# CHAPTER IV

RESULTS

1. Optimization of fluorescence microplate assay for detecting DNA relaxation caused by topoisomerase I

1.1 Effect of dyes on supercoiled and relaxed plasmid from enzyme reaction

Four fluorescent dyes which can interact with DNA such as H 33258, H 33342, Picogreen and Sybr Green I were used in this experiment to determine the difference of fluorescence intensity between supercoiled and relaxed form of pBR322 that converted by topoisomerase I. The pBR322 was aliquot from enzyme reaction mixture (25µg/ml of DNA concentration) and diluted to 200 ng/ml 100 µl by TE buffer pH 7.4 and then 100 µl of dye solution was added to keep 100 ng/ml DNA final concentration.

From Figure 2, there was no significant different in fluorescence intensity between supercoiled and relaxed pBR322 compared with blank and dye solution when using 12.5 µg/ml of H 33258 and H 33342 in TE buffer to stain DNA in fluorescence microplate assay. The TEN buffer was used instead of TE buffer for the dye dilution to increase fluorescence intensity at the same concentration of both dyes. The fluorescence intensity was increased extremely in all groups. However, the different fluorescence intensity of the two forms of DNA was not change. When the dye concentration was decreased to 1 µg/ml in TEN buffer, the fluorescence intensity of both form of DNA were increased significantly compared with blank and dye solution. The fluorescence intensity of relaxed form of DNA was slightly more than supercoiled form.

The overall the fluorescence intensity between supercoiled and relaxed pBR322 of each 4 dyes was shown in Figure 3. Two cyanine dyes including Picogreen and Sybr Green I showed different fluorescence intensity of the two forms of DNA (Figure 3C, 3D). In contrast with bisbenzimide dyes, the fluorescene intensity of supercoiled DNA which complexed with the cyanine dyes was higher than relaxed DNA. In addition, Picogreen exhibited greater difference fluorescence inhtensity between two forms of DNA over Sybr Green I.
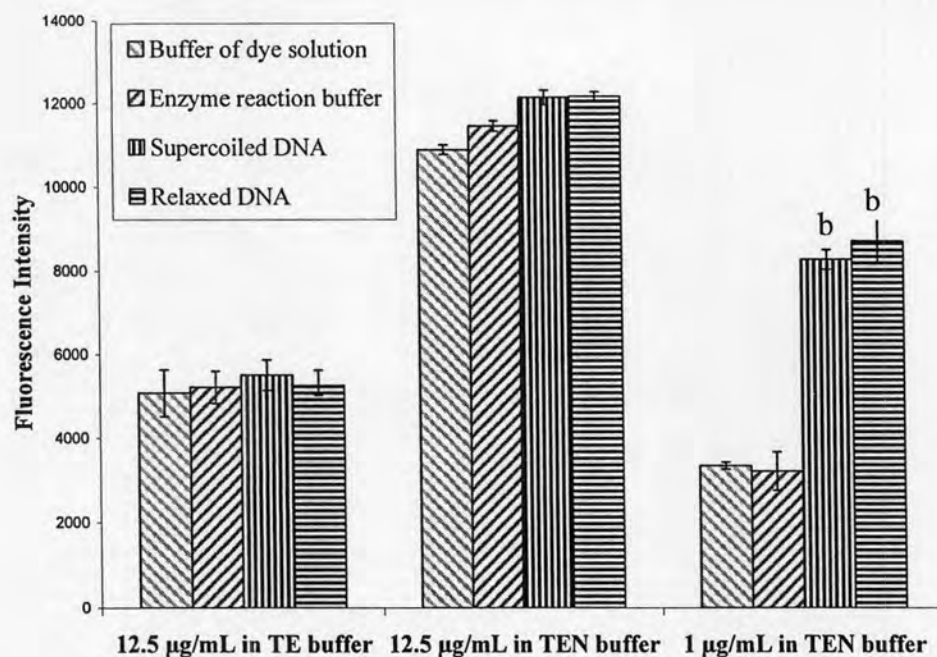
(A)
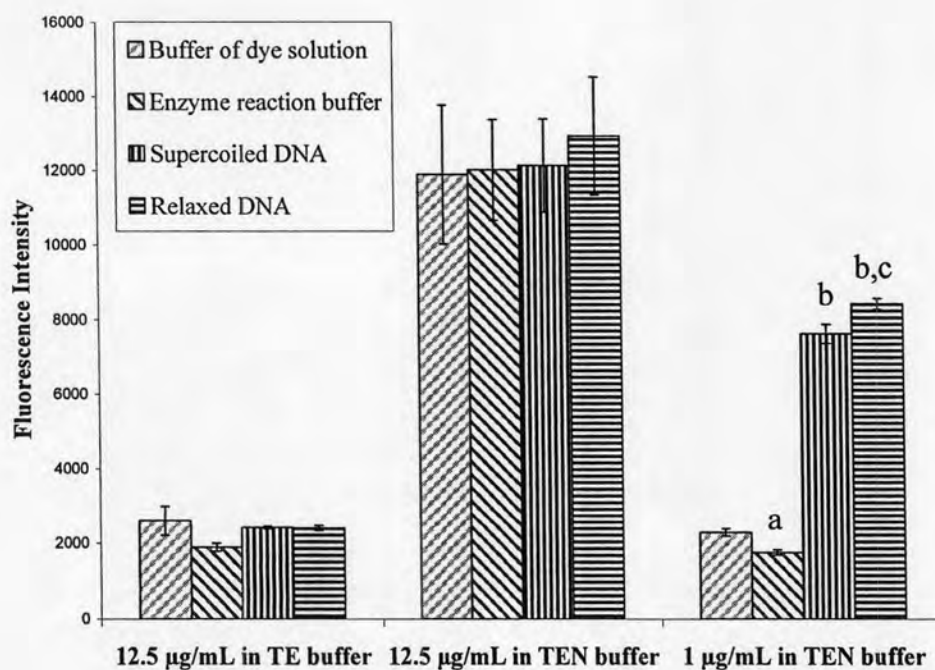
(B)

Figure 2 Fluorescence Effect of buffer and dye concentration on fluorescence intensity of (A) H 33258 and (B) H 33342. Final DNA concentration in 96-well microplate was 100 ng/ml in each experiments. Each data column was the mean ± S.E.M. (n = 2 or 3 independent experiments). [a]$p < 0.01$, compared with buffer of dye solution. [b]$p < 0.01$, compared with enzyme reaction buffer. [c]$p < 0.01$, compared with supercoiled DNA.
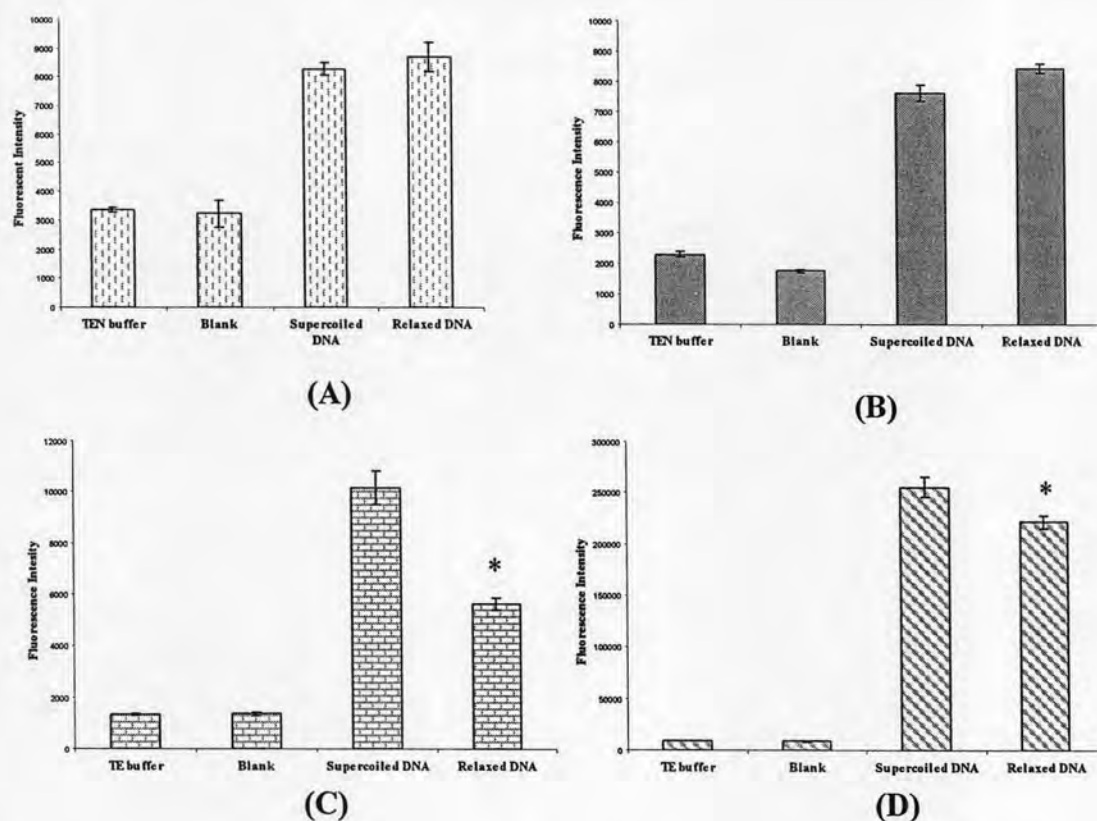
Figure 3 Fluorescence effect of dyes on supercoiled and relaxed plasmid from enzyme reaction (intraday assay). The plasmid DNA from enzyme reaction mixture was diluted to 96-well microplate at 100 ng/ml final concentration incubated with each dye for 5 min before detection. (A) 1 µg/ml H 33258 (B) 1 µg/ml H 33342 (C) 1:400 Picogreen and (D) 1:10000 Sybr Green I. Each data column was the mean ± S.E.M. (n = 2 or 3 independent experiments). *P < 0.01, compared with supercoiled DNA.

1.1.1 Intraday assay precision

To validate the precision of the assay, the enzyme reaction was performed. The fluorescence intensity at time of initial reaction and time of complete reaction of 4 dyes were determined and calculated the %CV (Table 4). The %CV of all data was not more than the acceptant value, 20%. Only the blank of H 33258 had %CV more than 10%. To compare the ability in separation of maximum and minimum signal between dyes, the Z′ value must be used (the equation represented in Material and Method). The Z′ value that more than 0.4 was accepted (Eli Lilly and Company and NIH Chemical Genomics Center, 2008). The Z′ value of H 33258, H 33342 and Sybr Green I were minus values. In contrast, Picogreen had only positive Z′ value (0.42).

