CHAPTER III
RESULTS

3.1 Extraction and fractionation of curcuminoids

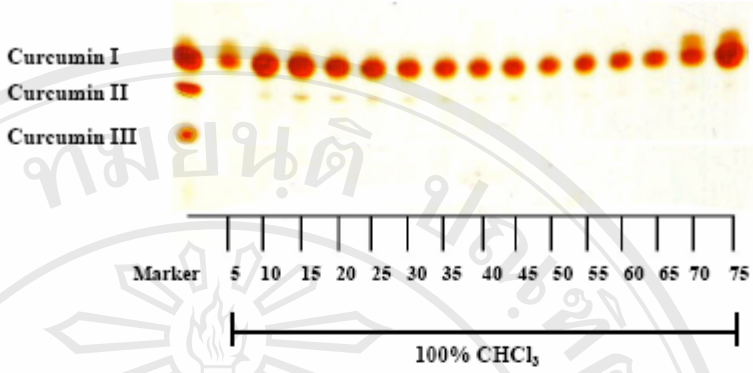
Three major forms of curcuminoids from turmeric, curcumin I, II and III were purified following the purification method which was previously reported in Ref [27, 128]. Turmeric powder was extracted with 95% ethanol then the resultant crude extract was further subjected to purification by silica gel 60 column chromatography. To yield pure fractions of curcumin I, II and III, the column was eluted using 100% $CHCl_3$ then $CHCl_3$/methanol (98:2) and $CHCl_3$/methanol (95:5) respectively. The purity of each fraction was determined by Thin Layer Chromatography (TLC) aluminum sheets, as shown in Figure 15 (A-F), the fractions that showed the same pattern on TLC were pooled and rechecked the purity (Figure 16). The result showed three pure forms of curcuminoids were attained after the column.

The purity of curcumin I, II and III was confirmed by HPLC analysis; single peak was detected from each form with the retention time at 6.91, 7.92, and 9.14 min, respectively [28]. Also as expected, the curcumin mixture showed three peaks, with a retention time of 7.06, 8.11 and 9.31 min corresponding to curcumin I, II, and III [28]. The result demonstrated curcumin I, II and III purified from turmeric after the silica gel 60 column are pured, and curcumin mixture has three major forms of curcuminoids; curcumin I, II and III.

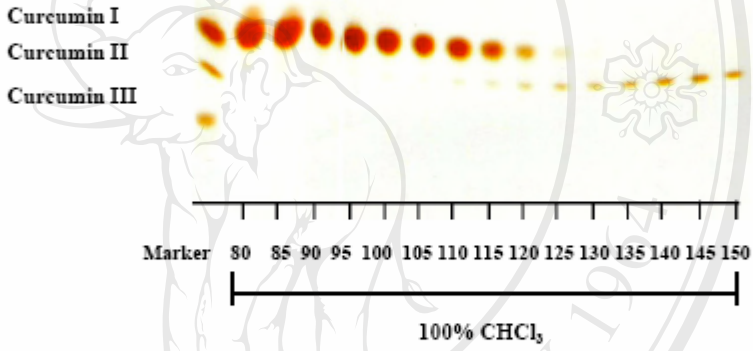
The purity analysis by IR, MS, and NMR revealed that all samples had high purity (99%) and accurate identification [28]. The data reported here are consistent with the earlier published spectroscopic analyses of curcumin I, II, and III reported by Chowdhury [148].

The HPLC chromatograms of curcuminoids as well as their IR, MS and NMR spectrums are given in Appendix A.

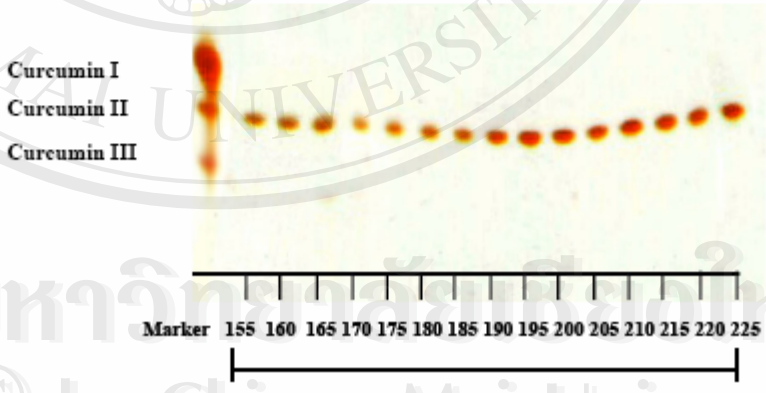
A. TLC chromatograms of curcuminoids from fraction eluted using 100% $CHCl_3$



B. TLC chromatograms of curcuminoids from fraction eluted using 100% $CHCl_3$



C. TLC chromatograms of curcuminoids from fraction eluted using $CHCl_3$/methanol (98:2)



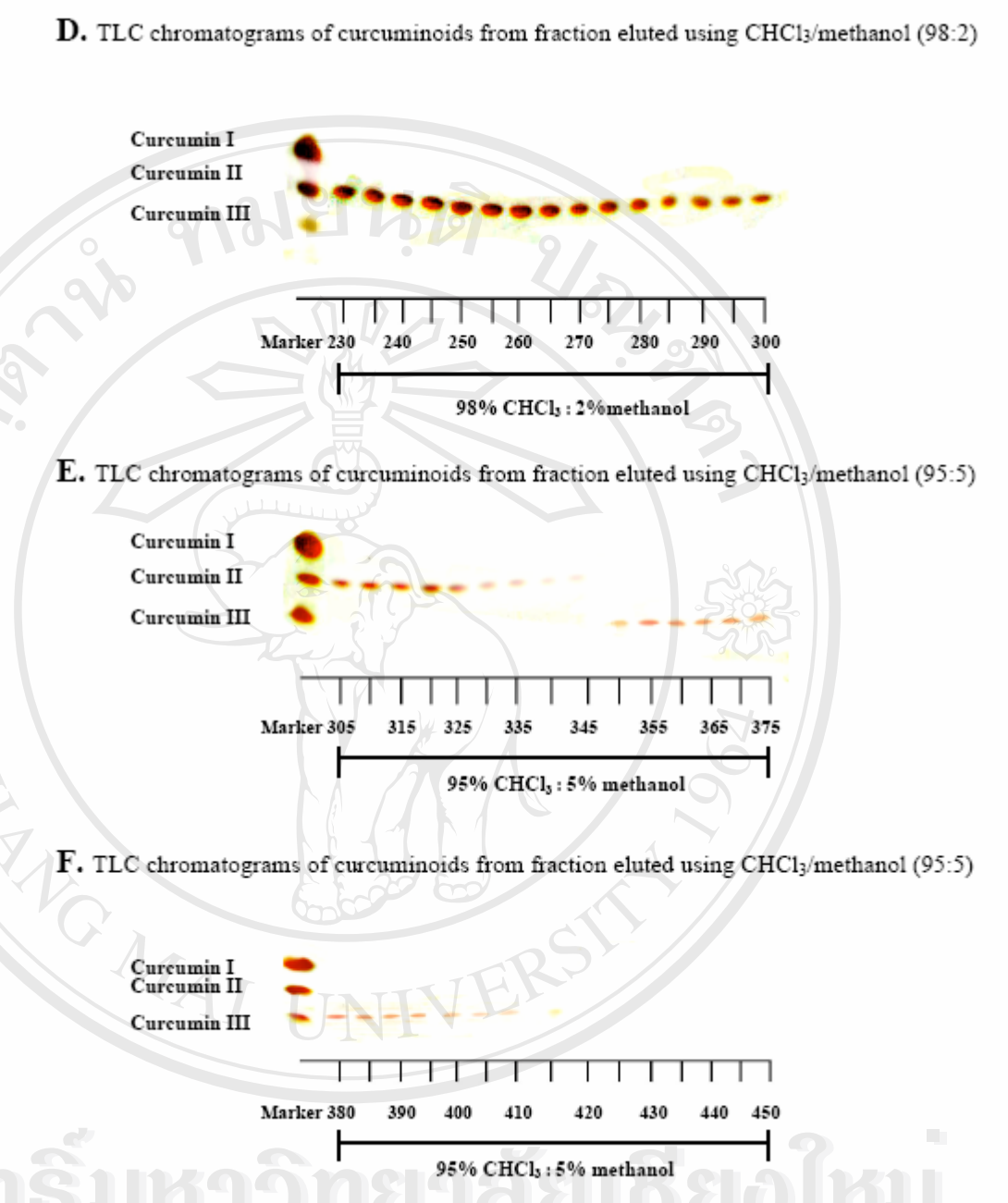


Figure 15: TLC Chromatograms of curcuminoids separating from silica gel 60 column chromatography. Three grams of curcuminoids in 30 ml acetone was loaded onto a silica gel 60 column (45x3.6cm) and each of curcuminoids eluted using 100% $CHCl_3$ at first and then $CHCl_3$:Methanol with increasing polarity; 98:2 and 95:5 respectively. The fractions were collected and the purity checked by TLC aluminum sheets coated with Silica gel 60 F254. TLC chromatograms of curcuminoids from fraction eluted using 100% $CHCl_3$, $CHCl_3$/methanol (98:2) and $CHCl_3$/methanol (95:5) are shown in Figure A-B, C-D and E-F respectively. Curcumin mixture from

Sigma was spotted as a marker and the fraction numbers were labeled under the TLC chromatogram.

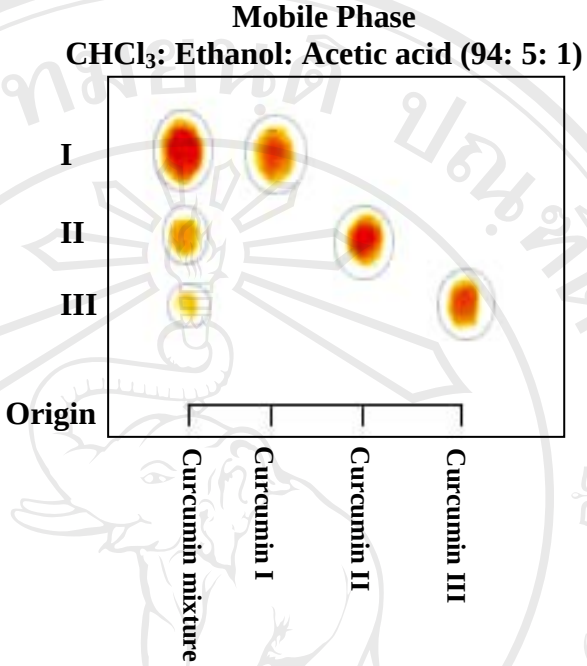


Figure 16: TLC Chromatograms of curcumin I, II and III from the pooled fractions. Fractions that showed the same pattern on TLC were pooled and the purity rechecked by TLC. Single TLC spot was observed from the pooled fractions of curcumin I, II and III indicating each form is pure. Curcumin mixture from Sigma Aldrich, in which the results from HPLC showed that it contains 77% curcumin I, 17% curcumin II and 3% curcumin III (Appendix A), was spotted as a marker.

