13656660	100.00%	75.406%
16	47.713	7694	7727	7761	rBV2	9729	79745	0.58%	0.440%
17	48.313	7796	7831	7881	rBV2	220021	1639317	12.00%	9.052%

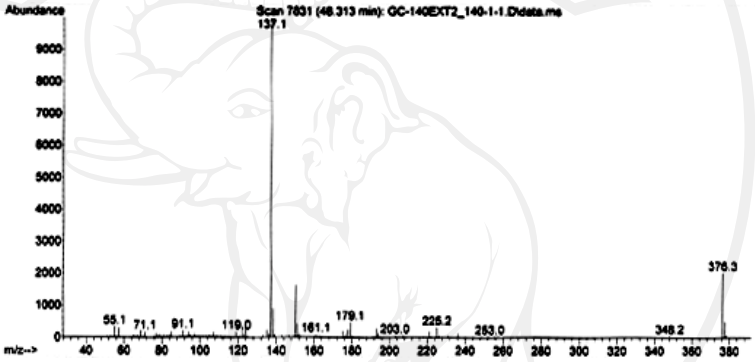
```
                        Sum of corrected areas:    18110907
```

Figure 3.16 GC chromatogram data from E2.1 subfraction.

A.



B.



C.

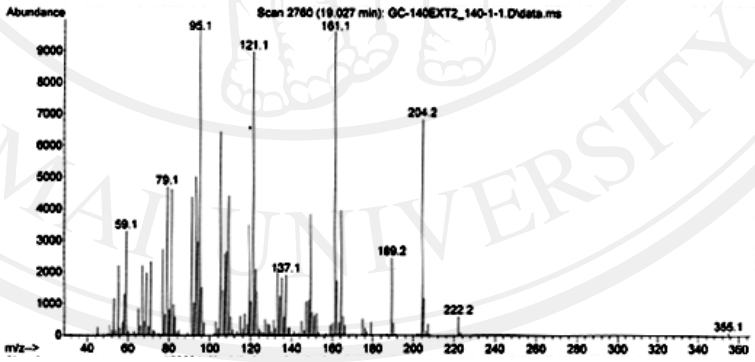


Figure 3.17 Selected GC/MS spectra from E2.1 subfraction. The spectra are from the 3 most abundance peaks: A) Peak #15 (75.41%), B) Peak #17 (9.05%), and C) Peak #7 (7.11%). The compounds are identified as 11-paradol, 13-paradol, and β-bisabolene, respectively.

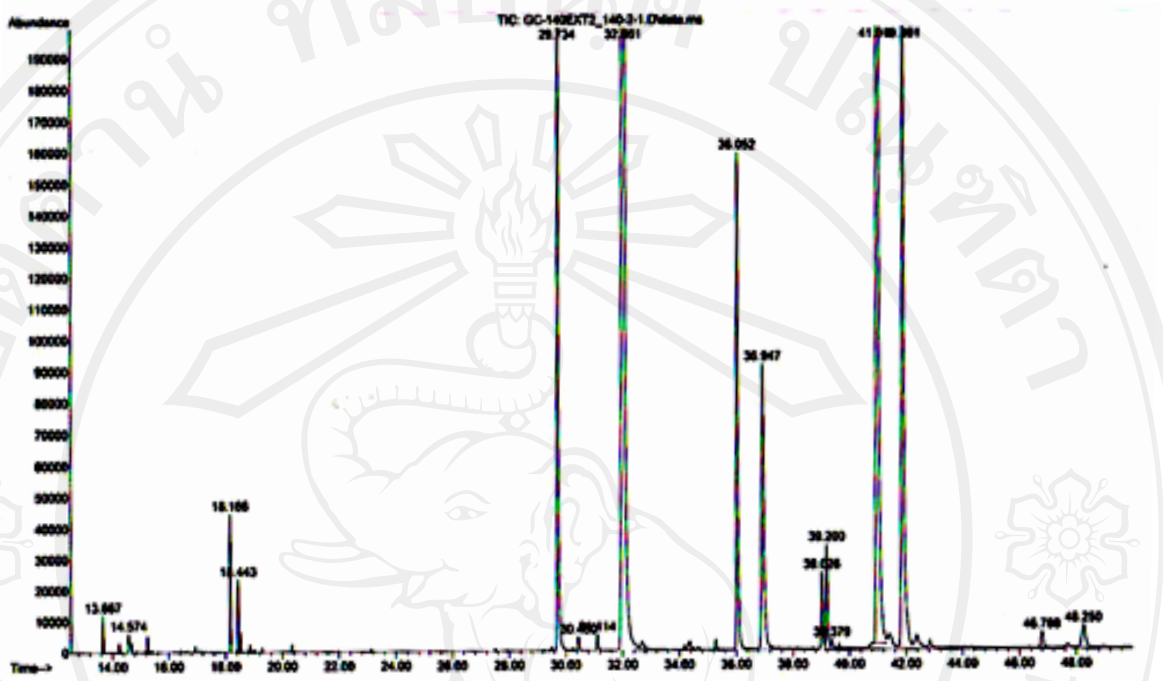


Figure 3.18 GC/MS chromatogram of E2.2.1 subfraction. GC-MS data were recorded with a GC 7890A from Agilent Technologies and MSD 5975 (EI). The gas chromatograph was fitted with a HP5-MS column (30 m × 0.25 mm ID × 0.25 μm film thickness) and used the following temperature programming (50 °C, 5 min; to 180 °C at 10 °C/min; to 250 °C at 3 °C/min; and 250 °C, 10 min), ionizing voltage 70 eV, and 1 μl split injection (split ratio 25:1). Helium was used as the carrier gas at a flow rate of 1.5 ml/min.