Table 4 Intraday precision data and Z′ value from the fluorescent assay validation

	number of replication	% CV[a]			Z′value[b]
		Blank	**Supercoiled**	**Relaxed**	
H 33258	4	14.09	2.78	5.91	-4.21
H 33342	4	3.95	3.37	1.75	-0.50
Picogreen	6	5.68	6.22	4.19	0.42
Sybr Green I	6	1.43	3.69	2.78	-0.37

[a] $\% \, CV \; = \; \dfrac{(SD/\sqrt{n})}{average}$

[b] $Z' \, value \; = \; \dfrac{(average_{max} - 3SD_{max}/\sqrt{n}) - (average_{min} + 3SD_{min}/\sqrt{n})}{(average_{max} - average_{min})}$

1.1.2 Interday assay precision

Since there were significantly different of fluoresecence intensity of Picongreen and Sybr Green I on the form of DNA, they were selected for the 3 days repeating experiment to confirm assay reproducibility. Table 5 showed %CV and Z′ value of blank, supercoiled and relaxed DNA in each dye. The %CV of all data was not more than 10%. Z′ value of Picogreen was positive value and met the acceptance criterion (The Z′ value $\geq$ 0.4). The Z′ value of Sybr Green I was still minus value. Therefore, Picogreen was chosen to determine optimum concentrations of DNA for using in the proposed assay.

Table 5 Interday precision data and Z′ value from the fluorescent assay validation

	day	% CV[a]			Z′ value[b]
		Blank	Supercoiled	Relaxed	
Picogreen	1	5.68	6.22	4.19	0.42
	2	5.42	2.51	4.66	0.42
	3	4.63	3.53	3.44	0.40
Sybr Green I	1	1.43	3.69	2.78	-0.37
	2	1.99	1.86	8.99	-0.48
	3	2.31	1.25	7.01	-0.22

$$^{a}\% \text{CV} = \frac{(SD/\sqrt{n})}{\text{average}}$$

$$^{b}\text{Z}′ \text{ value} = \frac{(\text{average}_{max}-3SD_{max}/\sqrt{n})-(\text{average}_{min}+3SD_{min}/\sqrt{n})}{(\text{average}_{max}-\text{average}_{min})}$$

1.1.3 Dynamic range and limit of detection

Picogreen was usually determined DNA concentration as only single form of DNA including supercoiled DNA but never determined concentration of relaxed form (Singer *et al.*, 1997). In this study, relaxed DNA from enzyme reaction mixture was diluted in various DNA concentrations and detected by Picogreen compared with supercoiled DNA from enzyme reaction mixture. The fluorescence intensity of relaxed DNA increased proportionally with the increasing DNA concentration (Figure 4). To assess assay performance, Z′ value was calculated in each DNA concentration (Table 6). Almost Z′ value of each DNA concentrations passed the acceptance criterion (Z′ value ≥ 0.4), except for 10 ng/ml DNA. However, the Z′value was not directly correlate with the increasing DNA concentration.
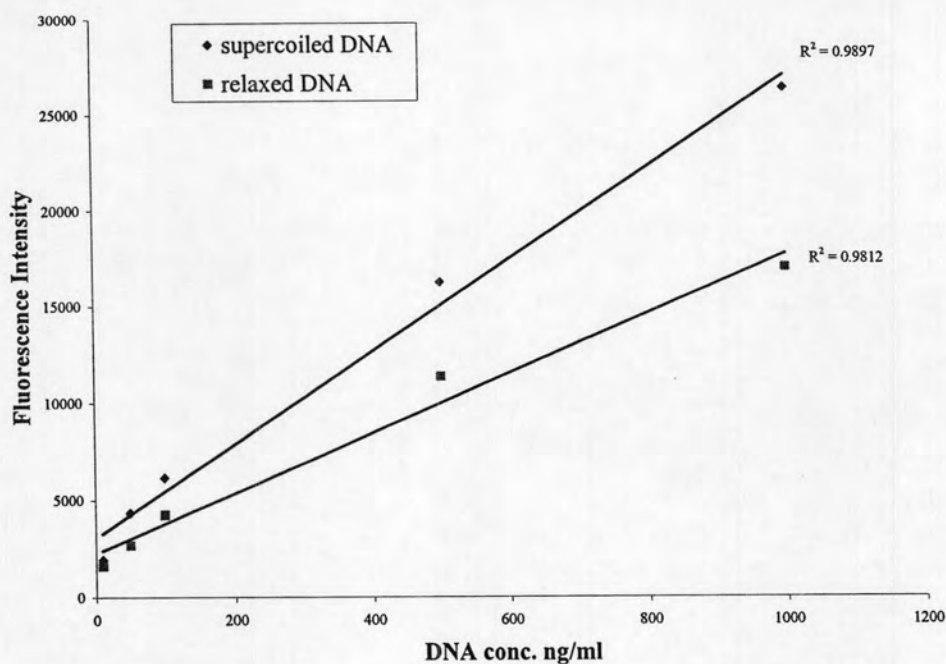
Figure 4 The fluorescence intensity of supercoiled and relaxed DNA determined by Picogreen as increasing DNA concentrations.

Table 6 The Z′ value of assay determined by Picogreen at different DNA concentrations

DNA conc. (ng/ml)	10	50	100	500	1000
Z′ value[a]	0.39	0.71	0.48	0.69	0.89

$$^{a}\ Z'\ value\ =\ \frac{(average_{max} - 3SD_{max}/\sqrt{n}) - (average_{min} + 3SD_{min}/\sqrt{n})}{(average_{max} - average_{min})}$$

1.2 Time-course analysis of topoisomerase I activity: conventional method compared with Picogreen fluorescence-based assay

To determine topoisomerase I activity, time-course assay was demonstrated by conventional method compared with using Picogreen to detect topology alteration of plasmid by the enzyme. The Figure 5 represented gel electrophoresis of time-course assay. Supercoiled DNA bands were measured by Scion Image at various times and were plotted as function of time (Figure 6A). For fluorescence microplate assay, the sample was measured at the same manner. Fluorescence intensity was plotted as function of time (Figure 6B). The band intensity and fluorescence intensity were converted to % relaxation and was plotted versus time (Figure 6C).

At the initial time of incubation, the % relaxation change rate was observed in fluorescence microplate assay higher than gel-based assay that might suggest the sensitivity of fluorescence microplate higher than that of gel-based assay.

The curve fit was a non-linear relationship. The correlation analysis presented a good relationship between conventional and proposed assay (Figure 7). The correlation is significant at $p < 0.01$ with the R value of 0.872.
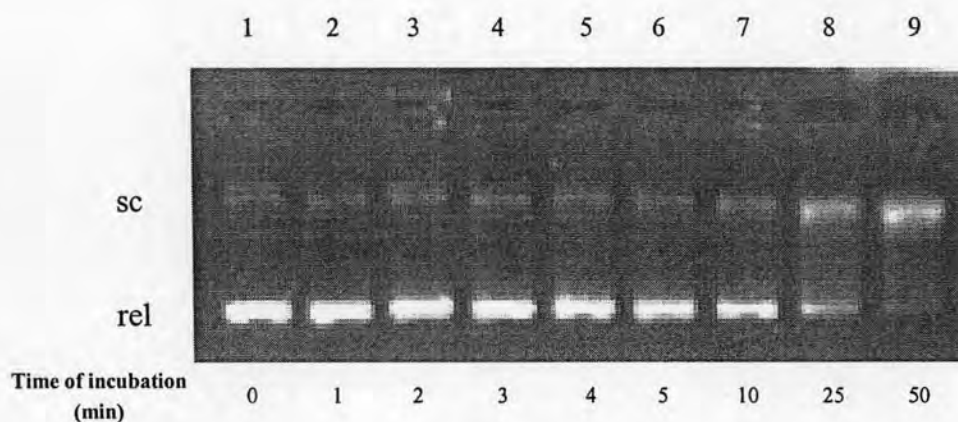

Figure 5 Time-course study of enzyme activity by gel-based assay. Samples were collected from the same enzyme reaction at various times of incubation. DNA amount was 50 ng per well.
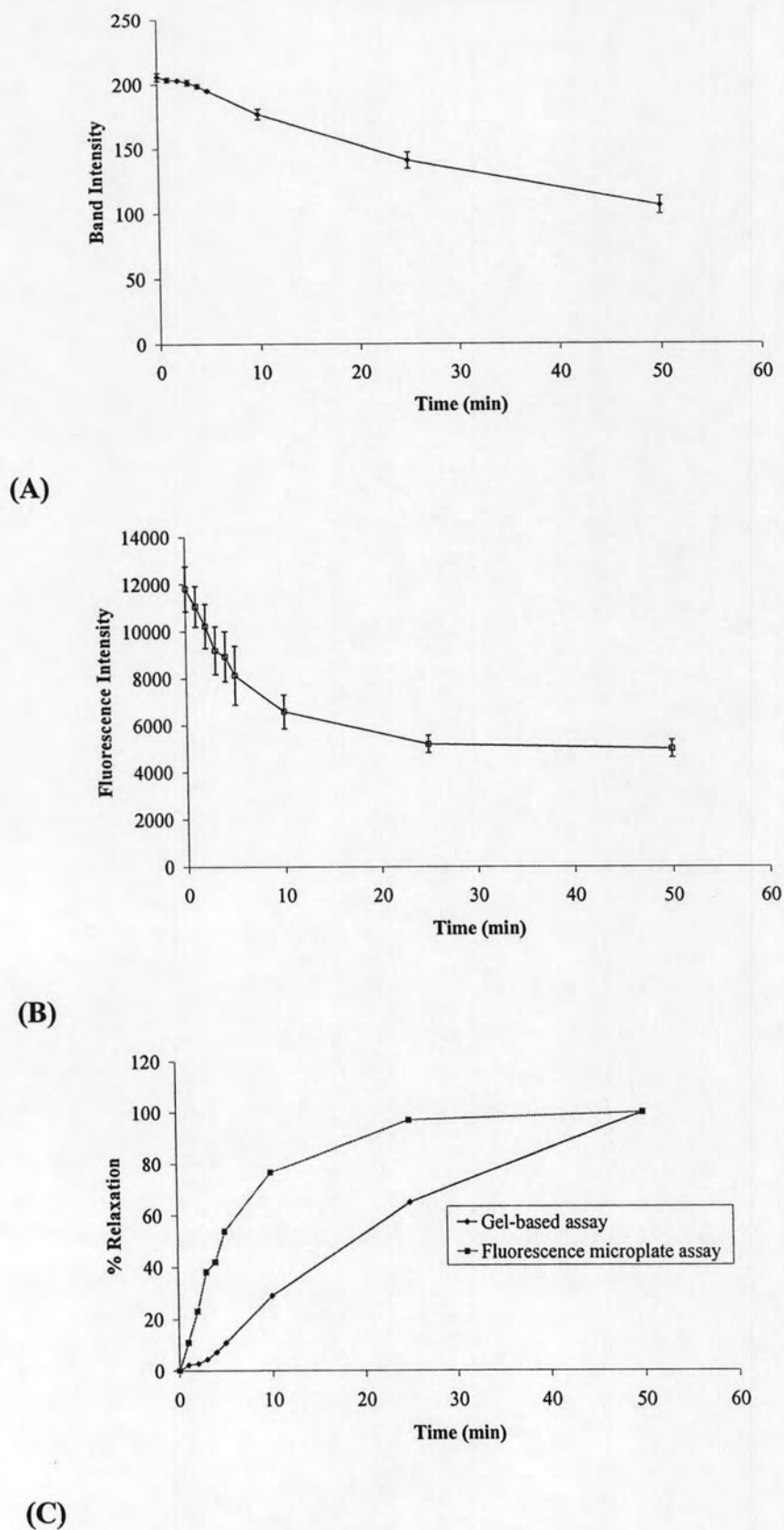
(A)