Modulation of human P-glycoprotein (P-gp, ABCB1) function by curcuminoids

3.2 Effect of curcuminoids on P-gp function in human cervical carcinoma cells

P-glycoprotein (P-gp) is a 170-kilodalton-membrane glycoprotein which is known to play a major role in multidrug resistance in cancer cells [8]; many anticancer drugs e.g. vinblastine, vincristine, doxorubicin, paclitaxel have been described to be substrates for this efflux pump [3, 8, 18, 149]. In the first part of the study, three major curcuminoids purified from turmeric (Section 3.1) were evaluated for their effects on P-gp function using human multidrug resistant KB-V1 cells and their drug sensitive KB-3-1 cells (see Section 2.3 for the detail of these two cell lines).

3.2.1 Expression and transport capability of P-gp in KB-V-1 and KB-3-1 cells

As mentioned above, the research was carried out the first part of the study in a vinblastine-resistant KB-V-1 cell lines and its wild type, KB-3-1. However, to assure that batch of KB-3-1 and KB-V-1 cell lines that had been used in this study, the P-gp is expressed highly in KB-V-1 and no endogenous P-gp background in KB-3-1. Thus the expression of P-gp was evaluated with two monoclonal antibodies directed to P-gp intracellular epitopes by Western blot analysis (monoclonal antibody C219) and P-gp extracellular epitope by flow cytometry analysis (monoclonal antibody MRK-16). The results shown in Figure 17, highly expressed P-gp was detected in KB-V-1 cells and no endogenous background of P-gp was observed in KB-3-1 cells. Also, the transport capability of P-gp has been determined by calcien AM accumulation assay (Figure 18), in which calcein was effluxed effectively by KB-V-1 cells, not by KB-3-1(the histogram shift to the left hand side indicating lesser fluorescence intensity). The efflux was completely inhibited by CsA, a potent modulator of P-gp (the histogram shift to the right, the same position at the drug sensitive KB-3-1 cells). All the results verified that these two cell lines are appropriate to use as a tool to study the function of P-gp transporter.

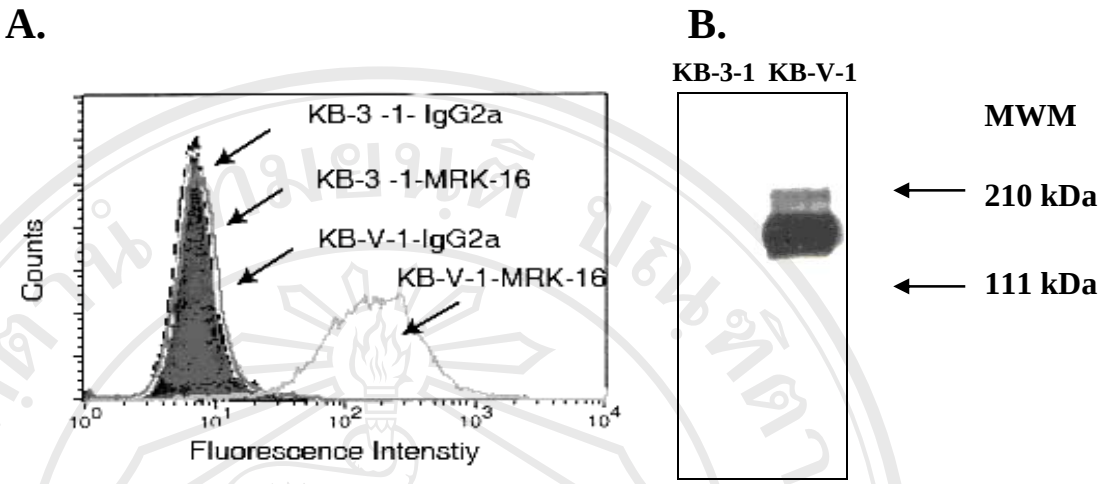


Figure 17. Expression level of P-gp detected in KB-3-1 and KB-V-1 cells. A; Flow cytometry analysis detecting P-gp by using MRK-16. Each histogram shows the overlay of the specific antibody (solid line) with the isotype control (filled gray). B; Western blot analysis stained the P-gp with specific antibody to P-gp, C219. The arrows show the position of molecular weight marker presenting that P-gp had a molecular weight about 170 kDa. MWM, molecular weight marker.

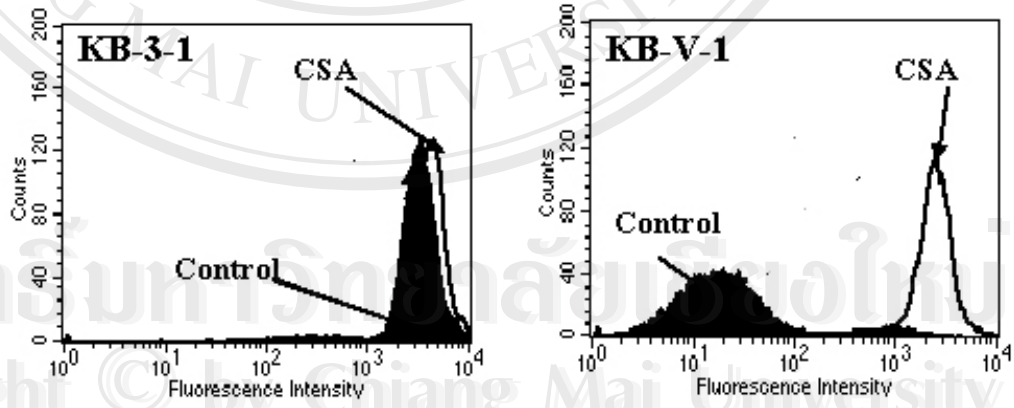


Figure 18. Functional assay for P-gp in KB-3-1 and KB-V-1 cells. Accumulation assay was performed with calcein AM. The cells were incubated for 10 min at 37°C with calcein AM in the presence or absence of 10 μM cyclosporin A (CsA). After that the cells were washed and analyzed as described in Section 2.8. Each histogram shows the overlay of the presence of CsA (solid line) with the DMSO control (0.1%) (filled gray).

3.2.2 Cytotoxicity of curcuminoids in KB-3-1 and KB-V-1 cells.

To examine whether each form of curcuminoids affects the viability of cells, KB-3-1 and KB-V-1 cells were exposed to various concentrations of curcuminoids (without drug) for 72 h and cytotoxicity was determined by MTT assay. Dose response cytotoxicity profiles for curcuminoids was observed as shown in Figure 19. The percentage of viable cells was calculated to determine the IC_{50}, the results shown in Table 14. The effect of curcumin mixture, curcumin I, II and curcumin III shows no significant difference ($P>0.05$) in IC_{50} values for cytotoxicity in KB-3-1 and KB-V-1 cells. The relative resistance calculated by dividing the IC_{50} value of the curcuminoids in KB-V-1 cells by the IC_{50} value in KB-3-1 cells is about 1, suggesting that these three forms of curcuminoids; curcumin I, II and III may not be substrate for the P-gp transporter. The concentration of curcuminoids at 10 μM (> 80% cell survival) was chosen for the MDR phenotype study.

Verapamil was the first compound found to reverse MDR in vitro by inhibiting the efflux function of P-gp. To determine the cytotoxicity of verapamil in KB-3-1 and KB-V-1, both cell lines were incubated with various concentrations of verapamil (0-100 μM). Then the survival cells were detected by MTT assay as described in Materials and Methods (Section 2.4). The data are represented as mean values ± SE of three-independent experiments performed in triplicate. The IC_{50} value was calculated and shown in Table 14. The concentration at 20 μM (> 80% cell survival) was chosen for the MDR phenotype study (Section 3.2.4)

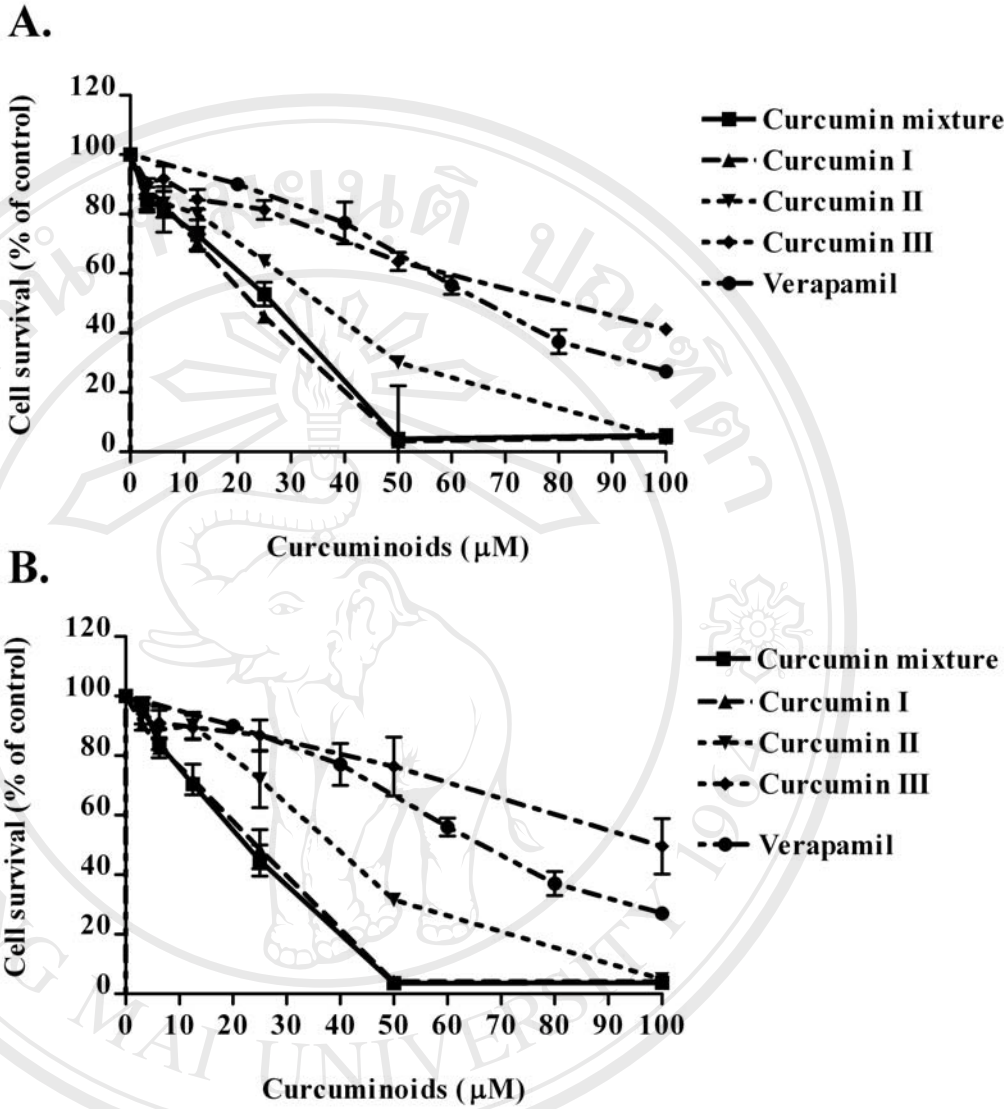


Figure 19. Cytotoxicity of curcuminoids and verapamil in KB-3-1 and KB-V-1 cells. KB-3-1 and KB-V-1 cells ($5.0X10^3$cells) were seeded into 96 well plates and cultured overnight. Various concentrations of curcuminoids or verapamil (0-100 μM) were then added and incubated further for 72 h in a 37 °C incubator. The number of viable cells was determined by MTT assay as described in Materials and Methods (Section 2.4). The experiments were conducted in triplicate and the values represent mean ± SE of three independent experiments. The data of KB-3-1 and KB-V-1 are shown in Panel A and B, respectively.