Integration Parameters: rteint.p
Integrator: RTE
Smoothing : ON Filtering: 5
Sampling : 1 Min Area: 7000 Area counts
Start Thrs: 0.5 Max Peaks: 17
Stop Thrs : 0 Peak Location: TOP

If leading or trailing edge < 100 prefer < Baseline drop else tangent >
Peak separation: 5

Method : C:\msdchem\1\METHODS\GC-140EXT2.M
Title :

Signal : TIC: GC-140EXT2_140-2-1.D\data.ms

peak #	R.T. min	first scan	max scan	last scan	PK TY	peak height	corr. area	corr. % max.	% of total
1	13.667	1826	1832	1840	rBB	12099	14938	0.26%	0.108%
2	14.574	1983	1989	2001	rBV	6193	16777	0.29%	0.121%
3	18.166	2605	2611	2620	rBB	44738	62682	1.08%	0.452%
4	18.443	2654	2659	2665	rBB	23628	31504	0.54%	0.227%
5	29.734	4592	4614	4651	rBV	246093	773445	13.34%	5.580%
6	30.450	4726	4738	4751	rBB2	4568	14913	0.26%	0.108%
7	31.114	4842	4853	4877	rBB2	5282	18576	0.32%	0.134%
8	32.061	4979	5017	5078	rBV	1090380	5799982	100.00%	41.842%
9	36.052	5690	5708	5739	rBV	159505	560858	9.67%	4.046%
10	36.947	5830	5863	5905	rBV2	91749	528546	9.11%	3.813%
11	39.026	6207	6223	6238	rBV	25301	98033	1.69%	0.707%
12	39.200	6240	6253	6273	rVV	33974	137299	2.37%	0.991%
13	39.379	6277	6284	6310	rVB3	2890	12273	0.21%	0.089%
14	41.019	6543	6568	6619	rBV	1048066	4546273	78.38%	32.798%
15	41.891	6694	6719	6772	rBV	218509	1185027	20.43%	8.549%
16	46.788	7547	7567	7592	rBV4	5384	32223	0.56%	0.232%
17	48.250	7799	7820	7822	rBV2	6842	28205	0.49%	0.203%

Sum of corrected areas: 13861554

Figure 3.19 GC chromatogram data from E2.2.1 subfraction.