(B)

(C)

Figure 6 Time-course analysis of of enzyme activity compared between gel-based assay and fluorescence microplate assay. (A) Band intensity, (B) fluorescence intensity and (C) % relaxation were plotted as function of time of incubation. Each data point was the mean ± S.E.M. (3 replicates).
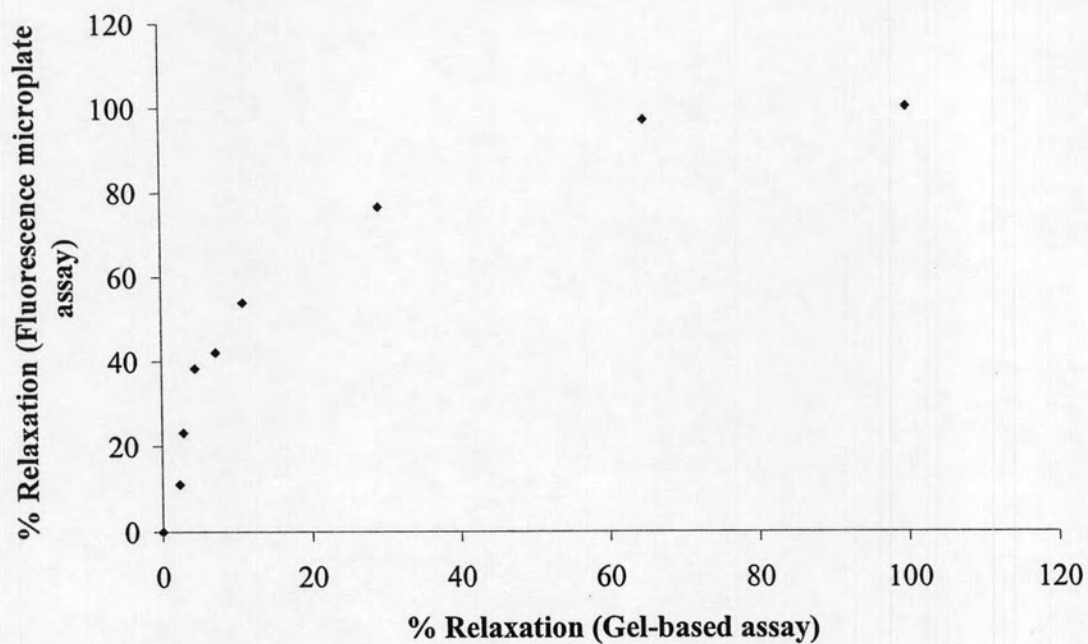
Figure 7 Correlation analysis between the % relaxation obtained from fluorescence microplate assay and the % relaxation obtained from gel-based assay. Pearson Correlation is significant at 0.01 levels (2-tailed), R = 0.872.

2. Evaluation of the ability of fluorescence-based assay to detect known topoisomerase I inhibitors

In this study, some known topoisomerase inhibitors were selected to examine the assay system including camptothecin, heparin, quercetin and menadione as topoisomerase I inhibitor and etoposide and ellipticine as topoisomerase II inhibitor.

2.1 Topoisomerase I inhibitors

2.2.1 Camptothecin

Camptothecin is a selective inhibitor of topoisomerase I which was taken into the assay to confirm the detection ability of the proposed method. The sample was obtained from the same enzyme reaction to test for both assays. As show in Figure 8, the supercoiled DNA increased with increasing camptothecin concentration. The inhibition of 5% DMSO, a solvent control was negligible (Lane 3). The IC_{50} value of both assays was determined by fitting log dose-response curve (Figure 9). The IC_{50} values of each assay were 3.46 ± 1.20 and 11.6 ± 9.20 μM, respectively. Correlation analysis of camptothecin inhibition between the two assays was significant ($p<0.01$) with the R value of 0.983.
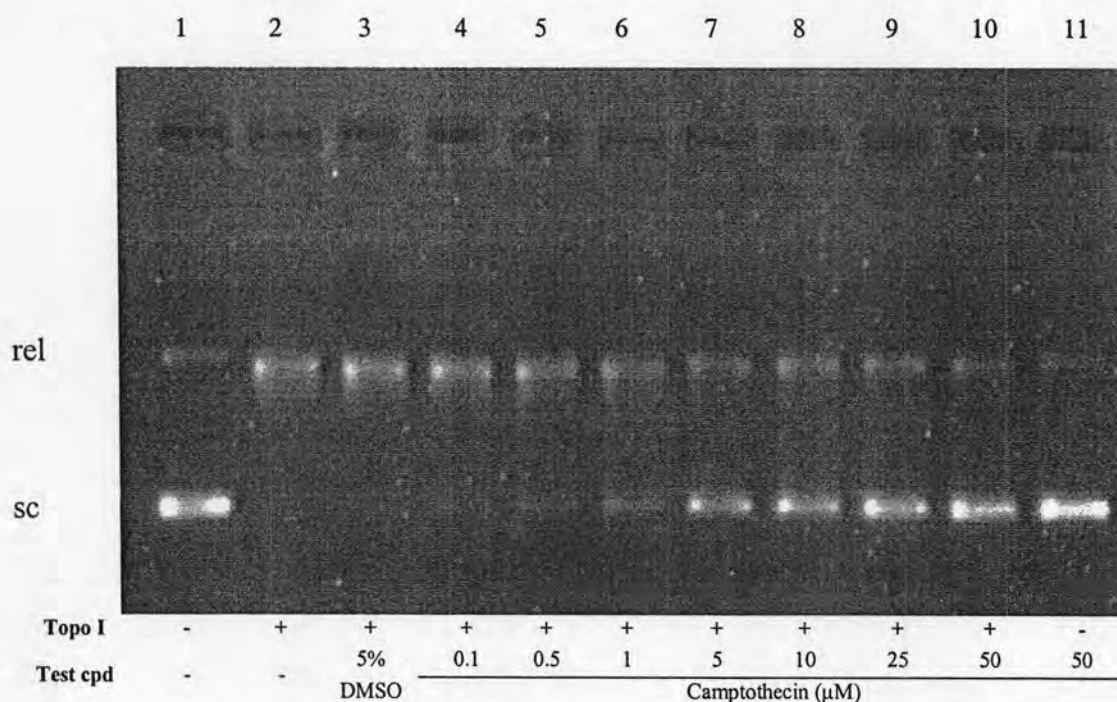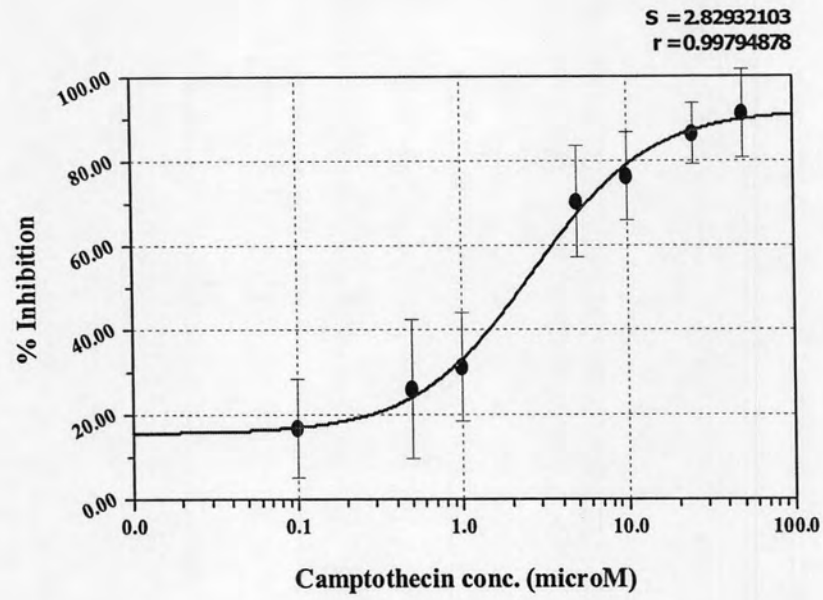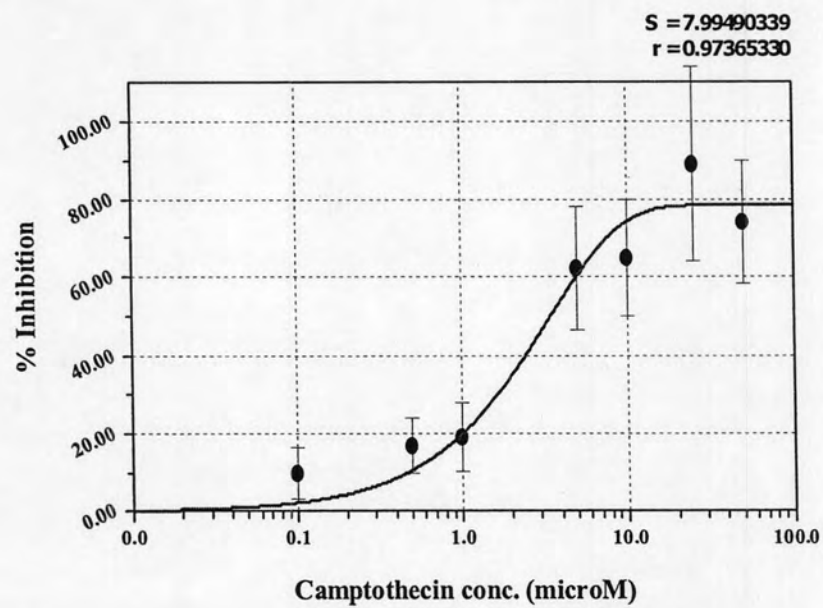

Figure 8 Agarose-gel electrophoresis of camptothecin in a dose-response manner.

S = 2.82932103
r = 0.99794878

(A)

S = 7.99490339
r = 0.97365330

(B)

Figure 9 Camptothecin dose-response curve. (A) gel-based assay and (B) fluorescence microplate assay. Each data point was the mean ± S.E.M. (n = 4 independent experiments).
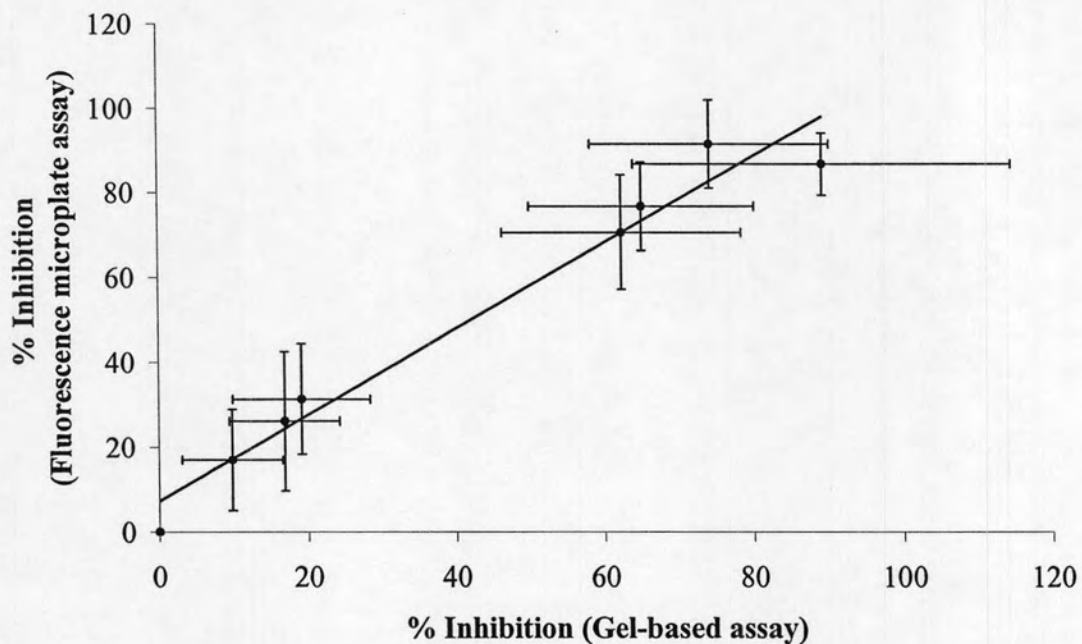
Figure 10 Correlation analysis of camptothecin inhibitory effect between the % inhibition obtained from fluorescence microplate assay and the % inhibition obtained from gel-based assay. Pearson Correlation is significant at 0.01 levels (2-tailed), R = 0.983. Each data point was the mean ± S.E.M. (n = 4 independent experiments).