Table 14. IC_{50} values of curcuminoids and verapamil on cytotoxicity in KB-3-1 and KB-V-1 cells. The data represent the mean values±SE of three independent experiment perform in triplicate.

Curcuminoids	IC_{50} Values (μM)		Relative resistance [a]
	KB-3-1	KB-V-1	
Curcumin mixture	26.3 ± 4.7	22.8 ± 4.4	0.9
Curcumin I	24.0 ± 1.7	23.5 ± 5.6	1.0
Curcumin II	33.3 ± 2.9	35.8 ± 3.7	1.1
Curcumin III	85.0 ± 8.7	93.0 ± 9.5	1.1
Verapamil	67.0 ± 7.0	72.0 ± 5.0	1.1

[a] Relative resistance values were obtained by dividing the IC_{50} value of curcuminoids in the KB-V-1 cells by the IC_{50} value of curcuminoids in the KB-3-1 cells.

3.2.3 Modulating effect of curcuminoids on anticancer drugs sensitivity in KB-3-1 and KB-V-1 cell lines

3.2.3.1 Effect of curcuminoids on cytotoxicity of vinblastine in KB-3-1 and KB-V-1 cells.

To study the effect of curcuminoids on vinblastine cytotoxicity in the drug resistant KB-V-1 cells and the drug sensitive KB-3-1 cells, the growth inhibition of cells was investigated in response to increasing concentrations of vinblastine with and without addition of curcuminoids. Based on Figure 19 and Table 14**,** 10 μM of curcuminoids, which was the concentration at IC_{20}-IC_{10} in KB cells, was used. In this study verapamil, a well known modulator of P-gp was chosen as positive control (inhibitor).

The effects of curcuminoids on vinblastine sensitivity, as shown in Figures 20, revealed that curcumin I, II, and III at 10 μM increased the vinblastine cytotoxicity only in KB-V-1 cells. The IC_{50} shifted drastically from 1.1 μM to 0.3 μM for curcumin I or to 0.65 μM for curcumin II and to 0.68 μM for curcumin III, respectively (Figure 20B). However, this effect of curcuminoids was not observed in

parental KB-3-1 cells (Figure 20A). Furthermore, these results showed that among curcuminoids, curcumin I is the most effective form in increasing the sensitivity to vinblastine in the KB-V-1 cells (Table 15).

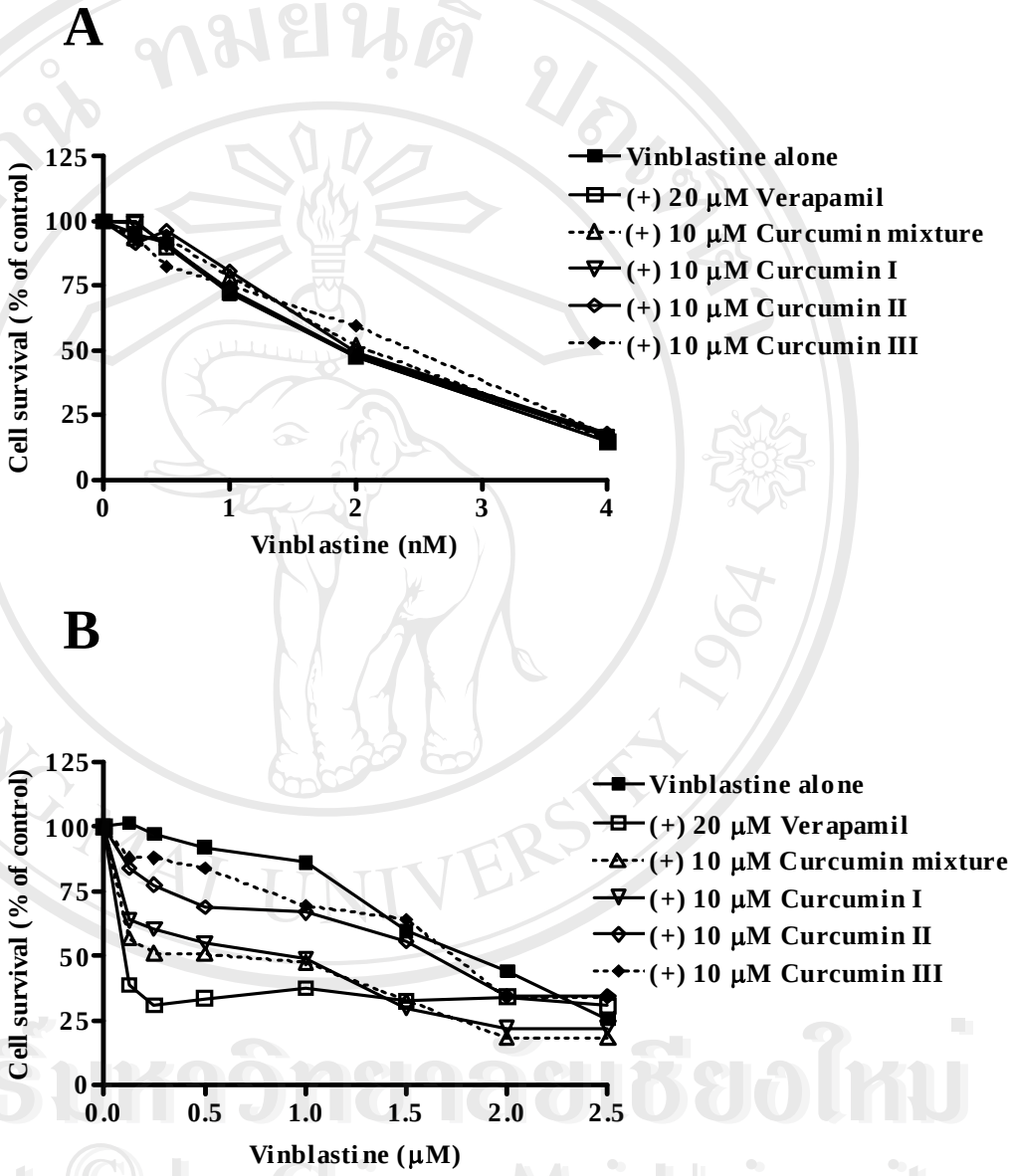


Figure 20. Effect of curcuminoids on vinblastine cytotoxicity in KB-3-1 cells (Panel A) and KB-V-1 cells (Panel B). The cells were grown in the presence of various concentrations of vinblastine as indicated alone or with 10 μM of curcumin mixture, curcumin I, curcumin II and curcumin III and 20 μM verapamil. The number of viable cells was determined by MTT assay . The result of one typical experiment from three independent experiments is depicted

3.2.3.2 Effect of curcuminoids on cytotoxicity of paclitaxel in KB-3-1 and KB-V-1 cells.

To study whether curcuminoids are able to modulate other anticancer drugs such as paclitaxel, the KB-3-1 and KB-V-1 cells were incubated with various concentrations of paclitaxel in the presence of 10 μM curcuminoids. The viable cells were determined by MTT assay as described in Materials and Methods (Section 2.4). As shown in Figure 21, curcuminoids are able to increase the sensitivity of cancer drug not only vinblastine but also paclitaxel which is the anticancer drug that has been reported about the high affinity substrate for the P-gp transporter. The IC_{50} of paclitaxel in the presence of curcumin mixture, curcumin I, II, III was significantly ($P<0.05$) decreased from 14 μM to 7.5, 8, 8.2 and 8.3 μM respectively. Verapamil was included in the experiment as a positive control. The IC_{50} and the relative resistance values for paclitaxel cytotoxicity in KB-3-1 and KB-V-1 cells are shown in Table 15.