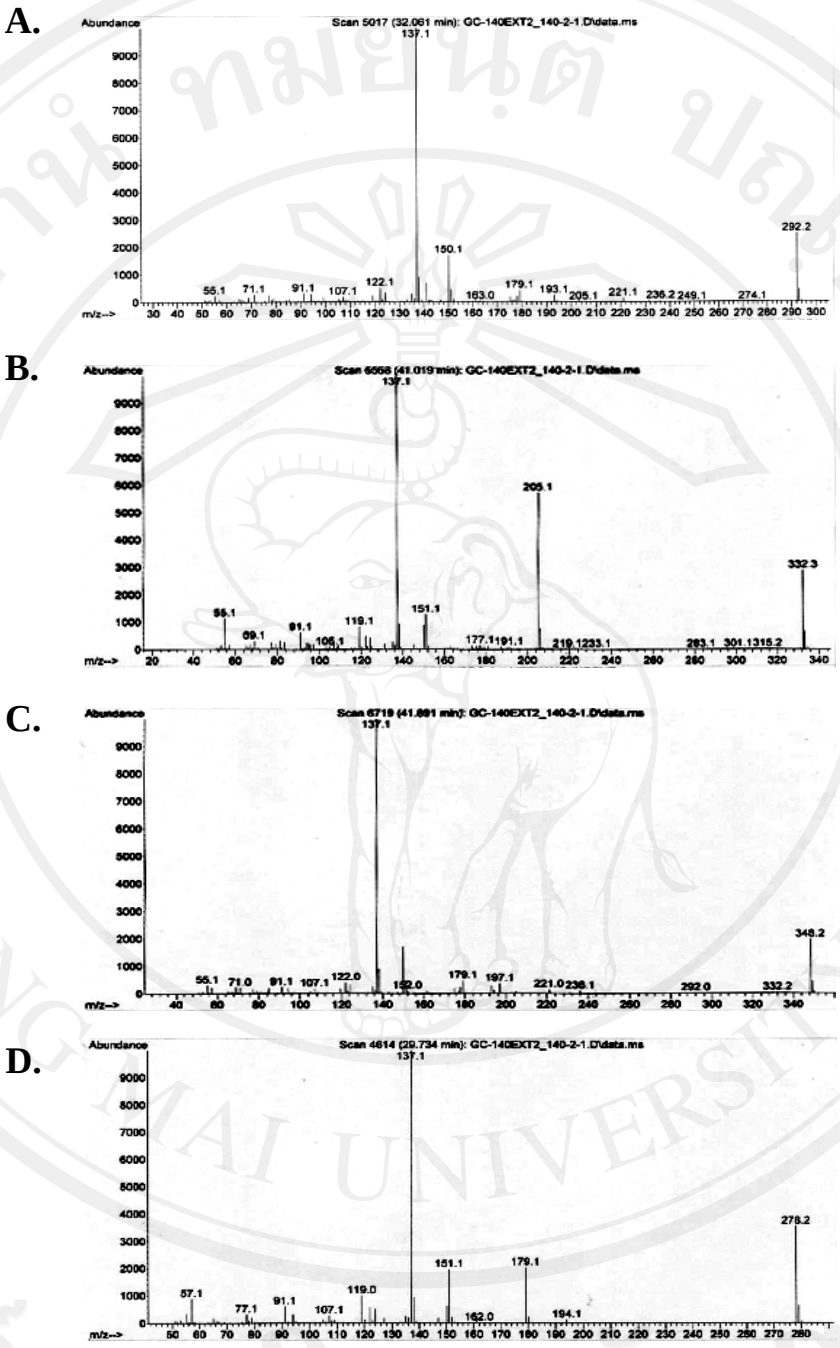


Figure 3.20 Selected GC/MS spectra from E2.2.1 subfraction. The spectra are from the four most abundance peaks: A) Peak #8 (41.8%), B) Peak #14 (32.8%), C) Peak #15 (8.5%), and D) Peak #5 (5.6%). The compounds are identified as 7-paradol, 10-shogaol, 11-paradol, and 6-paradol, respectively.

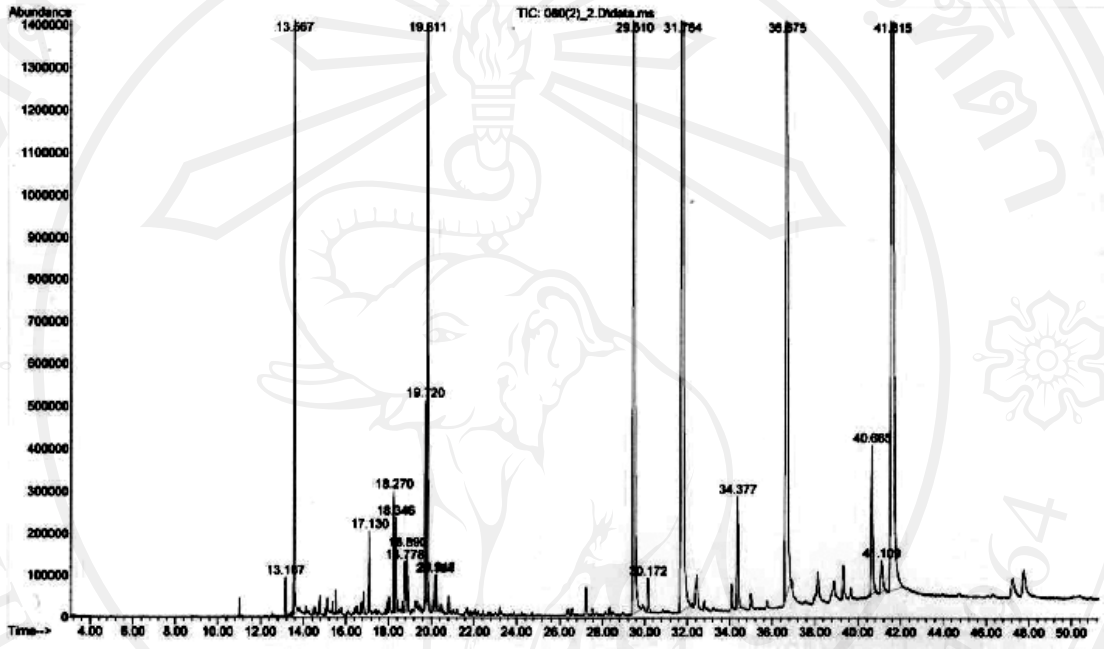


Figure 3.21 GC/MS chromatogram of E2.2.2 subfraction. GC-MS data were recorded with a GC 7890A from Agilent Technologies and MSD 5975 (EI). The gas chromatograph was fitted with a HP5-MS column (30 m × 0.25 mm ID × 0.25 μm film thickness) and used the following temperature programming (50 °C, 5 min; to 180 °C at 10 °C/min; to 250 °C at 3 °C/min; and 250 °C, 10 min), ionizing voltage 70 eV, and 1 μl split injection (split ratio 25:1). Helium was used as the carrier gas at a flow rate of 1.5 ml/min.