2.2.2 Heparin

Figure 11 showed gel electrophoresis of topoisomerase I inhibition by heparin. The IC_{50} values of both assays were obtained by fitting log dose-response curve (Figure 12) and were 2.45 ± 1.02 and 4.43 ± 1.24 µg/ml, respectively. Correlation analysis of heparin inhibition was significant ($p<0.01$) with the R value of 0.943 (Figure 13).

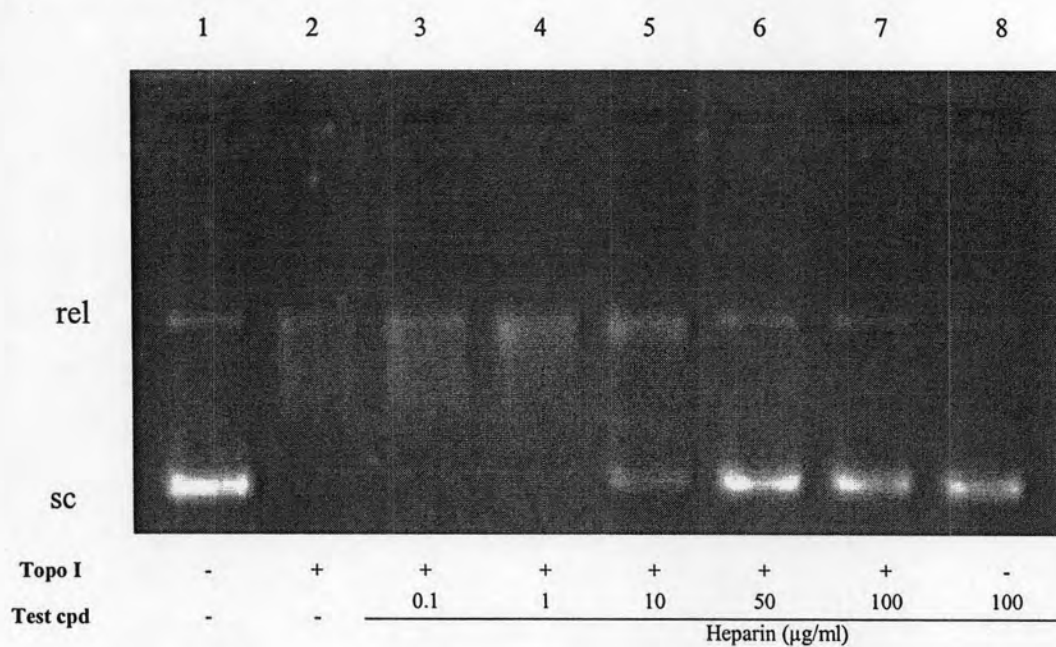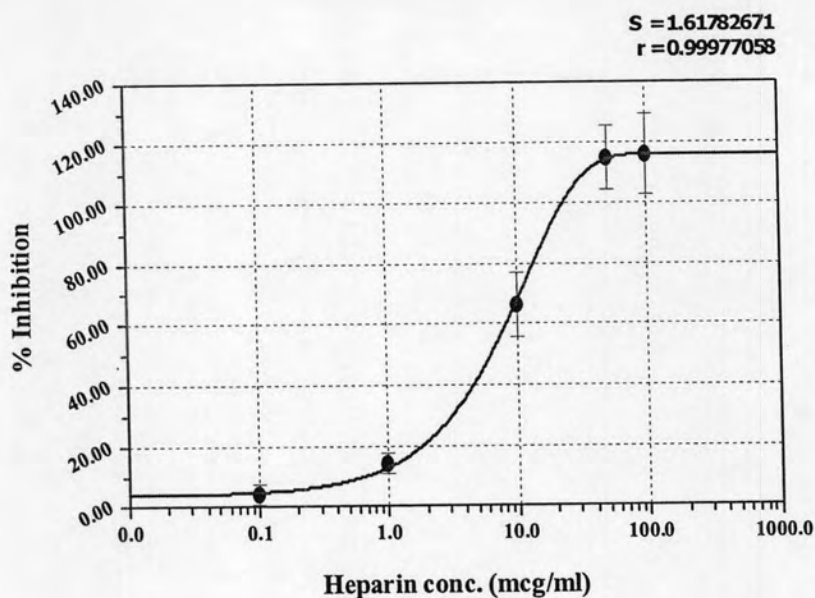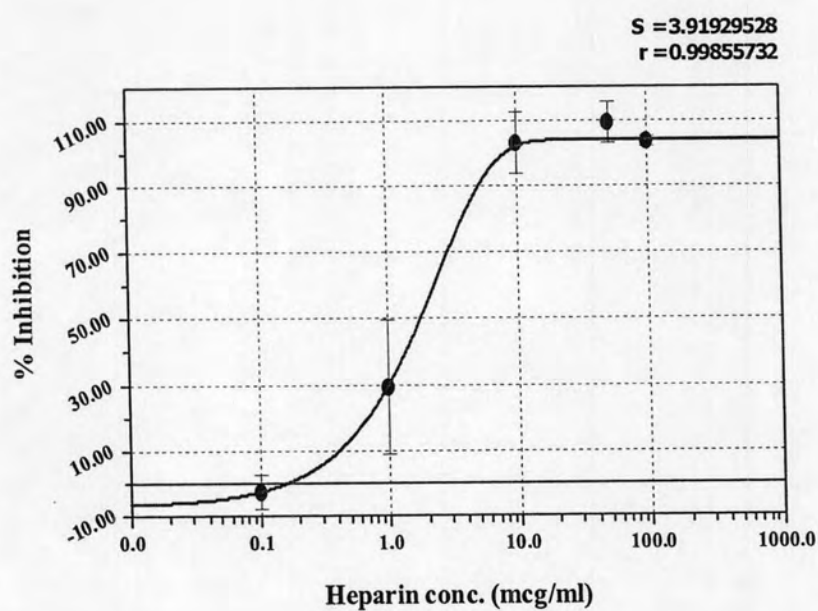**Figure 11 Agarose-gel electrophoresis of heparin in a dose-response manner.**

31

(A)

(B)

Figure 12 Heparin dose-response curve (A) gel-based assay and (B) fluorescence microplate assay. Each data point was the mean ± S.E.M. (n = 3 independent experiments).
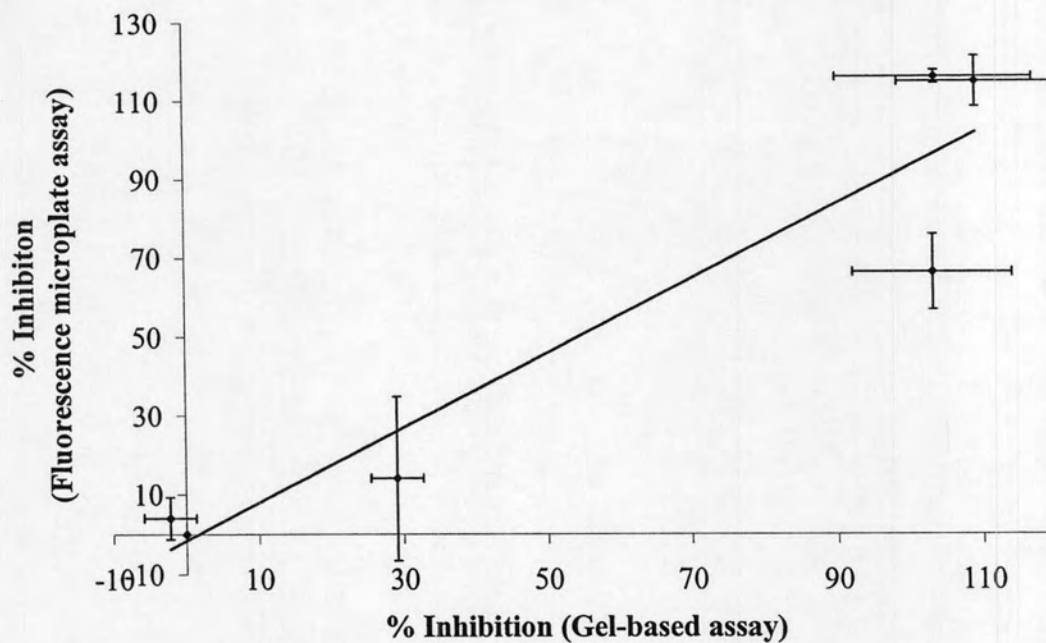
32

Figure 13 Correlation analysis of heparin inhibitory effect between the % inhibition obtained from fluorescence microplate assay and the % inhibition obtained from gel-based assay. Pearson Correlation is significant at 0.01 levels (2-tailed), R = 0.943. Each data point was the mean ± S.E.M. (n = 3 independent experiments).