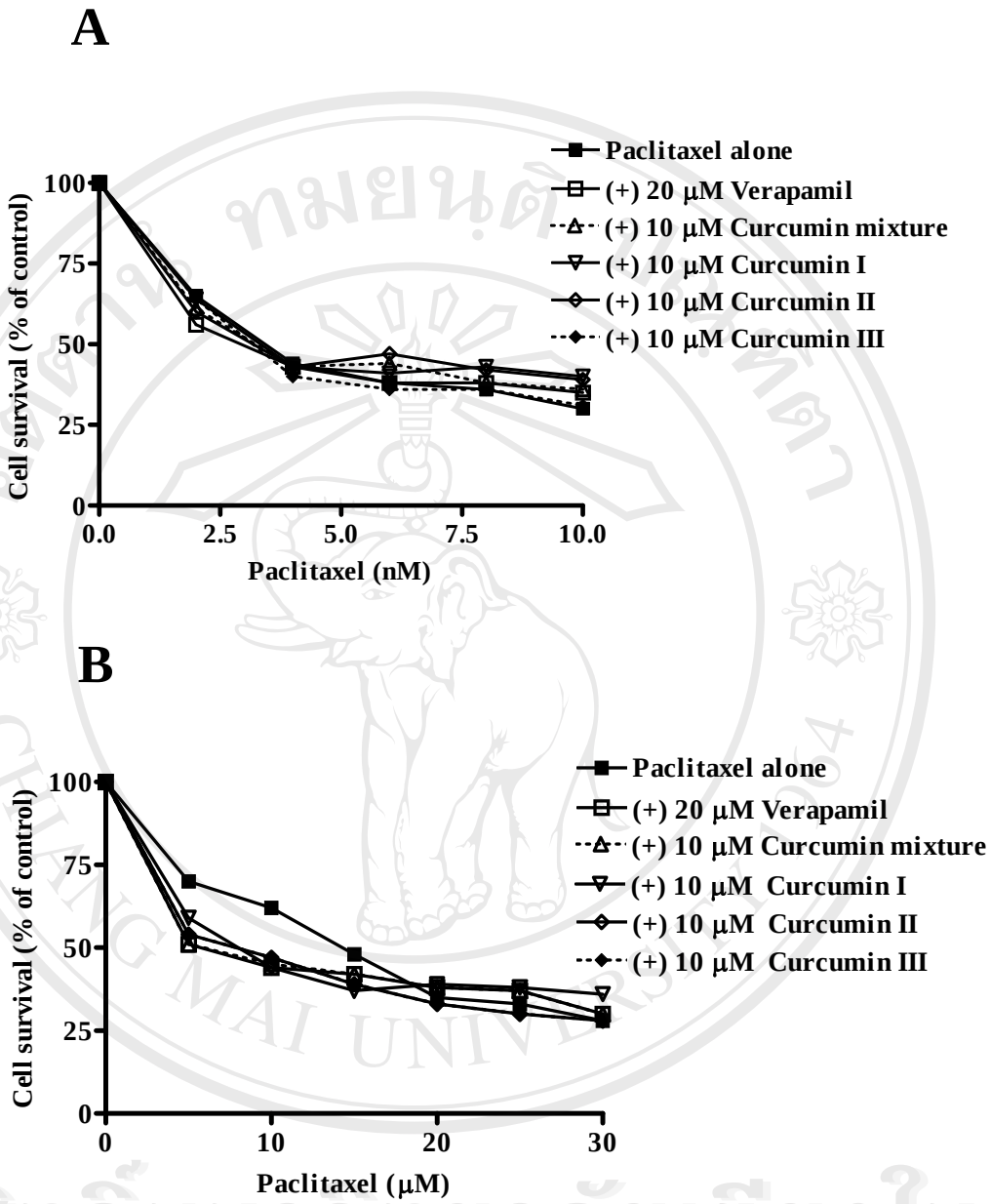


Figure 21. Effect of curcuminoids on paclitaxel cytotoxicity in KB-3-1 cells (Panel A) and KB-V-1 cells (Panel B). The cells were grown in the presence of various concentrations of paclitaxel alone or with 10 μM of curcumin mixture, curcumin I, curcumin II and curcumin III and 20 μM verapamil. The number of viable cells was determined by MTT assay. The result of one typical experiment from two independent experiments is depicted

Table 15. IC_{50} values of curcuminoids on vinblastine and paclitaxel cytotoxicity in KB-3-1 and KB-V-1 cells. The data represent the mean values±SE of three independent experiment perform in triplicate.

Compounds	IC_{50} Values		RR[a]	Degree of MDR reversal activity[b]
	KB-3-1 (nM)	KB-V-1 (μM)		
Vinblastine (0.5%DMSO)	**1.90 ± 0.2**	**1.1 ± 0.3**	**578.9**	**1**
(+) 20 μM Verapamil	2.0 ± 0..4	0.10 ± 0.2	51.3**	11.3
(+) 10 μM Curcumin mixture	2.0 ± 0.1	0.25 ± 0.1	128.2**	4.5
(+)10 μM Curcumin I	2.0 ± 0.2	0.30 ± 0.1	150**	3.9
(+)10 μM Curcumin II	2.1 ± 0.4	0.61 ± 0.1	290.5**	2.0
(+)10 μM Curcumin III	2.3 ± 0.3	0.68 ± 0.1	295.6**	1.9
Paclitaxel (0.5%DMSO)	**3.1 ± 0.5**	**14.0 ± 0.7**	**4516.1**	**1**
(+) 20 μM Verapamil	2.9 ± 0.1	5.0± 0.2	1724.1**	2.6
(+) 10 μM Curcumin mixture	3.0 ± 0.4	7.5± 0.6	2500.5**	1.8
(+)10 μM Curcumin I	3.0 ± 0.1	8.0 ± 0.9	2666.7**	1.7
(+)10 μM Curcumin II	3.1 ± 0.5	8.2± 1.0	2645.2**	1.7
(+)10 μM Curcumin III	3.1 ± 0.8	8.3± 1.3	2677.4**	1.6

[a] : Relative resistance values were obtained by dividing the IC_{50} value of the tested compounds in KB-V-1 cells by the IC_{50} value of the tested compounds the KB-3-1 cells.

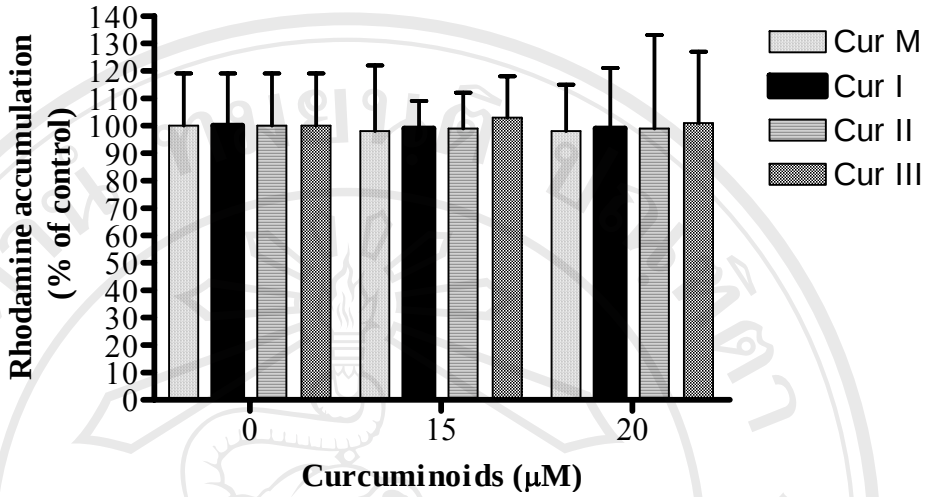
[b] : Degree of reversal activity were obtained by dividing the relative resistance (RR) value of vinblastine or paclitaxel alone (0.5% DMSO) by the relative resistance value of vinblastine or paclitaxel in the presence of curcuminoids or verapamil

** Asterisk denotes values that were significantly different from the control ($P<0.05$)

3.2.4 Effect of curcuminoids on P-gp mediated drug transport.

The effect of curcuminoids on P-gp function was confirmed by accumulation assay. Three fluorescent substrates of P-gp: calcein-AM, rhodamine123, and bodipy-FL-vinblastine were used in this experiment. The activity of P-gp was assessed by measuring the intracellular accumulation of the fluorescence substrate in P-gp expressing KB-V-1 cell compared with its vehicle control (0.1% DMSO). Flow cytometry was carried out to investigate the amount of the fluorescence intensity of the fluorescence substrate. The results showed that curcumin mixture, curcumin I, II, and III increased the accumulation of fluorescent substrates, rhodamine123 (Figure 22), calcien (Figure 23) and bodipy-FL-vinblastine (Figure 24) in a dose dependent manner (0-25 μM). Figure 25 shows the effect of 20 μM curcumin mixture and curcumin I, II, III on the accumulation of the fluorescent substrates in comparison to the cyclosporin A, a potent inhibitor of P-gp. The results showed the effective accumulation of the fluorescent substrate by curcuminoids, and curcumin I is the most effective form. Taken together, these results further confirmed previous findings (MTT assays) curcuminoids inhibit the efflux function of P-gp and curcumin I was the most active form present in the curcumin mixture.

A. Effect of curcuminoids at 20 µM on rhodamine123 accumulation in KB-3-1



B. Effect of curcuminoids (0-25 µM) on rhodamine123 accumulation in KB-V-1

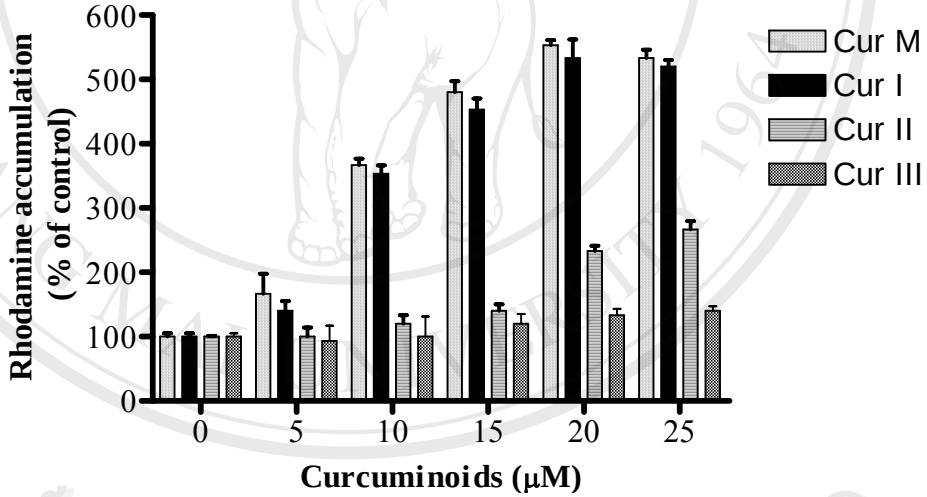
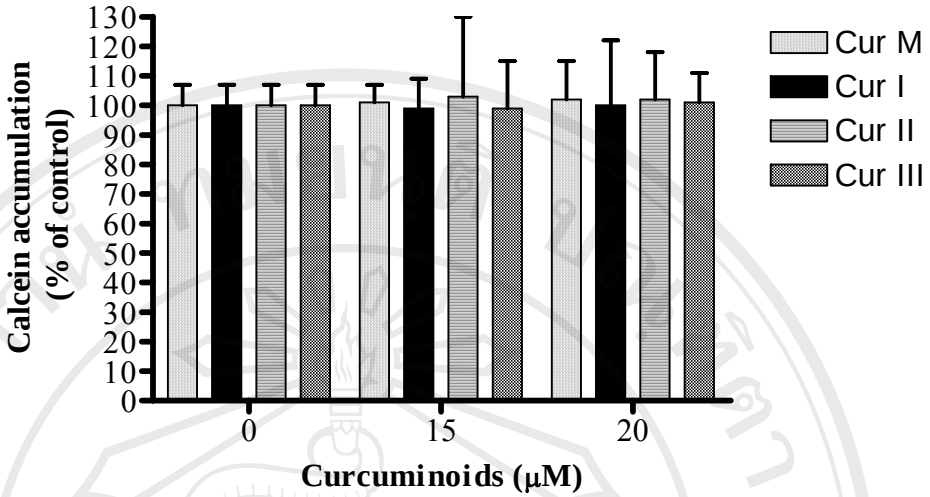


Figure 22. Effect of curcuminoids on rhodamine123 accumulation in KB-3-1 and KB-V-1. Cells were incubated at 37°C for 45 min in dark condition with 0.5 µg/ml rhodamine123 in the presence of various concentrations of the curcuminoids (0-25 µM). The accumulation of the rhodamine123 was analyzed immediately by FACS. Mean fluorescence intensity values was collected and representated as histogram (% of control). The experiments were conducted in A. KB-3-1 and**.** B. KB-V-1 cells.