Data Path : D:\data\2011\Mar\9\
Data File : 080(2)_2.D
Acq On : 9 Mar 2011 15:52
Operator :
Sample : 080(2)_200x
Misc :
ALS Vial : 57 Sample Multiplier: 1 Samp. Amt.: 1

Integration Parameters: autoint1.e
Integrator: ChemStation

Method : C:\msdchem\1\METHODS\MeOH_ext.M
Title :

Signal : TIC: 080(2)_2.D\data.ms

peak #	R.T. min	first scan	max scan	last scan	PK TY	peak height	corr. area	corr. % max.	% of total
1	13.167	1706	1714	1725	BB	91844	1471508	0.64%	0.167%
2	13.567	1766	1782	1810	BB	1675895	25247765	10.97%	2.869%
3	17.130	2383	2388	2396	VV	187873	2849011	1.24%	0.324%
4	18.270	2561	2581	2588	PV	283285	4895902	2.13%	0.556%
5	18.346	2588	2594	2610	VV 3	223400	4595450	2.00%	0.522%
6	18.778	2657	2668	2674	PV 4	120740	2678803	1.16%	0.304%
7	18.890	2674	2687	2702	VV 9	150218	5905791	2.57%	0.671%
8	19.720	2815	2828	2835	PV	490715	9532364	4.14%	1.083%
9	19.811	2835	2843	2853	VV	1349816	24980402	10.85%	2.838%
10	20.164	2898	2903	2911	VV 2	85413	1991588	0.87%	0.226%
11	20.242	2911	2917	2933	VB 2	86910	2019875	0.88%	0.229%
12	29.510	4470	4492	4549	BB	5031797	192394913	83.60%	21.860%
13	30.172	4586	4605	4629	BB 2	82435	3217681	1.40%	0.366%
14	31.764	4847	4875	4944	BV 3	2825624	197241771	85.70%	22.410%
15	34.377	5289	5320	5352	VB	269431	11217088	4.87%	1.274%
16	36.675	5681	5710	5744	BV	2254444	139532635	60.63%	15.854%
17	40.665	6346	6389	6416	BV 2	349818	15481843	6.73%	1.759%
18	41.109	6447	6464	6491	BV 2	76182	4732959	2.06%	0.538%
19	41.615	6513	6550	6634	BV 2	3370277	230148009	100.00%	26.149%

Sum of corrected areas: 880135358

MeOH_ext.M Thu Mar 10 15:45:53 2011

Figure 3.22 GC chromatogram data from E2.2.2 subfraction.