2.2.3 Quercetin

Quercetin was a putative of intercalator that exhibited topoisomerase I inhibition. Figure 14 shown gel electrophoresis of topoisomerase I inhibition by quercetin. The band intensity of supercoiled DNA was not relatively increased with increasing quercetin concentration. There was an interference of band or fluorescence intensity of supercoiled and relaxed DNA in both assays. In the case of fluorescence microplate assay was significantly interfered by quercetin ($p < 0.01$).
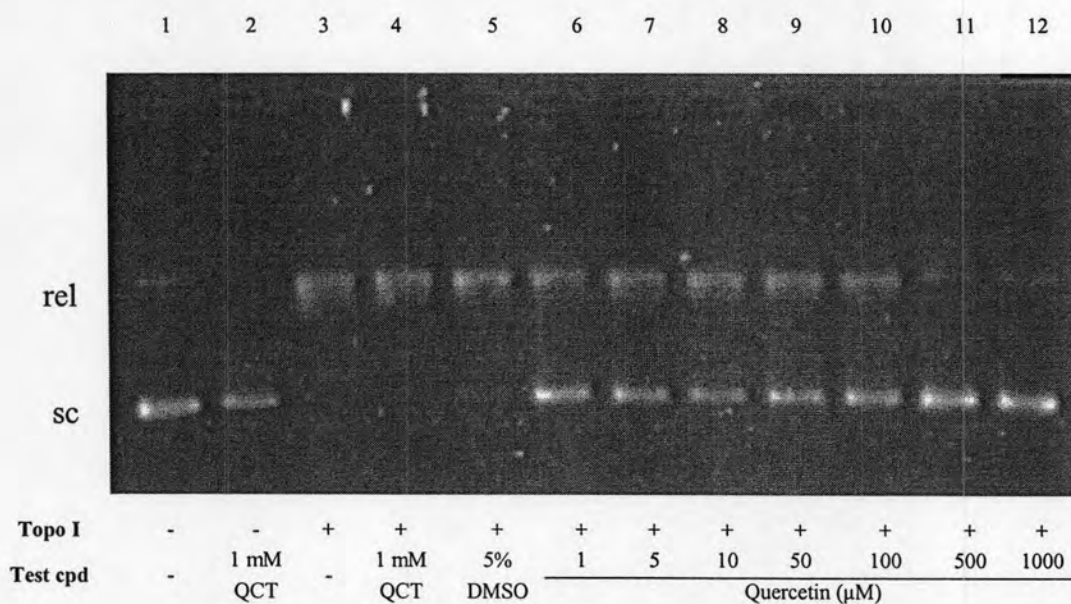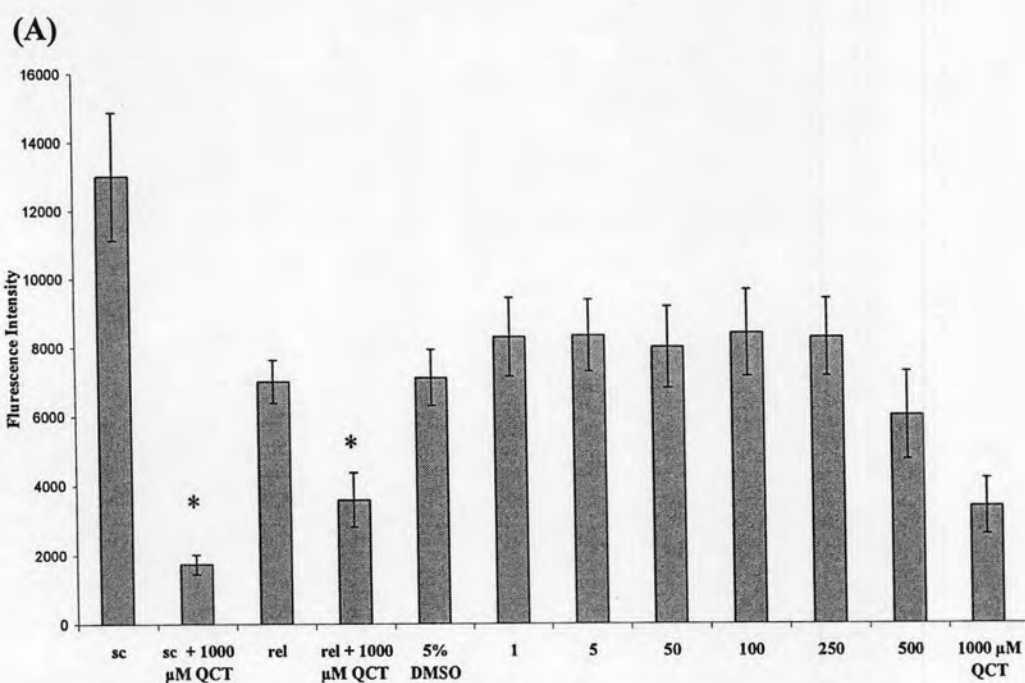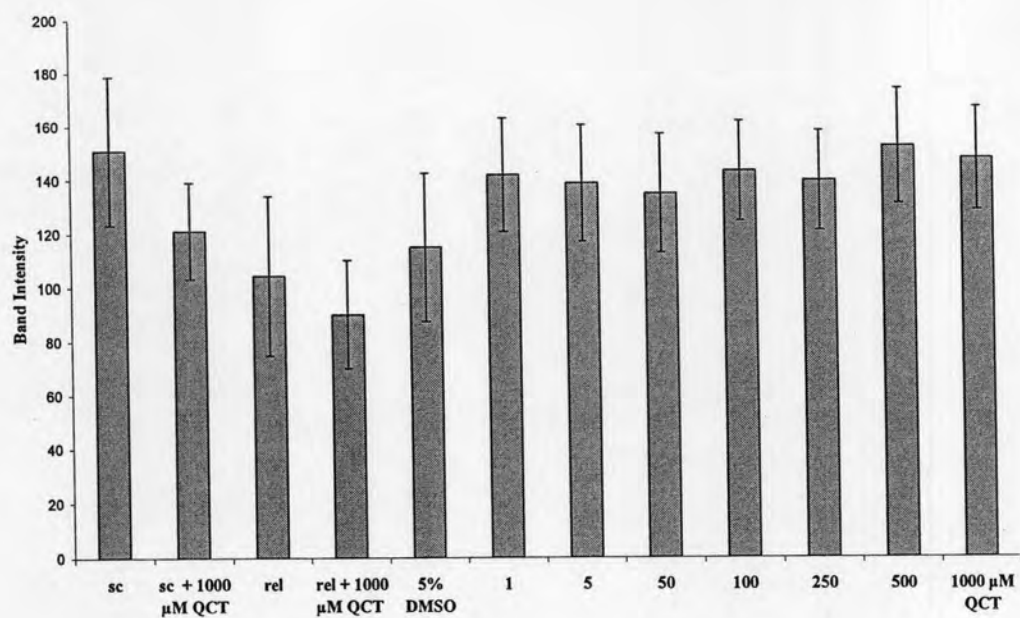
Figure 14 Agarose-gel electrophoresis of quercetin.

34

(A)

(B)

Figure 15 Histogram of response of quercetin showing (A) band intensity obtained from gel-based assay and (B) fluorescence intensity obtained from fluorescence microplate assay. Fluorescence intensity of supercoiled and relaxed DNA with 1 mM quercetin was statistically significant different from control (*p<0.01). Each data column was the mean ± S.E.M. (n = 4 independent experiments)

2.2.4 Menadione

Menadione was the only one that is a catalytic inhibitor using in this study. Preincubation of topoisomerase I with menadione was necessary. Gel electrophoresis shown inhibition in a dose-response manner (Figure 15). The IC_{50} value was calculated by fitting log dose-response curve obtained form gel-based assay and fluorescence microplate assay were 51.0 ± 28.7 and 58.1 ± 6.3 μM, respectively (Figure 16). Correlation analysis of heparin inhibition was significant ($p<0.01$) with the R value of 0.957 (Figure 16).

The IC_{50} values of all inhibitors were summarized in Table 7. All IC_{50} values of the inhibitors obtained from fluorescence assay were higher than that from gel-based assay.
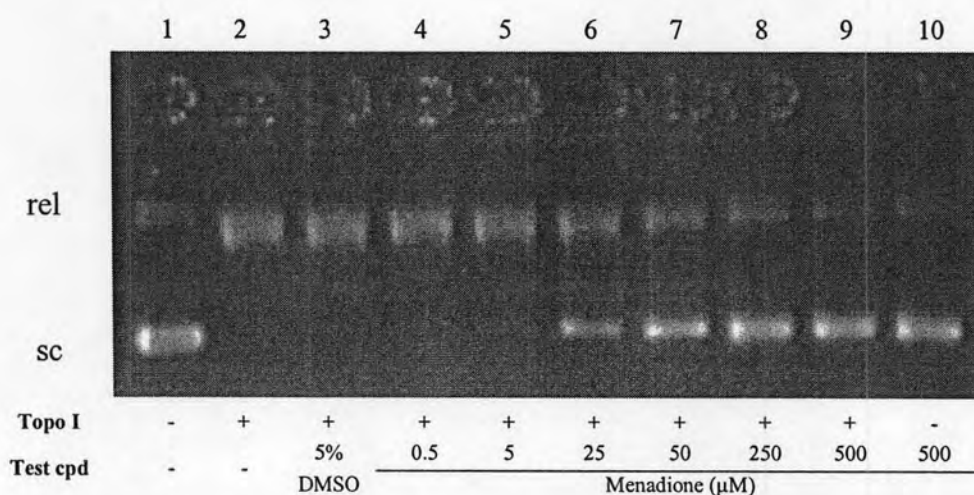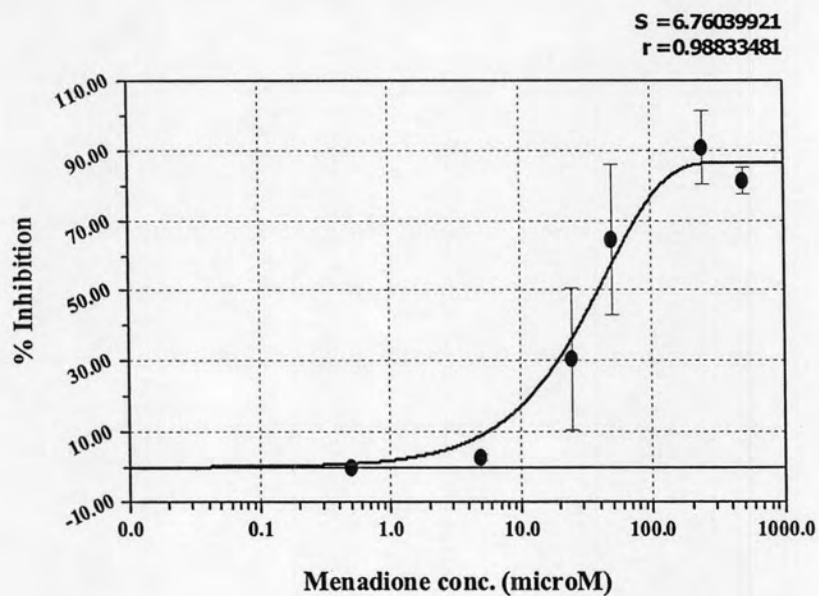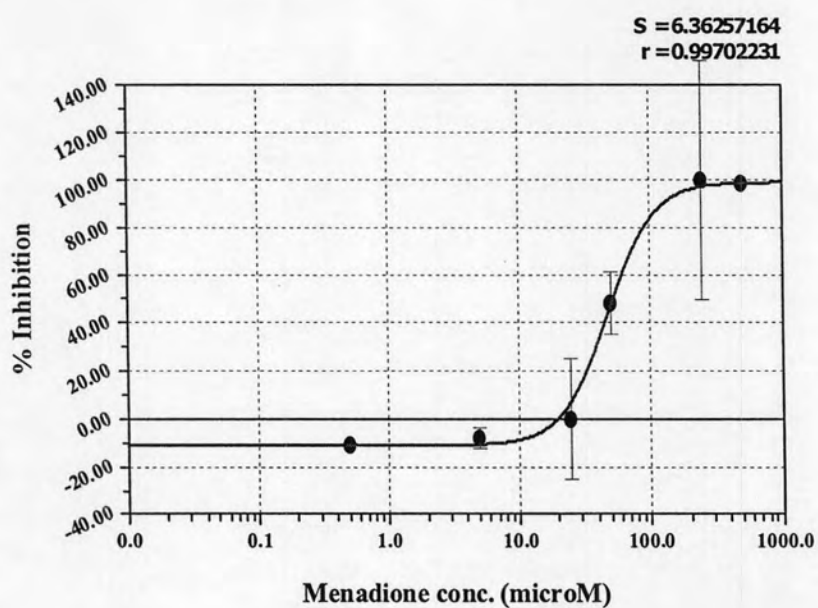

Figure 16 Agarose-gel electrophoresis of menadione in a dose-response manner. Topoisomerase I was preincubated with menadione for 30 min.