A. Effect of curcuminoids at 20 µM on calcein accumulation in KB-3-1



B. Effect of curcuminoids at 0-25 µM on calcein-AM accumulation in KB-V-1

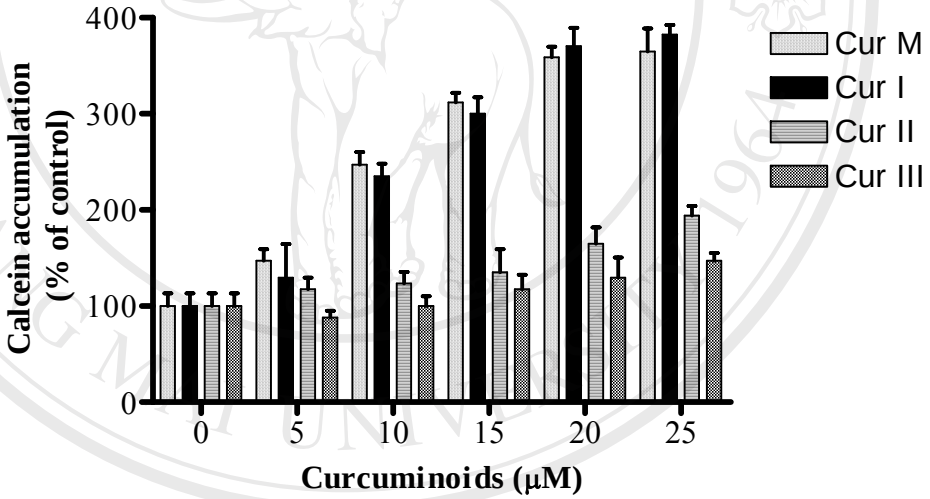
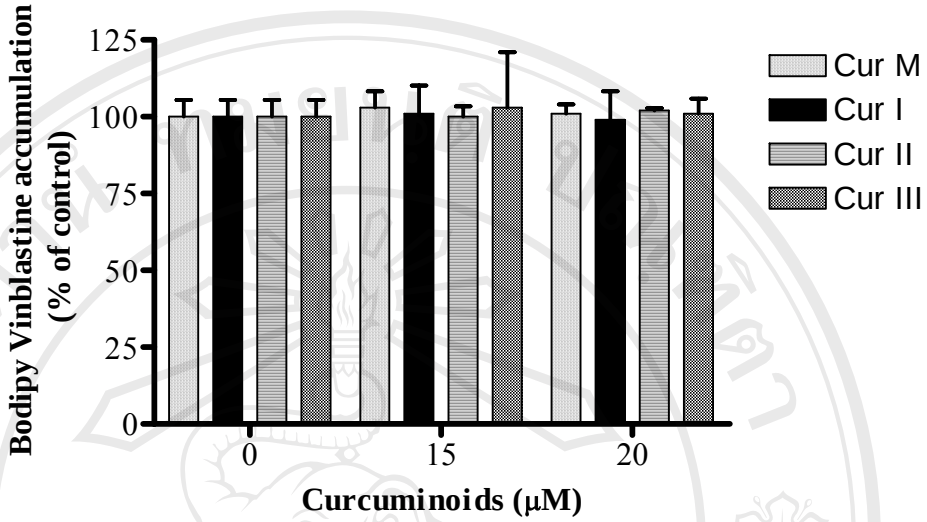


Figure 23. Effect of curcuminoids on calcein accumulation in KB-3-1 and KB-V-1. To study the effect of curcuminoids on P-gp transport function, the activity of P-gp was assessed by measuring the intracellular accumulation of calcein AM. Cells were incubated at 37°C for 10 min in dark condition with 0.25 µM calcein AM in the presence of various concentrations of the curcuminoids (0-25 µM). The accumulation of the calcein was analyzed immediately by FACS. Mean fluorescence intensity values was collected and represented as histogram (% of control). The experiments were conducted in A. KB-3-1 and**.** B. KB-V-1 cells.

A. Effect of curcuminoids at 20 μM on bodipy-FL-vinblastine accumulation in KB-3-1



B. Effect of curcuminoids (0-25 μM) on bodipy-FL-vinblastine accumulation in KB-V-1

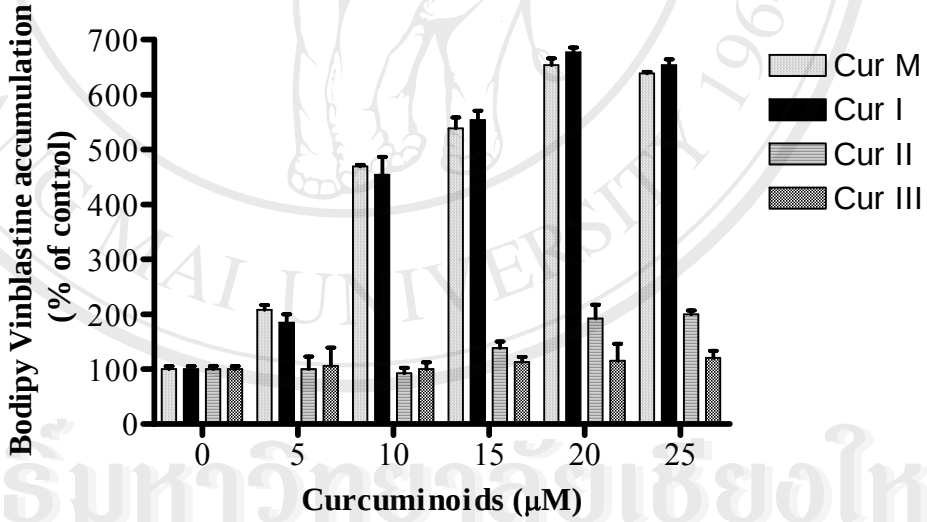


Figure 24. Effect of curcuminoids on bodipy-FL-vinblastine accumulation KB-3-1 and KB-V-1. To study the effect of curcuminoids on P-gp transport function, the activity of P-gp was assessed by measuring the intracellular accumulation of bodipy-FL-vinblastine. Cells were incubated at 37°C for 45 min in dark condition with 0.5 μM bodipy-FL-vinblastine in the presence of various concentrations of the curcuminoids (0-25 μM). The accumulation of the bodipy-FL-vinblastine was analyzed immediately by FACS. Mean fluorescence intensity values was collected

and representated as histogram (% of control). The experiments were conducted in A. KB-3-1 and**.** B. KB-V-1 cells.

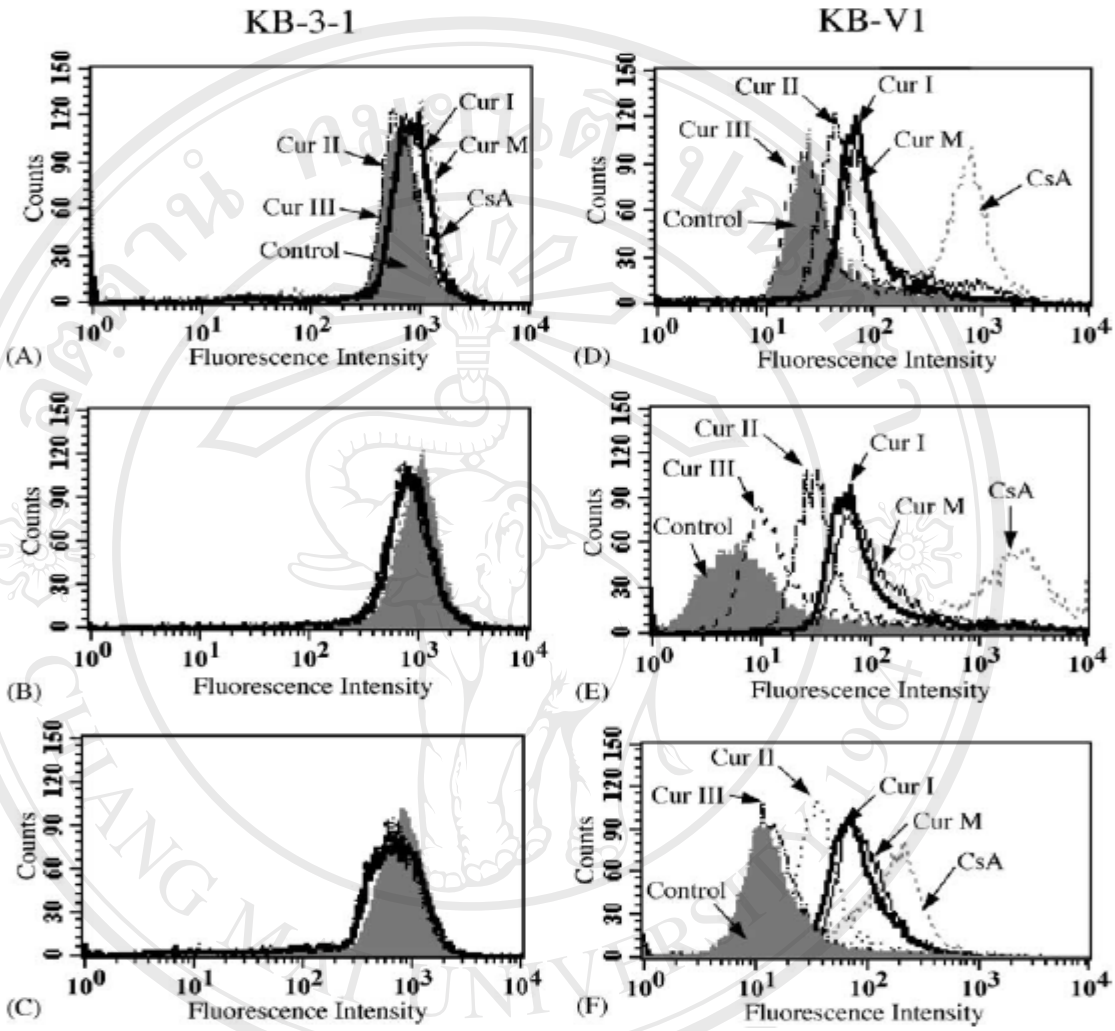


Figure 25. Effect of curcuminoids on rhodamine123, calcein-AM and bodipy-FL-vinblastine accumulation in KB-3-1 and KBV-1 cells. Cells were resuspended in IMDM supplemented with 5% FBS. Rhodamine123 (0.5 μg/mL) or bodipy-FL-vinblastine (0.5 μM) or calcein-AM (0.25 μM) was added to 5 X 10^5 cells in 4 ml of IMDM in the presence or absence of reversing agent cyclosporin A (10 μM) or indicated curcuminoid (20 μM). The cells were incubated at 37 ^{0}C in dark. After 45 min for rhodamine123 and bodipy-FL-vinblastine or 10 min incubation time for calcein-AM, the cells were pelleted by centrifugation at 500 x g and resuspended in 300 μl of PBS containing 0.1% BSA. Samples were analyzed immediately by using flow cytometer. A-C, and D-E represented the results in KB-3-1 and KB-V-1 respectively

3.2.5 Effect of curcuminoids on ATPase activity of P-gp

The transport of a drug substrate by P-gp is coupled to ATP hydrolysis and there is evidence for stimulation of ATPase activity of P-gp by drug-substrates or modulators from diverse systems [150, 151]. This has led to the use of the stimulation by substrate or modulator of ATP hydrolysis by P-gp as a surrogate assay to determine drug-P-gp interaction.