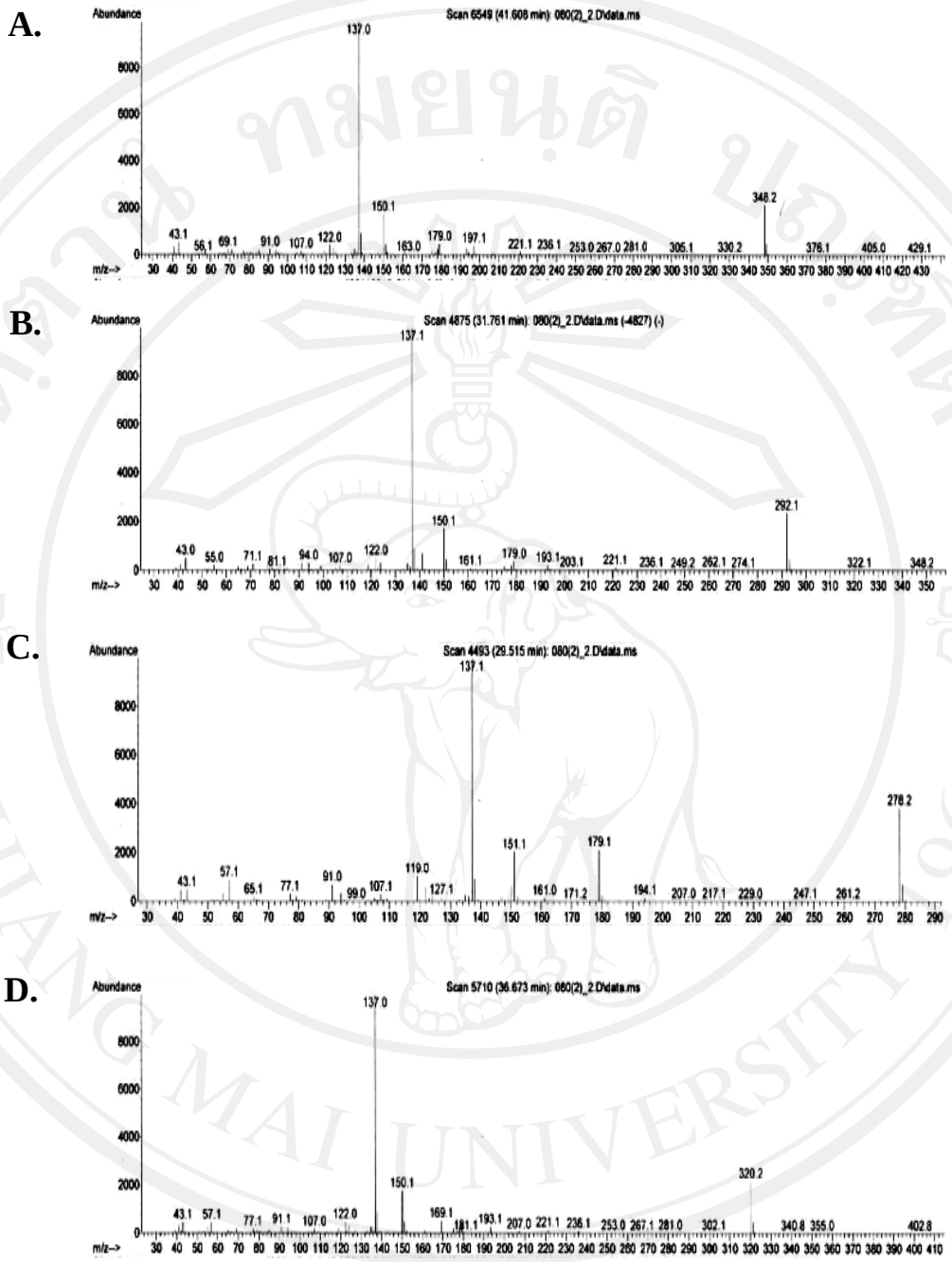


Figure 3.23 Selected GC/MS spectra from E2.2.2 subfraction. The spectra are from the 4 most abundance peaks: A) Peak #19 (26.1%), B) Peak #14 (22.4%), C) Peak #12 (21.9%), and D) Peak #16 (15.8%). The compounds are identified as 11-paradol, 7-paradol, 6-paradol, and 9-paradol, respectively.

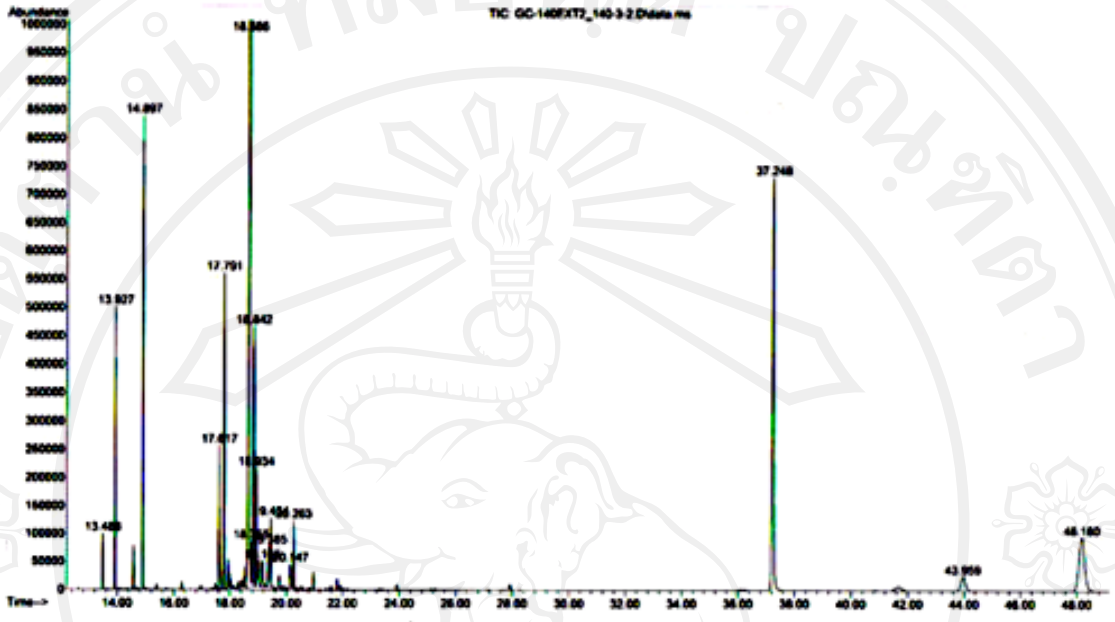


Figure 3.24 GC/MS chromatogram of E2.2.3 subfraction. GC-MS data were recorded with a GC 7890A from Agilent Technologies and MSD 5975 (EI). The gas chromatograph was fitted with a HP5-MS column (30 m × 0.25 mm ID × 0.25 μm film thickness) and used the following temperature programming (50 °C, 5 min; to 180 °C at 10 °C/min; to 250 °C at 3 °C/min; and 250 °C, 10 min), ionizing voltage 70 eV, and 1 μl split injection (split ratio 25:1). Helium was used as the carrier gas at a flow rate of 1.5 ml/min.