S = 6.76039921
r = 0.98833481

% Inhibition

Menadione conc. (microM)

(A)

S = 6.36257164
r = 0.99702231

% Inhibition

Menadione conc. (microM)

(B)

Figure 17 Menadione dose-respons curve (A) gel-based assay (B) fluorescence microplate assay. Each data point was the mean ± S.E.M. (n = 2 independent experiments).
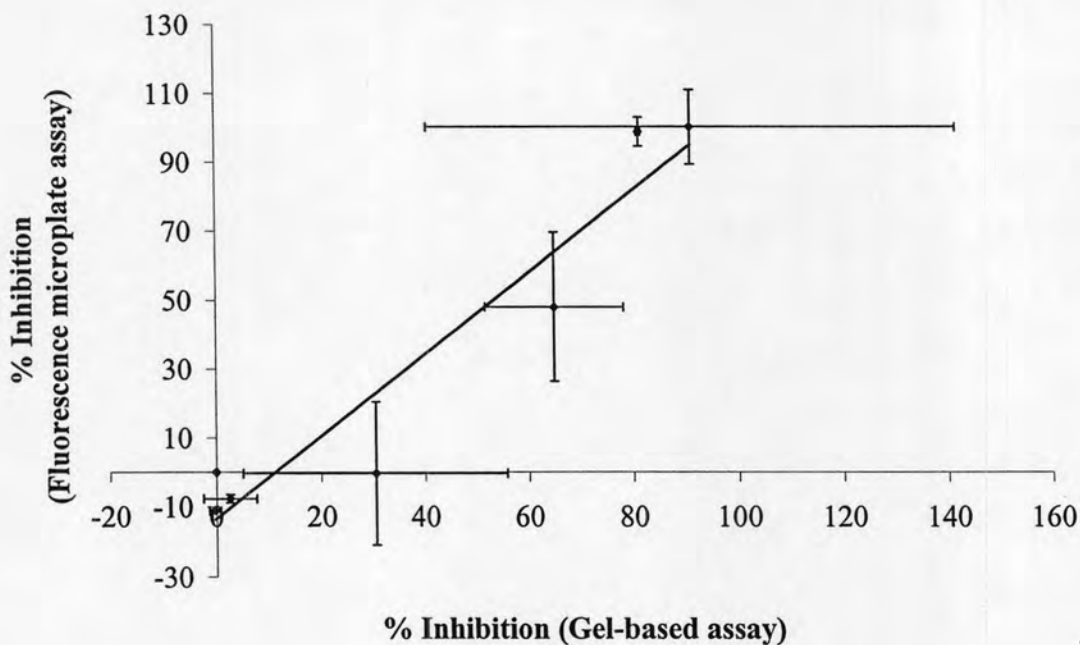
Figure 18 Correlation analysis of camptothecin inhibitory effect between the % inhibition obtained from fluorescence microplate assay and the % inhibition obtained from gel-based assay. Pearson Correlation is significant at 0.01 levels (2-tailed), R = 0.957. Each data point was the mean ± S.E.M. (n = 2 independent experiments).

Table 7 Comparison of IC$_{50}$ value of topoisomerase I inhibitor using gel-based and fluorescence microplate assay

	Gel-based assay	Fluorescence microplate assay
Camptothecin (n=4)[a]	3.46±1.20	11.6±9.2
Heparin (n=3)[b]	2.45±1.02	4.43±1.24
Menadione (n=2)[a]	51.0±28.7	58.1±6.3

[a] IC$_{50}$ value was reported in µM

[b] IC$_{50}$ value was reported in µg/ml

Data shown in mean ± S.E.M.

2.2 Topoisomerase II inhibitors

To investigate the selectivity of this assay for detecting topoisomerase I inhibitors, some known topoisomerase II inhibitor such as etoposide and ellipticine were carried out. The Figure 19 and 20 demonstrated no inhibitory effect of etoposide at 1 mM on topoisomerase I. In contrast, ellipticine at 20 μM exhibited inhibitory effect on topoisomerase I as shown in gel electrophoresis (Figure 19). For fluorescence assay (Figure 20), no effect on fluorescence signal of supercoiled or relaxed DNA by etoposide but ellipticine reduced fluorescence signal of supercoiled DNA significantly ($p < 0.01$).
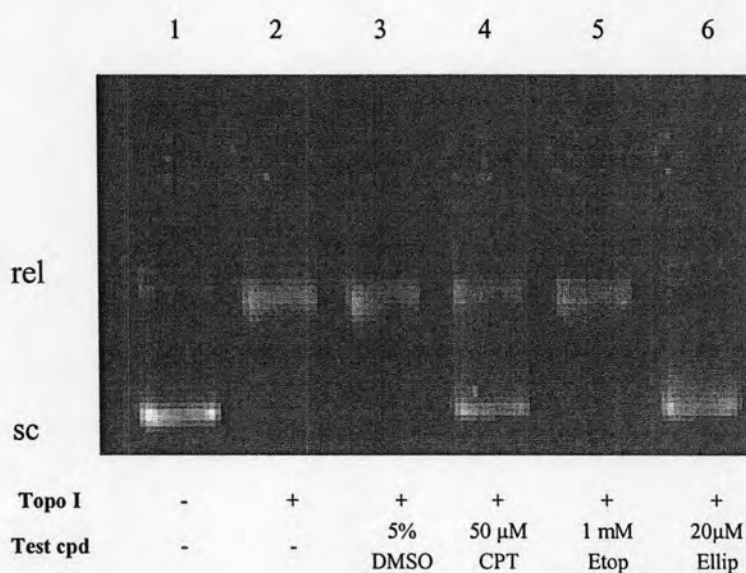

	1	2	3	4	5	6
Topo I	-	+	+	+	+	+
Test cpd	-	-	5% DMSO	50 μM CPT	1 mM Etop	20μM Ellip

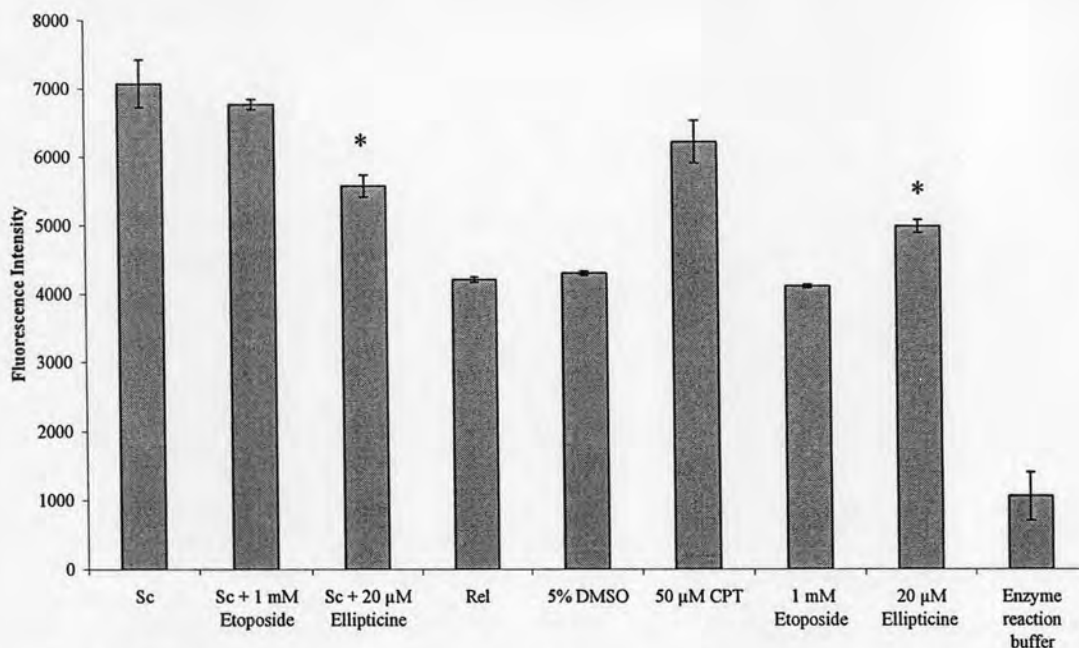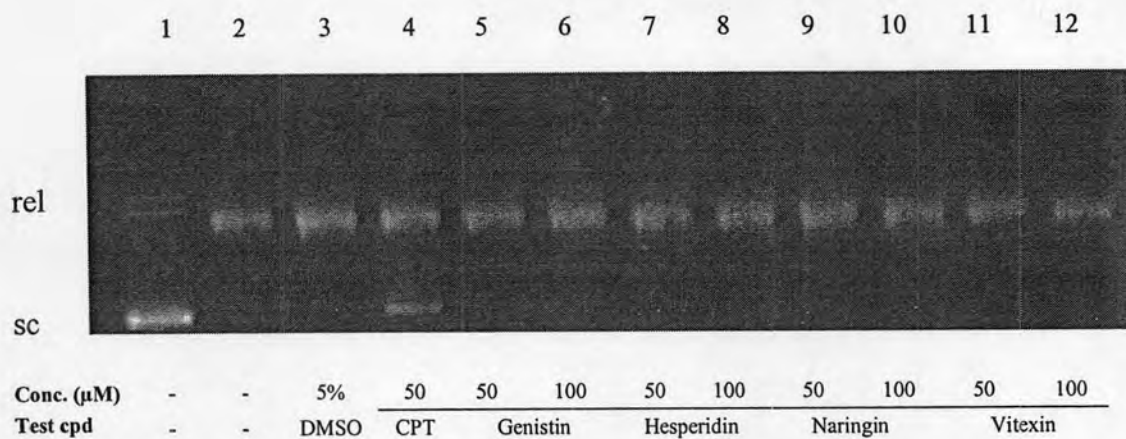**Figure 19 Agarose-gel electrophoresis of topoisomerase II inhibitor, etoposide and ellipticine.**

Figure 20 Histogram of response of topoisomerase II inhibitors showing fluorescence intensity obtained from fluorescence microplate assay. Fluorescence intensity of supercoiled DNA with 20 µM ellipticine was statistically significant different from control (*$p<0.01$). Each data column was the mean ± S.E.M. (n = 3 replicates).