In this study, the ATPase activity of P-gp in crude membrane was measured by endpoint, Pi assay, based on the principle of the ATP hydrolysis yields inorganic phosphate (Pi) thus the amount of Pi liberated by the P-gp is proportional to the activity of the P-gp. The assay measured the amounts of Pi released over 20 min at 37^{o}C in the ATPase assay buffer (see Appendix D). The assay was initiated by the addition of 5 mM ATP in the presence and absence of the tested compounds and quenched with SDS (2.5% final concentration). The amount of Pi released was quantified using a colorimetric reaction as described in Material and Methods, Section 2.13.

As shown in Figure 26 and 27, curcumin mixture, curcumin I, II, and III, stimulate the ATPase activity at low concentrations (0.5-1 μM) and inhibit the ATPase activity to the level of basal at higher concentrations. In addition, curcuminoids inhibited verapamil-stimulated ATPase activity in a concentration-dependent manner (Figure 26 and 27). IC_{50} values for curcumin I, II and III in the presence of 5 μM verapamil are in the ranged from 5-15 μM. The results in Table 16, revealing that, among curcuminoids, curcumin I was as effective as the curcumin mixture (IC_{50} values 5.90 and 5.24 μM, respectively), and curcumin II and III are less effective.

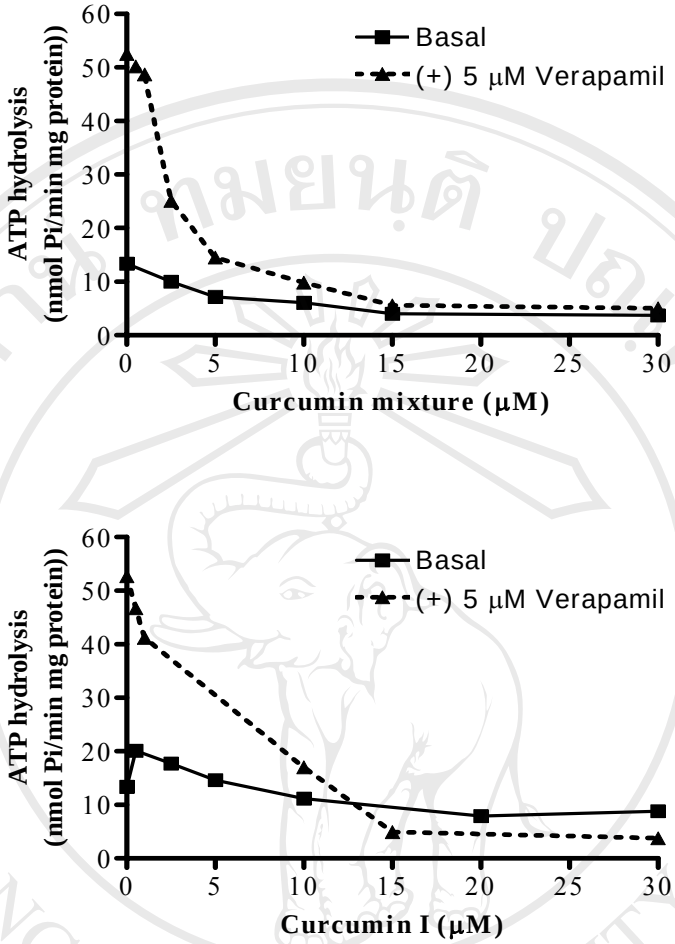


Figure 26. Effect of curcumin mixture and I on basal and verapamil –stimulated of P-gp ATPase activity. Crude membranes of High Five insect cells expressing *MDR*1 (100 μg protein/ml) were incubated with increasing concentrations of curcumin mixture and curcumin I (0-30 μM) in the presence and absence of 5 μM verapamil. P-gp-specific activity was recorded as the vanadate-sensitive ATPase activity as described in the Materials and Methods Section 2.13. ***Upper Panels*** ; (■) P-gp ATPase activity (DMSO control) and (▲) verapamil-stimulated P-gp ATPase activity in the presence of curcumin mixture. ***Lower Panel*;** (■) P-gp ATPase activity (DMSO control) and (▲) verapamil-stimulated P-gp ATPase activity in the presence of curcumin I. The results are representative of at least three independent experiments, one typical experiment is depicted.

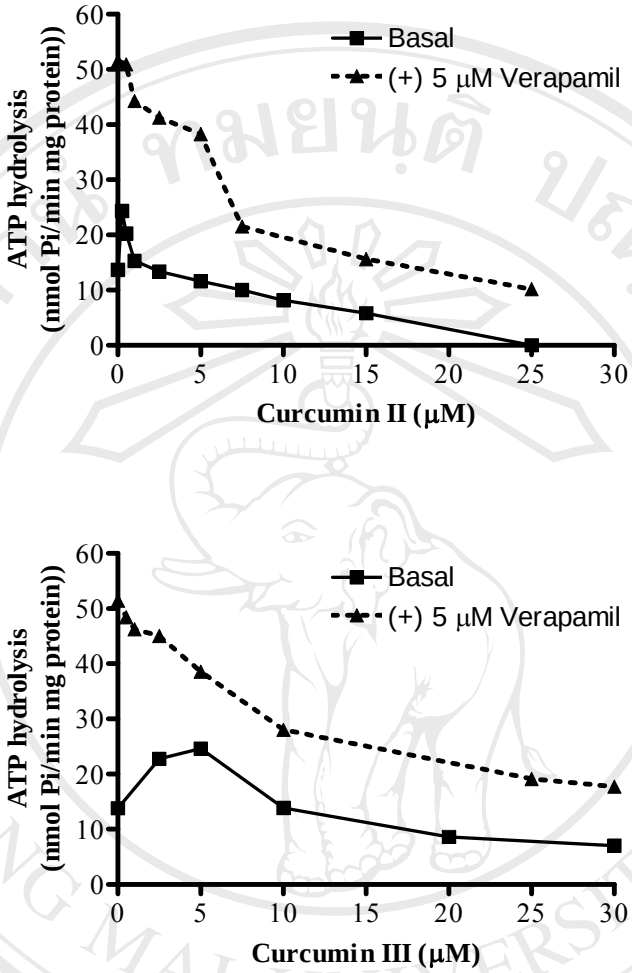


Figure 27. Effect of curcumin II and III on basal and verapamil –stimulated of P-gp ATPase activity. Crude membranes of High Five insect cells expressing *MDR*1 (100 μg protein/ml) were incubated with increasing concentrations of curcumin II and III (0-30 μM) in the presence and absence of 5 μM verapamil. P-gp-specific activity was recorded as the vanadate-sensitive ATPase activity as described in the Materials and Methods section 2.13. ***Upper Panels*** ; (■) P-gp ATPase activity (DMSO control) and (▲) verapamil-stimulated P-gp ATPase activity in the presence of curcumin II. ***Lower Panel***; (■) P-gp ATPase activity (DMSO control) and (▲) verapamil-stimulated P-gp ATPase activity in the presence of curcumin III. The results are representative of at least three independent experiments, one typical experiment is depicted.

Table 16. The IC_{50} values of curcuminoids on ATPase activity of P-gp.

Compound	ATP hydrolysis (nanomoles/min/mg protein)[a]	
	Basal (Fold-stimulation)	(+) 5 μM Verapamil IC_{50} (μM)[b]
Curcumin mixture	2.0 ± 0.9[c]	5.2 ± 1.0
Curcumin I	1.4 ± 0.1	5.9 ± 1.7
Curcumin II	2.5 ± 0.2	10.4 ± 2.1
Curcumin III	2.4 ± 1.2	14.6 ± 3.0

[a] The vanadate-sensitive ATPase activity was measured in the presence of curcuminoids.

[b] IC_{50} (concentration required for 50% inhibition) of verapamil (5 μM)-stimulated ATPase activity.

[c] Values are mean ± SE of three independent experiments.

3.2.6 Effect of curcuminoids on photoaffinity labeling of P-gp with [^{125}I]-iodoarylazidoprazosin

To assess whether curcuminoids interact directly with the substrate binding site(s) of P-gp, their effect on photoaffinity labeling of P-gp by IAAP was tested. The data shown in Figure 28-31 demonstrated that curcuminoids effectively inhibited photoaffinity labeling of P-gp with IAAP in a concentration– dependent manner. The IC_{50} values for inhibition of IAAP binding were 5.7, 9.7, 23.3 and 5.0 μM for curcumin I, II, III and curcumin mixture, respectively (Table 17). When compare the IC_{50} value (concentration required for 50% inhibition), it was revealed that curcumin I is the most effective form among curcuminoids but slightly less effective than curcumin mixture (Table 17). It is also clear that the IC_{50} values for the inhibition of both IAAP binding and verapamil-stimulated ATPase activity are in a similar range (see Table 16 and 17) suggesting that the curcuminoids exert their inhibitory effect most likely by binding to the substrate-binding sites on the transporter.