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  Sample    : 53/140
  Misc      :
  ALS Vial  : 98   Sample Multiplier: 1

  Integration Parameters: rteint.p
  Integrator: RTE
  Smoothing : ON                          Filtering: 5
  Sampling  : 1                           Min Area: 7000 Area counts
  Start Thrs: 0.5                         Max Peaks: 17
  Stop Thrs : 0                       Peak Location: TOP

  If leading or trailing edge < 100 prefer < Baseline drop else tangent >
  Peak separation: 5

  Method    : C:\msdchem\1\METHODS\GC-140EXT2.M
  Title     :

  Signal      : TIC: GC-140EXT2_140-3-2.D\data.ms

 peak   R.T. first  max last  PK   peak      corr.    corr.    % of
   #    min  scan scan scan  TY  height      area    % max.   total
 ---  ------ ----- ---- ---- --- -------  -------  ------  -------
  1   13.488  1795 1801 1808 rBV   100784   130997   1.20%   0.624%
  2   13.927  1867 1877 1898 rBB   501143   718673   6.56%   3.422%
  3   14.897  2038 2045 2055 rBV   840147  1070260   9.76%   5.097%
  4   17.617  2510 2516 2528 rBV   257313   429562   3.92%   2.046%
  5   17.791  2533 2546 2565 rVB   561303   842565   7.69%   4.012%

  6   18.686  2680 2701 2709 rBV  4913822 10961073 100.00%  52.198%
  7   18.755  2710 2713 2721 rVB3   74345   113021   1.03%   0.538%
  8   18.842  2721 2728 2737 rVB   461755   840083   7.66%   4.001%
  9   18.934  2737 2744 2751 rBV2  212310   379605   3.46%   1.808%
 10   19.165  2773 2784 2793 rBV5   46909   156752   1.43%   0.746%

 11   19.385  2809 2822 2827 rBV2   72454   206629   1.89%   0.984%
 12   19.454  2827 2834 2843 rVB2  125250   260204   2.37%   1.239%
 13   20.147  2944 2954 2966 rBV4   45863   119838   1.09%   0.571%
 14   20.263  2966 2974 2984 rVV    122436   229402   2.09%   1.092%
 15   37.248  5892 5915 5960 rBV    732415  3046556  27.79%  14.508%

 16   43.959  7043 7077 7080 rBV     25129   151565   1.38%   0.722%
 17   48.180  7759 7808 7860 rBV     95642  1342219  12.25%   6.392%

                          Sum of corrected areas:    20999004

GC-140EXT2.M Mon May 17 16:56:17 2010
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Figure 3.25 GC chromatogram data from E2.2.3 subfraction.