### 3. Screening for unknown compounds using Picogreen-based assay.

Twelve compounds were selected which depend on related structure, cytotoxic effect or DNA damage data of each compounds to screen using both assay systems. Gel electrophoresis results were shown in Figure 21. All compounds were tested at 50 µM and 100 µM and compared with camptothecin 50 µM. The % inhibiotion was calculated by comparing the band intensity of supercoiled DNA in the presence or absence of test sample (Table 8). Oxostephanine and fagarine strongly inhibited topoiosmerase I. Fagarine was more potent than camptothecin, a positive control. The inhibitory effect of oxostephanine and fagarine was not dose-dependent. The % inhibition of sanguinarine and chelerythrine at 50 µM and 100 µM were 47.7±9.01 and 61.0±19.7, and 41.1±18.9 and 53.7±20.1, respectively. It was likely in a concentration-dependent manner.
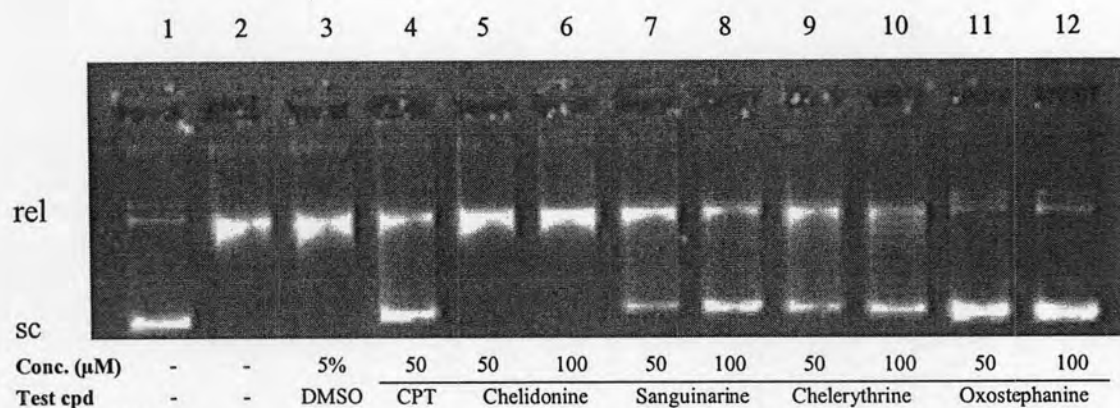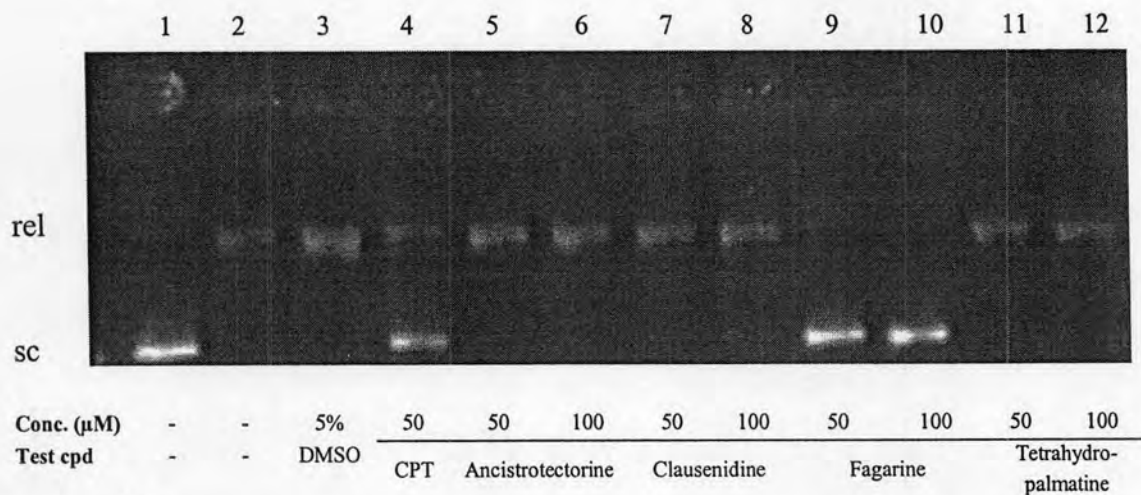
Figure 21 Agarose-gel electrophoresis of unknown compounds at 50 and 100 μM concentration. (A) genistin, hesperidine, naringin and vitexin (B) chelidonine, sanguinarine, chelerythrine and oxostephanin (C) ancistrotectorine, clausenidine, fagarine, and tetrahydropalmatine.

Table 8 The % inhibition of unknown compounds using gel-based assay

compound	% inhibition	
	50 µM	100 µM
camptothecin	90.5±16.7	ND
1. genistin	-	-
2. hesperidine	-	-
3. naringin	-	-
4. vitexin	-	-
5. chelidonine	-	-
6. sanguinarine	47.7±9.01	61.0±19.7
7. chelerythrine	41.1±18.9	53.7±20.1
8. oxostephanine	84.3±23.4	93.6±20.7
9. ancistrotectorine	-	-
10. clausenidine	-	-
11. fagarine	106±8.5	96.9±3.5
12. tetrahydropalmatine	-	-

ND = not determined., Data shown in mean ± S.E.M. (n = 3 replicates).

Four compounds exhibited enzyme inhibition or not from gel-based assay such as vitexin, chelidonine, chelerythrine and sanguinarine were chosen to comfirm the gel electrophoresis results using fluorescence assay. As shown in Figure 22, the fluorescence intensity of supercoiled DNA incubated with 100 µM of sanguinarine and chelerythrine was significantly decreased compared with the control ($p < 0.01$). Consequently, the fluorescence intensity of the end reaction groups was interfered. The fluorescence intensity of sanguinarine was more decreased than of chelerythrine that implied stronger DNA binding of sanguinarine much more than chelerythrine. These results were corrected with the obtained form gel-based assay results.
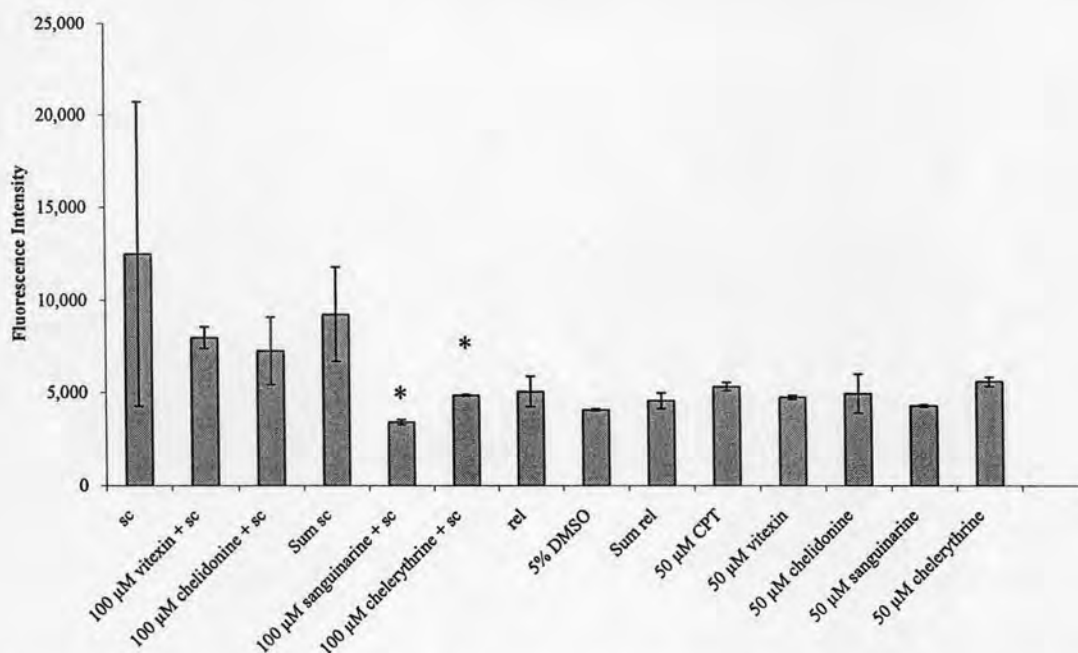
Figure 22 Histogram of response unknown compounds of showing fluorescence intensity obtained from fluorescence microplate assay. *P < 0.01, compared with the control. Data shown in mean ± S.E.M. (n = 3 replicates).

4. Modification of fluorescence ratio assay for detecting DNA cleavage caused by topoisomerase I

The fluorescence ratio assay was modified from Rock *et al.* (2003). They used their method for monitoring DNA strand breaks in plasmids that were prepared in industry. This study, the method was applied to determine single strand breaks induced by topoisomerase I or in the presence of enzyme poisons, a type of topoisomerase I inhibitors. The enzyme reactions were prepared as indicated in the DNA relaxation assay.

4.1 Evaluation of the stop processes of enzyme reaction

To investigate an appropriate stop processes for the fluorescence assay, the experiment designed in three groups: stopped by cooling down at 4° C or freezing, stopped by heat at 95° C for 1 min and stopped by adding 10% SDS following 1 µg/ml proteinase K incubated at 37° C for 1 hour. The result showed in Figure 23. The relative fluorescence of supercoiled DNA was less than relaxed DNA implicated that nicked DNA in the reaction was resealed to closed circular form by topoisomerase I. The data was consistent with gel electrophoresis. In positive control group, the decreased relative fluorescence was observed. This finding implied that the

enzyme activity might be inhibited by camptothecin. The relative fluorescence was not significantly different between cooling down stop process and chemical stop process. However, the relative fluorescence of the heat stop process was decreased compared with the other groups that indicated that nicked DNA was slightly destroyed by heat process.
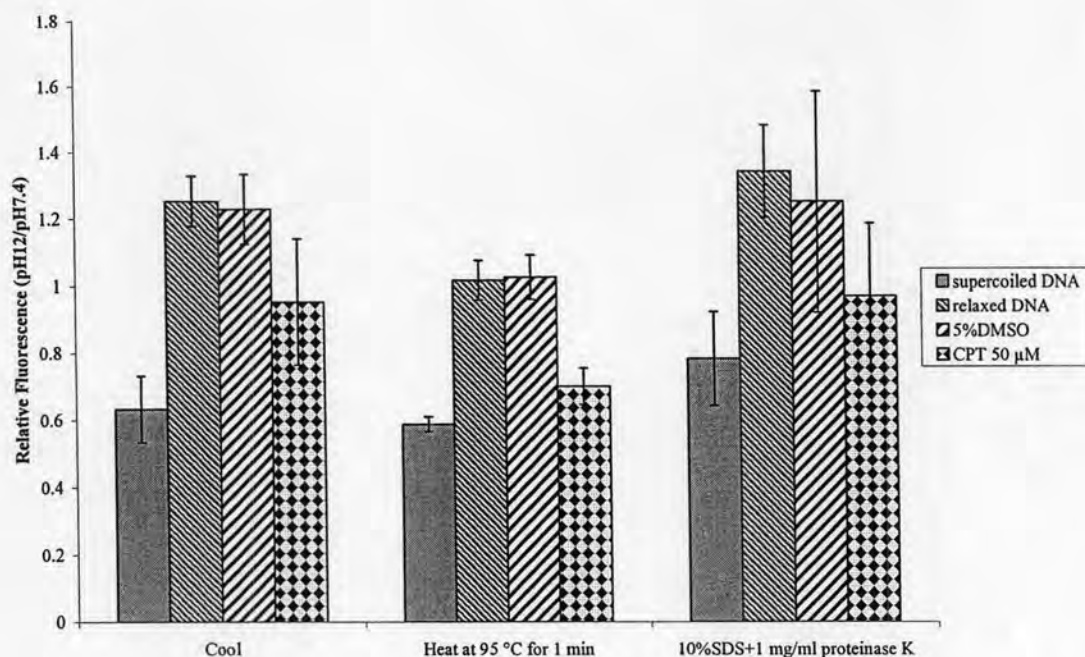

Figure 23 Relative fluorescence of Picogreen on supercoiled DNA, relaxed DNA, 5% DMSO and 50 camptothecin µM group of enzyme reaction. Compare between 3 stopped processes. Final DNA concentration was 250 ng/ml per well. Each data column was the mean ± S.D. (n=3-5 replicates).