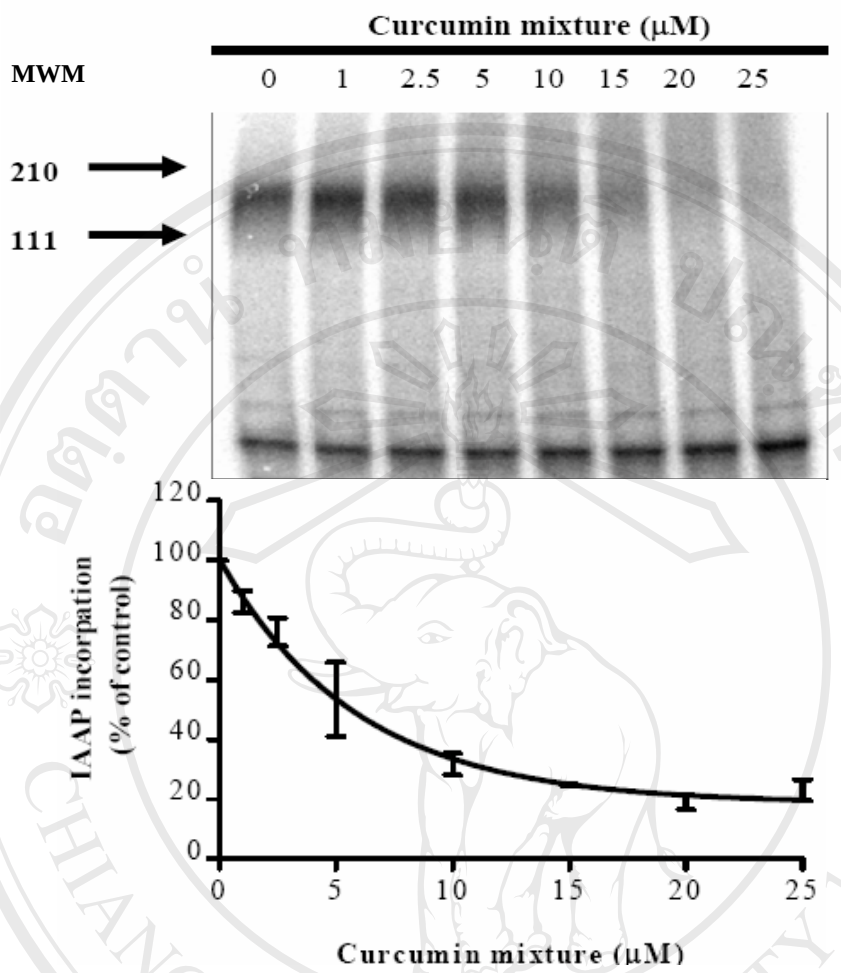


Figure 28. Effect of various concentrations of curcumin mixture on photoaffinity labeling of P-gp with IAAP. The crude membranes of High Five insect cells were incubated with increasing concentrations of curcumin mixture in the presence IAAP following SDS-PAGE. Then the gels were dried and exposed to Bio-Max MR film at –70 ^{o}C for 12-24 hr. The radioactivity incorporated into the P-gp band was quantified using the STORM 860 Phosphorimager system. ***Upper Panel***: he autoradiograms show incorporation of IAAP into the P-gp band in the presence of various concentrations of curcumin mixture. The arrow indicated the band occurring from the incorporation of [^{125}I]IAAP into Ppg. ***Lower Panel:*** the data were fitted by non-linear least squares regression analysis using the software GraphPad Prism 2.0 for the PowerPC Macintosh and are representative of three independent experiments. MWM, molecular weight marker.

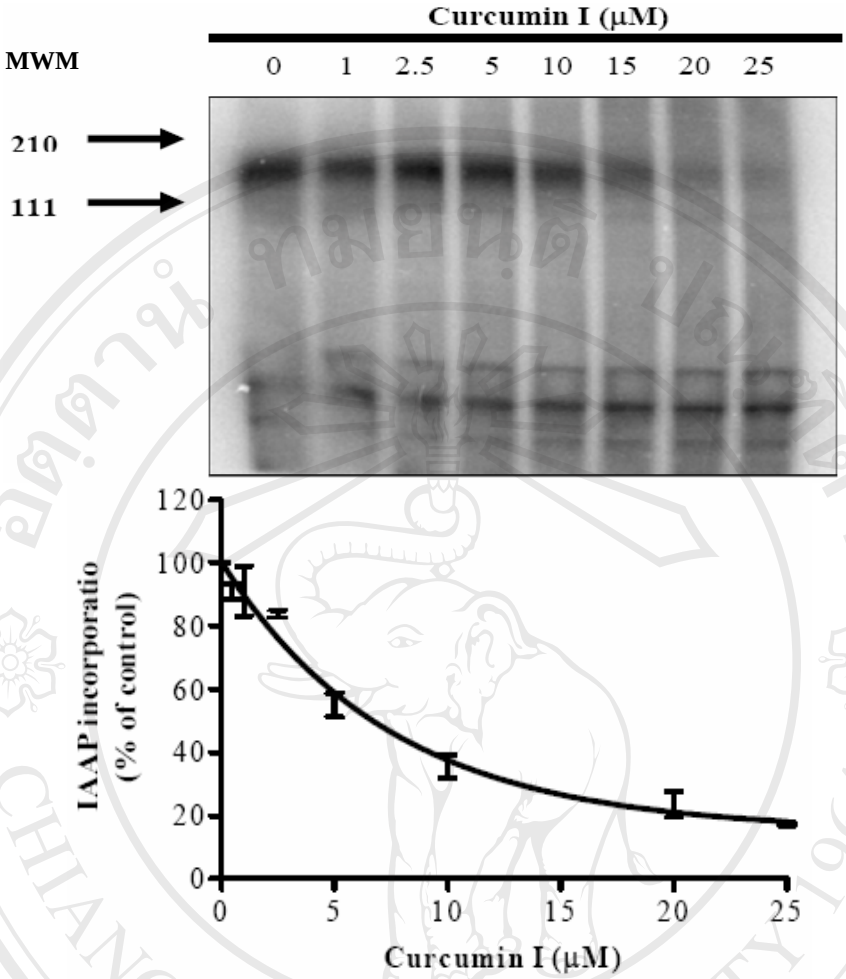


Figure 29. Effect of various concentrations of curcumin I on photoaffinity labeling of P-gp with IAAP. The crude membranes of High Five insect cells were incubated with increasing concentrations of curcumin I in the presence IAAP following SDS-PAGE. Then the gels were dried and exposed to Bio-Max MR film at –70 °C for 12-24 hr. The radioactivity incorporated into the P-gp band was quantified using the STORM 860 Phosphorimager system. ***Upper Panel***: the autoradiograms show incorporation of IAAP into the P-gp band in the presence of various concentrations of curcumin I. The arrow indicated the band occurring from the incorporation of [^{125}I] IAAP into Ppg. ***Lower Panel***: the data were fitted by non-linear least squares regression analysis using the software GraphPad Prism 2.0 for the PowerPC Macintosh and are representative of three independent experiments. MWM, molecular weight marker.

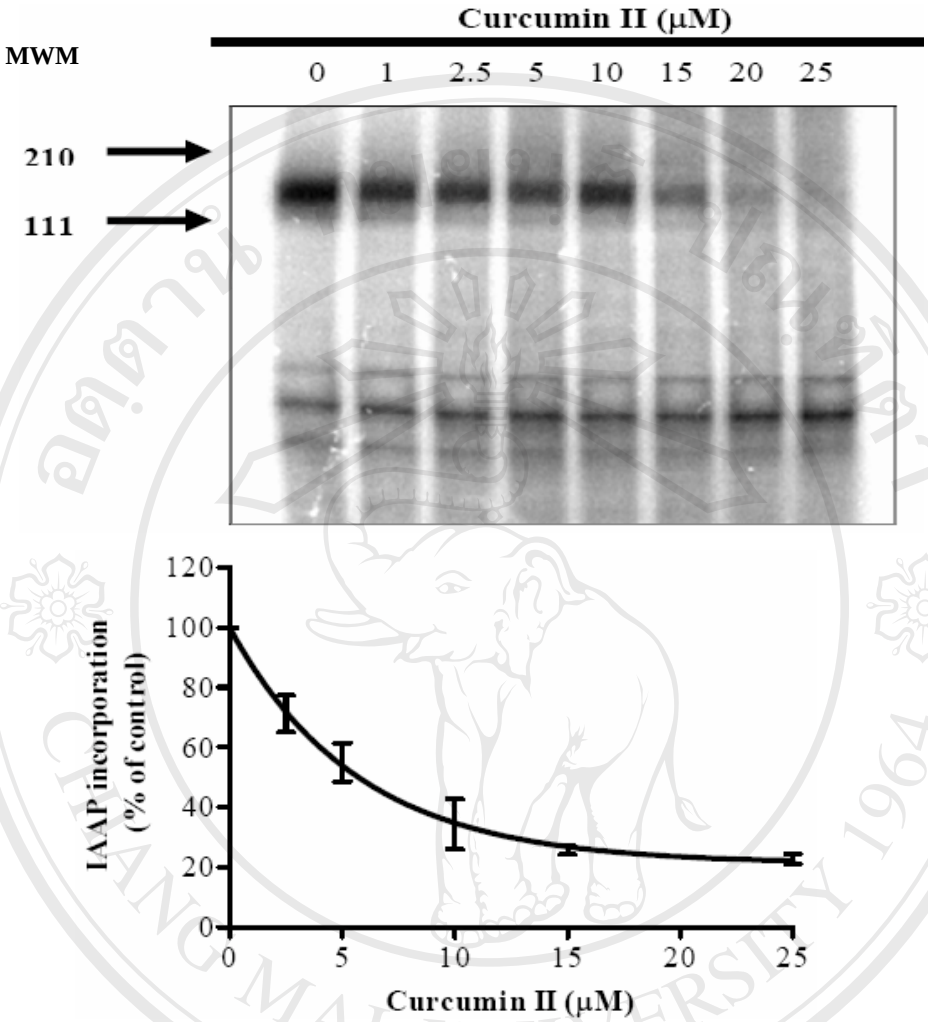


Figure 30. Effect of various concentrations of curcumin II on photoaffinity labeling of P-gp with IAAP. The crude membranes of High Five insect cells were incubated with increasing concentrations of curcumin II in the presence IAAP following SDS-PAGE. Then the gels were dried and exposed to Bio-Max MR film at –70 ^{o}C for 12-24 hr. The radioactivity incorporated into the P-gp band was quantified using the STORM 860 Phosphorimager system. ***Upper Panel:*** the autoradiograms show incorporation of IAAP into the P-gp band in the presence of various concentrations of curcumin II. The arrow indicated the band occurring from the incorporation of [^{125}I]IAAP into Ppg. ***Lower Panel:*** the data were fitted by non-linear least squares regression analysis using the software GraphPad Prism 2.0 for the PowerPC Macintosh and are representative of three independent experiments. MWM, molecular weight marker.