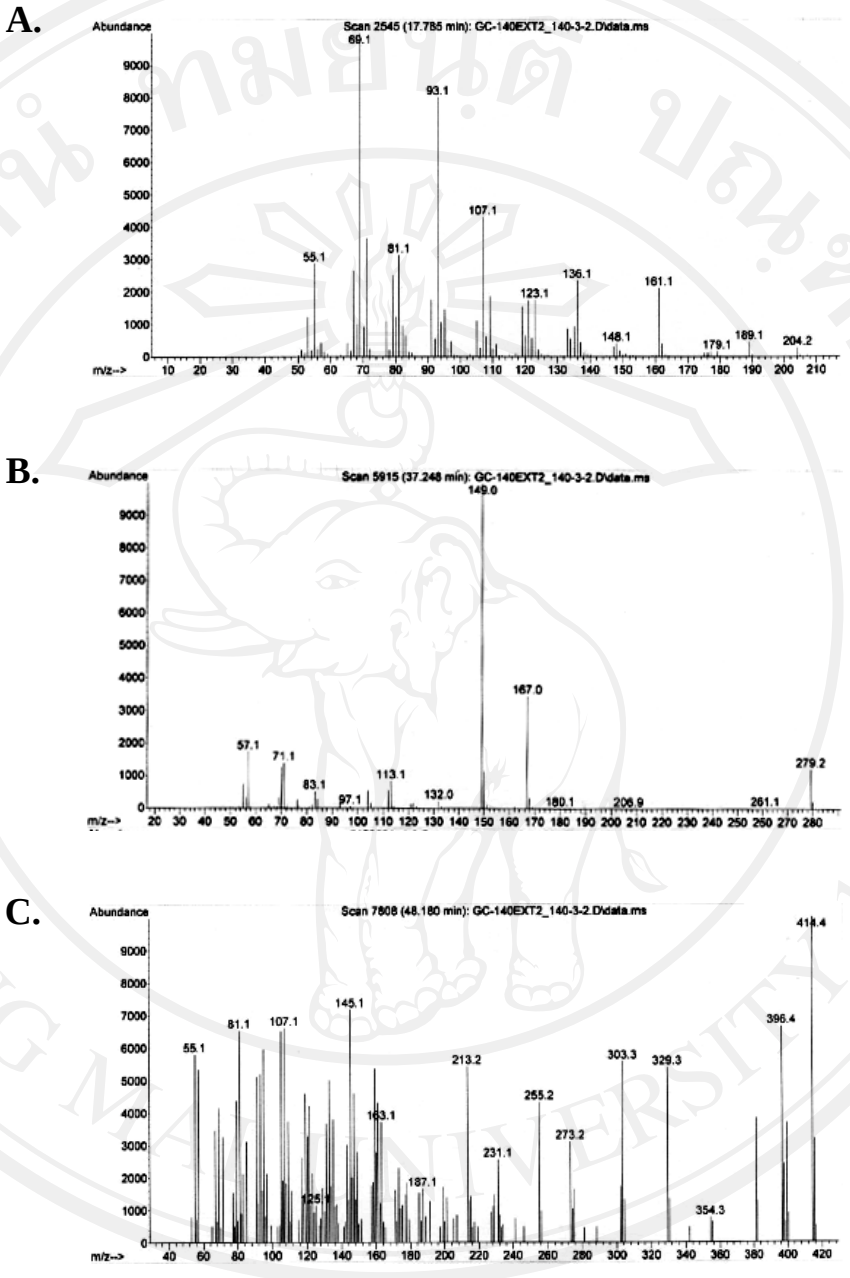

Figure 3.26 Selected GC/MS spectra from E2.2.3 subfraction. The spectra are from the 3 most abundance peaks: A) Peak #6 (52.2%), B) Peak #15 (14.5%), and C) Peak #17 (6.4%). The compounds are identified as γ-cadinene, 1,2-benzenedicarboxylic acid, diisooctyl ester, and stigmasterol, respectively.

Table 3.13 Major compounds from the E2 subfractions

E2.1	E2.2.1	E2.2.2	E2.2.3
11-paradol (75.4%) 13-paradol (9.1%) Bisabolene (7.1%)	7-paradol (41.8) 10-shogaol (32.8%) 11-paradol (8.5%) 6-paradol (5.6%)	11-paradol (26.1%) 7-paradol (22.4%) 6-paradol (21.9%) 9-paradol (15.9%)	Cadinene (52.2%) Benzene dicarboxylic diisooctyl ester (14.5%) Stigmasterol (6.4%)

According to Table 3.13, many subfractions contain paradols as their major compounds. We wonder whether these paradols could be responsible for the down-regulation of *hTERT* and *c-Myc* gene in A549 cells. Therefore, the effects of 6-paradol on hTERT and c-Myc mRNAs expression were tested using semi-quantitative RT-PCR in our next experiment.

3.9 Effect of 6-paradol on *hTERT* and *c-Myc* mRNAs expression in A549 cells using Semi-quantitative RT-PCR.

In the attempt to find active compound that suppress *hTERT* and *c-Myc* expression, we did the assay-guided purification of *Z. officinale* rhizome extract. It is known that in each subfractions consists of a few substances, but a major compound that is commonly found in many subfractions is paradol. Therefore, we are interested to test whether 6-paradol could suppress *hTERT* and *c-Myc* expression in A549 cells. Figure 3.27 and 3.28 showed that 6-paradol could significantly reduce *hTERT* and *c-Myc* mRNA expression in a dose-dependent manner. The *hTERT* and *c-Myc* expression were down-regulated about 73% and 23%, respectively, after A549 cells were treated with 32 μM 6-paradol for 24 h. At this concentration, 6-paradol exhibited less than 50% toxicity to A549 cells, as determined by SRB assay. Therefore, the reduction of *hTERT* and *c-Myc* mRNAs in cell treated with 6-paradol was not caused by the toxicity of this compound.