4.2 Effect of topoisomerase I amount on relative fluorescence

The experiment was performed to evaluate whether the enzyme amount could introduce nicked DNA quantity in the enzyme reaction. The enzyme was added 0.25, 1 and 5 units into the reaction for observing nicked DNA by Picogreen. As shown in Figure 24, no significant difference was noticed among three groups. This confirmed the result that mentioned above and demonstrated ineffectiveness of increased enzyme amount on nicked DNA quantity.
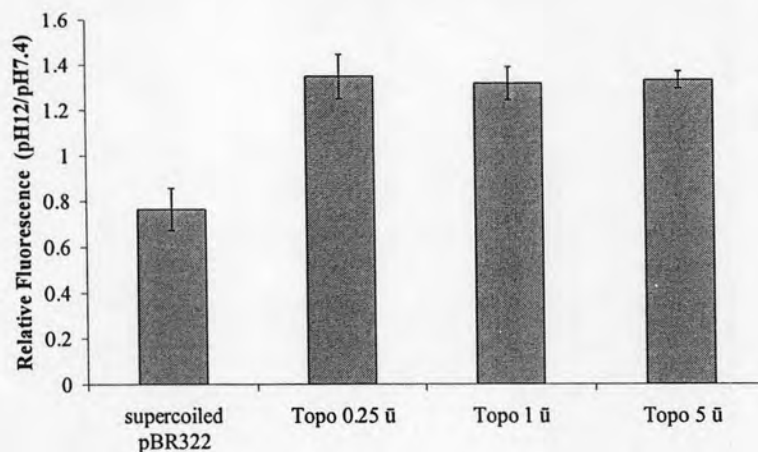
Figure 24 Effect of topoisomerase I amount on Relative fluorescence of Picogreen. All groups were stopped the reaction by cooling down the temperature. Final DNA concentration was 250 ng/ml per well. Each data column was the mean ± S.D. (n=3 replicates).

### 4.3 Time-course analysis of topoisomerase I by using fluorescence ratio assay

The difference relative fluorescence between supercoiled and relaxed DNA data was obtained from topic 4.1and 4.2, this parameter was taken in the time-course study. The Figure 25A represented the fluorescence intensity before and after added 0.1 M NaOH at difference time point. There was a fluctuation signal at the initial time of enzyme reaction. At t = 4 min, the relative fluorescence was significantly difference from t = 0 min (Figure 25B). The % conversion was shown in the Figure 25C. In the initial time of incubation, there was a fluctuation pattern like the relative fluorescence.
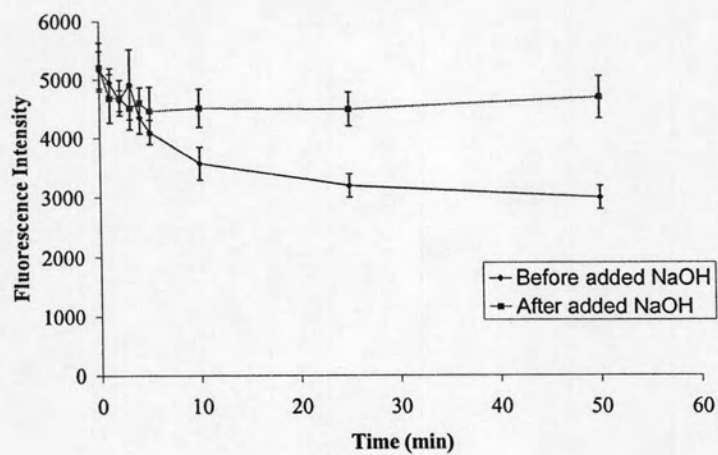
(A)
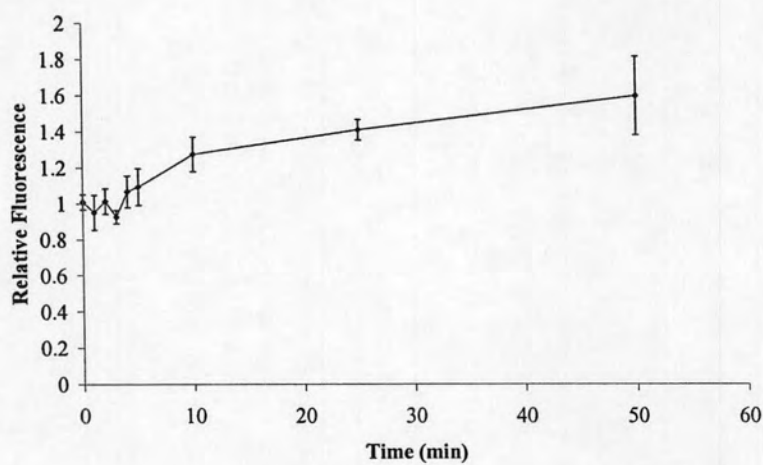
(B)
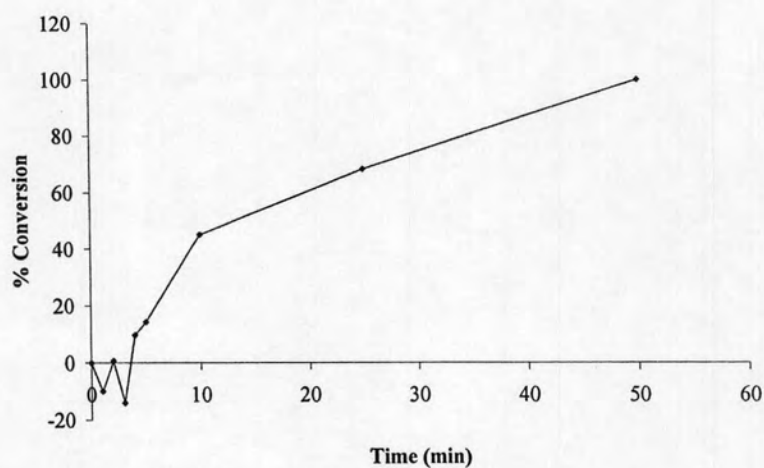
(C)

Figure 25 Time-course study using fluorescence ratio assay. (A) Fluorescence intensity of Picogreen before and after added 0.1 M NaOH. (B) Relative fluorescence of Picogreen and (C) % conversion.

4.4 Evaluation of fluorescence ratio assay to detect topoisomerase I inhibitors

Camptothecin and Menadione, a putative of a topoisomerase I poison and catalytic inhibitor were used to prove whether the fluorescence ratio assay could detect the cleavage complex induced by this enzyme. The results demonstrated dose-response manner for both inhibitors (Figure 26). The IC_{50} values of camptothecin and menadione was 7.31 ± 4.76 μM and 61.7 ± 16.9 μM, respectively. Because of menadione was a topoisomerase I catalytic inhibitor which did not induced the cleavage complex so the proposed fluorescence ratio assay should not detect this endpoint. However, in this study, the proposed assay could determined the IC_{50} value of medione demonstrated that this assay could only determined DNA relaxation assay but not DNA cleavage assay of topoisomerase I.

S = 4.71258272
r = 0.99334858

(A)

S = 10.22776206
r = 0.98633132
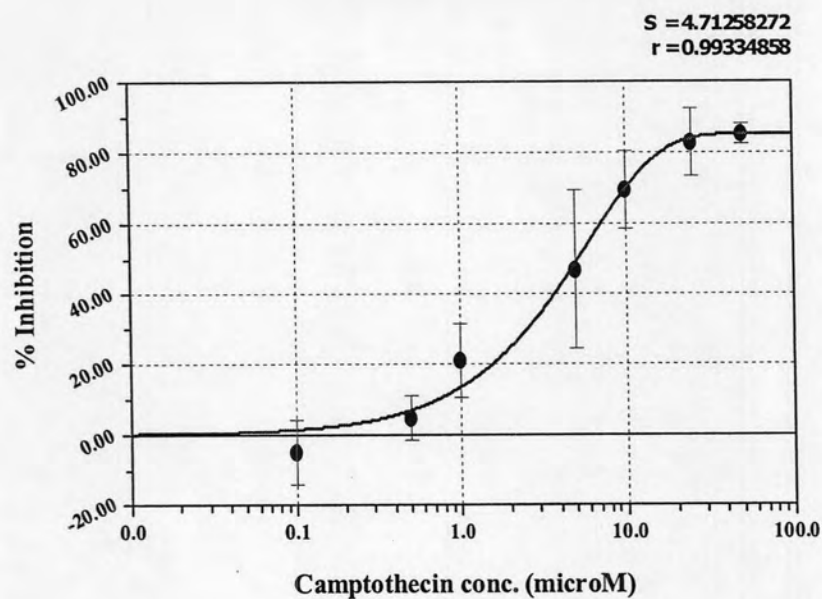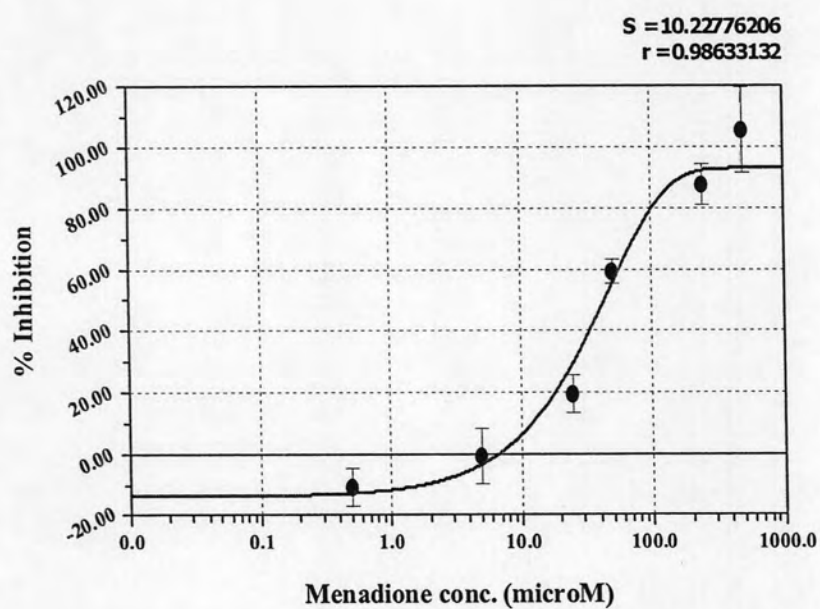
(B)

Figure 26 Dose-response curve obtained form fluorescence ratio assay. (A) Camptothecin (IC_{50} = 7.31±4.76 μM). (B) Menadione (IC_{50} = 61.7±16.9 μM). The results were presented as means ± SEM.