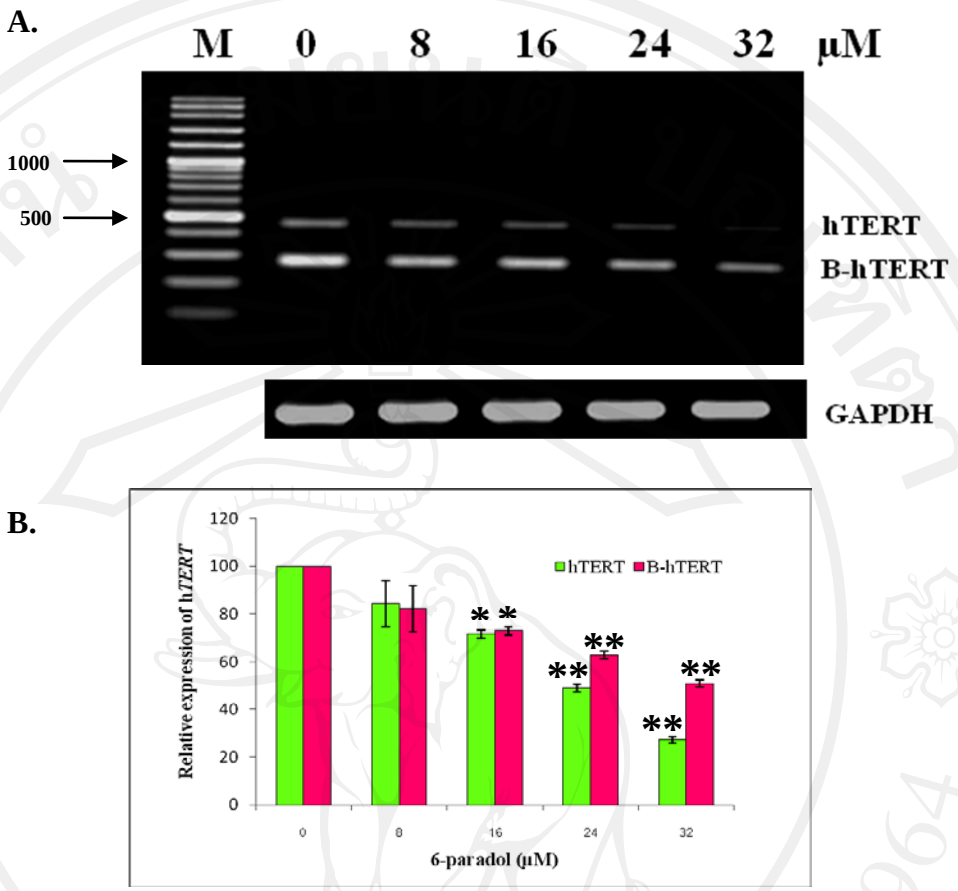

Figure 3.27 Effect of 6-paradol on *hTERT* mRNAs expression in A549 cells. **A.** A549 cells were treated with the indicated concentration of 6-paradol for 24 h. At the end of treatment, RNA was extracted, and RT-PCR assay was performed to detect *hTERT* (upper panel) or *GAPDH* (lower panel) mRNA. **B.** The density of the various bands was determined by scan densitometer. The *hTERT* mRNAs levels were normalized to the levels of *GAPDH* mRNAs. Results are expressed as the mean values ± standard derivation of three experiments. $*P < 0.05$ and $**P < 0.01$ is the statistical significance of the difference between the values for 6-paradol-treated and untreated cells.

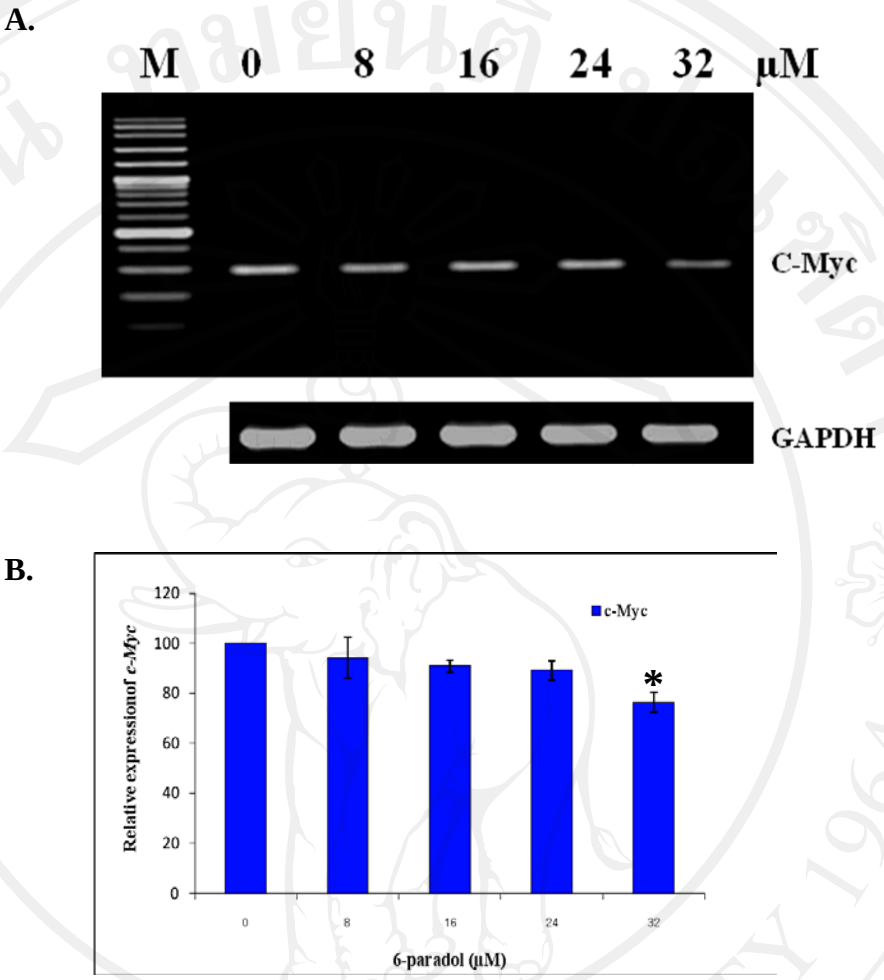

Figure 3.28 Effect of 6-paradol on *c-Myc* mRNAs expression in A549 cells. **A.** A549 cells were treated with indicated concentration of 6-paradol for 24 h. At the end of treatment, RNA was extracted, and RT-PCR assay was performed to detect *c-Myc* (upper panel) or *GAPDH* (lower panel) mRNA. **B.** The density of the various bands was determined by scan densitometer. The *c-Myc* mRNAs levels were normalized to the levels of *GAPDH* mRNAs. Results are expressed as the mean values ± standard derivation of three experiments. $*P < 0.05$ and $**P < 0.01$ is the statistical significance of the difference between the values for 6-paradol-treated and untreated cells.