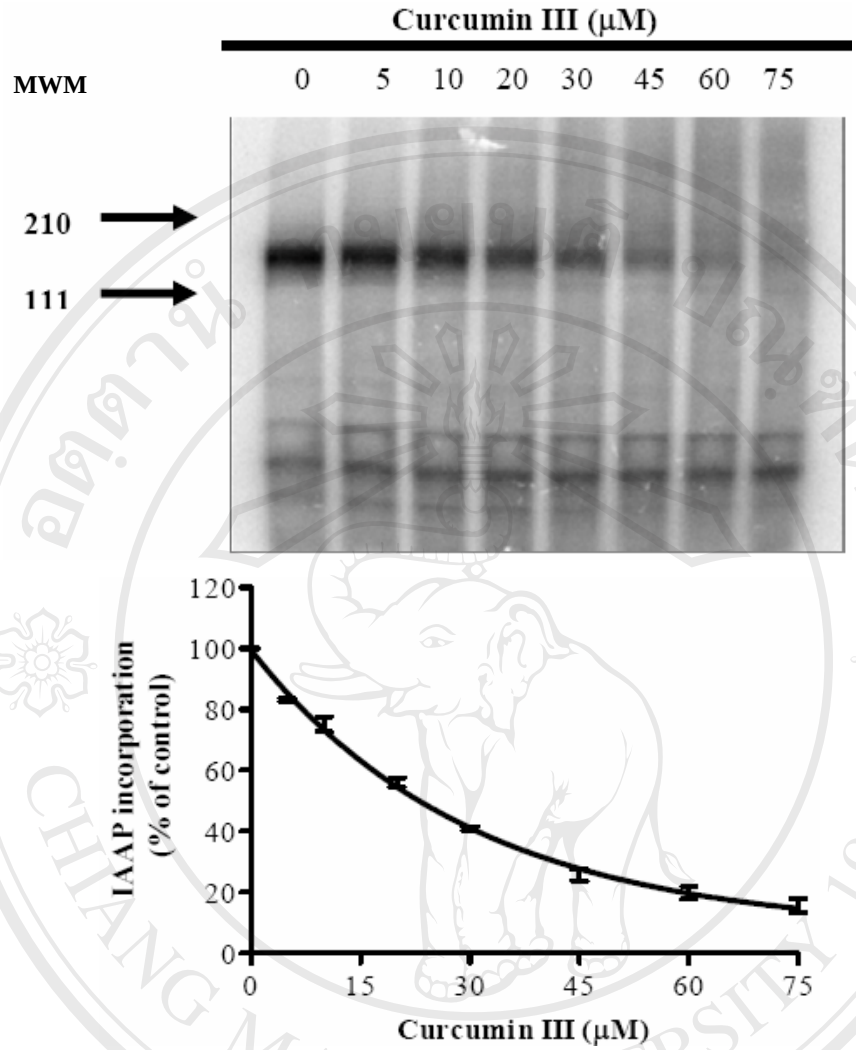


Figure 31. Effect of various concentrations of curcumin III on photoaffinity labeling of P-gp with IAAP. The crude membranes of High Five insect cells were incubated with increasing concentrations of curcumin III in the presence IAAP following SDS-PAGE. Then the gels were dried and exposed to Bio-Max MR film at –70 °C for 12-24 hr. The radioactivity incorporated into the P-gp band was quantified using the STORM 860 Phosphorimager system (27). ***Upper Panel:*** the autoradiograms show incorporation of IAAP into the P-gp band in the presence of various concentrations of curcumin III. The arrow indicated the band occurring from the incorporation of [^{125}I]IAAP into Ppg. ***Lower Panel***: the data were fitted by non-linear least squares regression analysis using the software GraphPad Prism 2.0 for the PowerPC Macintosh and are representative of three independent experiments. MWM, molecular weight marker.

Table 17. The IC_{50} values of curcuminoids on photoaffinity labeling of P-gp with IAAP

Compound	IC_{50} (μM)[a]
Curcumin mixture	5.0 ± 0.2[b]
Curcumin I	5.6 ± 0.4
Curcumin II	9.7 ± 1.0
Curcumin III	23.3 ± 1.5

[a] IC_{50} (concentration required for 50% inhibition).

[b] Values are mean ± SE of at least three independent experiments.

Modulation of human multidrug resistance protein (MRP1, ABCC1) function by curcuminoids

3.3 Effect of curcuminoids on MRP1 function

In the second part of the study, researcher focused the interest on the ABCC1 (or MRP1) transporter. In contrast to P-gp, MRP1 is primarily active transporter of GSH, oxidized GSH, glucuronate and sulfate conjugated of organic anions [152]. Many chemicals have been reported to inhibit MRP1-mediated transport, such as MK571, ONO-178 and glibenclamide [153]. Recently, the inhibitory effects of curcumin mixture on MRP1-mediated transport using isolated membrane vesicles of MRP1 expressing Sf9 insect cells was reported [154]. However, the mechanism of inhibition remains unknown. Moreover, it is unknown whether each pure form of curcuminoids exhibit the same effect. Previous part (Section 3.2), the research was reported that among the three major forms of the curcuminoids, curcumin I is the most active in modulating the function of P-gp by binding directly to the transporter P-gp, and perhaps to the same binding site as verapamil or prazosin. In the second part of the study, the research was compared the effects of the individual curcumin I, II and III and curcumin mixture with regard to their ability to inhibit MRP1 function and expression. Moreover, the research was probed the mechanism to clarify the nature of the modulatory effects of the curcuminoids on MRP1.

In this part of the study, the effect of curcuminoids were tested for their ability to modulate the function of MRP1 using human embryonic kidney 293 cells (HEK 293) transfected with MRP1-pcDNA3.1 and pcDNA3.1 vector alone.

3.3.1 Expression and transport capability of MRP1 in pcDNA 3.1- and MRP1 – HEK 293 cells

As preliminary data, the level of MRP1 in MRP1 transfected HEK 293 cells and its empty vector control pcDNA3.1 was determined by western blot analysis and the ability of the cells to transport calcein, a substrate of MRP1, was analyzed by flow cytometry. In the present study, by the western blot analysis- large amount of MRP1 protein was observed in MRP1-HEK 293 cells and it was not found in the empty vector control, pcDNA3.1-HEK 293 (Figure 32).

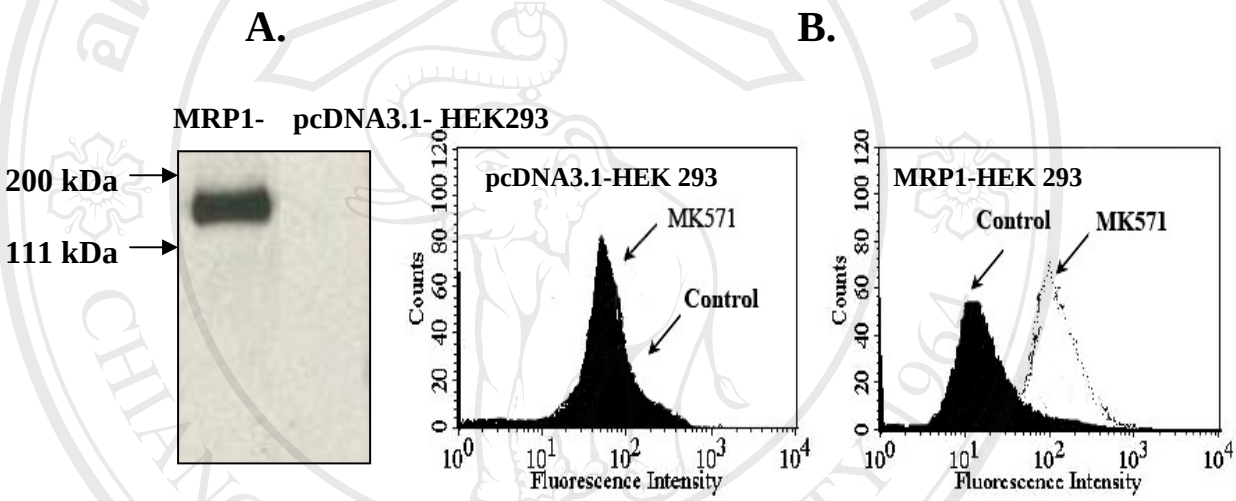


Figure 32. Expression and transport capability of MRP1 in MRP1-HEK 293 and its vector alone, pcDNA3.1-HEK 293 cells. MRP1 and pcDNA3.1-HEK 293 cells were cultured in DMEM medium with 800 μg/ml of Geniticin. A; The expression level of MRP1 were analyzed by western blot. Cell suspension ($1x10^6$) were transferred to an Eppendorf tube then centrifuged at 13,000 rpm for two-three min at 4°C. The supernatant was carefully aspirated then ice-cold TD buffer containing 2 mM DTT, 1% aprotini, 1 mM AEBSF and DNase were added. The pellet was resuspended well in TD buffer then incubated at 37 °C for 5 min. The lysate was quickly frozen-thawed then sonicated three times in a bath sonicator before adding 5X SDS-PAGE sample buffer. For Western blot The samples (12.5 μl) were separated by electrophoresis on NuPAGE gel (7% w/v) and the proteins were electroblotted on to nitrocellulose membrane. Immunoblotting was performed by using MRPm6 (1:4000) as first antibody and anti-mouse horseradish peroxidase

(HRP) conjugate (1:10000) as the secondary antibody. HRP-dependent luminescence was developed using ECL (Section 2.9) and exposed to Bio-Max MR film. B; Functional assay was performed with calcein AM. The cells were incubated for 10 min at 37^{o}C with calcein AM in the presence of DMSO control or 20 μM MK571. After that the cells were washed and analyzed by FACS as described in Section 2.8. Each histogram shows the overlay of the presence of MK571 (line) with the DMSO control (0.1%) (filled gray).

3.3.2 Cytotoxicity of curcuminoids for pcDNA3.1- and MRP1 –HEK 293 cells

Sensitivity to curcuminoids of parental pcDNA3.1-HEK293 cells and MRP1-overexpressing-HEK293 cells was determined by exposing cells to curcuminoids at various concentrations up to 50 μM for 72 h (Figure 33). The IC_{50} values were calculated from dose response curves obtained from three independent experiments (Table 18). The results showed that the IC_{50} values of curcuminoids in these cell lines were in the range of 14.5-39 μM. The curcumin mixture and curcumin I and II were more toxic compared to curcumin III in both control and MRP1 expressing cells (IC_{50} in the range of 14.5-20 and 34-40 μM, respectively). There was no significant difference between the IC_{50} in pcDNA3.1 control and MRP1-HEK293 cells for curcumin mixture or isolated curcuminoids I, II and III, the relative resistance value(s) are about 1 (Table 18) . Therefore, similar to P-gp, it can be suggested that curcuminoids are not MRP1 substrates. Moreover, as expected, only the MRP1-expressing HEK293 cells were resistant to etoposide and vinblastine (relative resistance factor 80.7 and 25.6 respectively, Table 18).

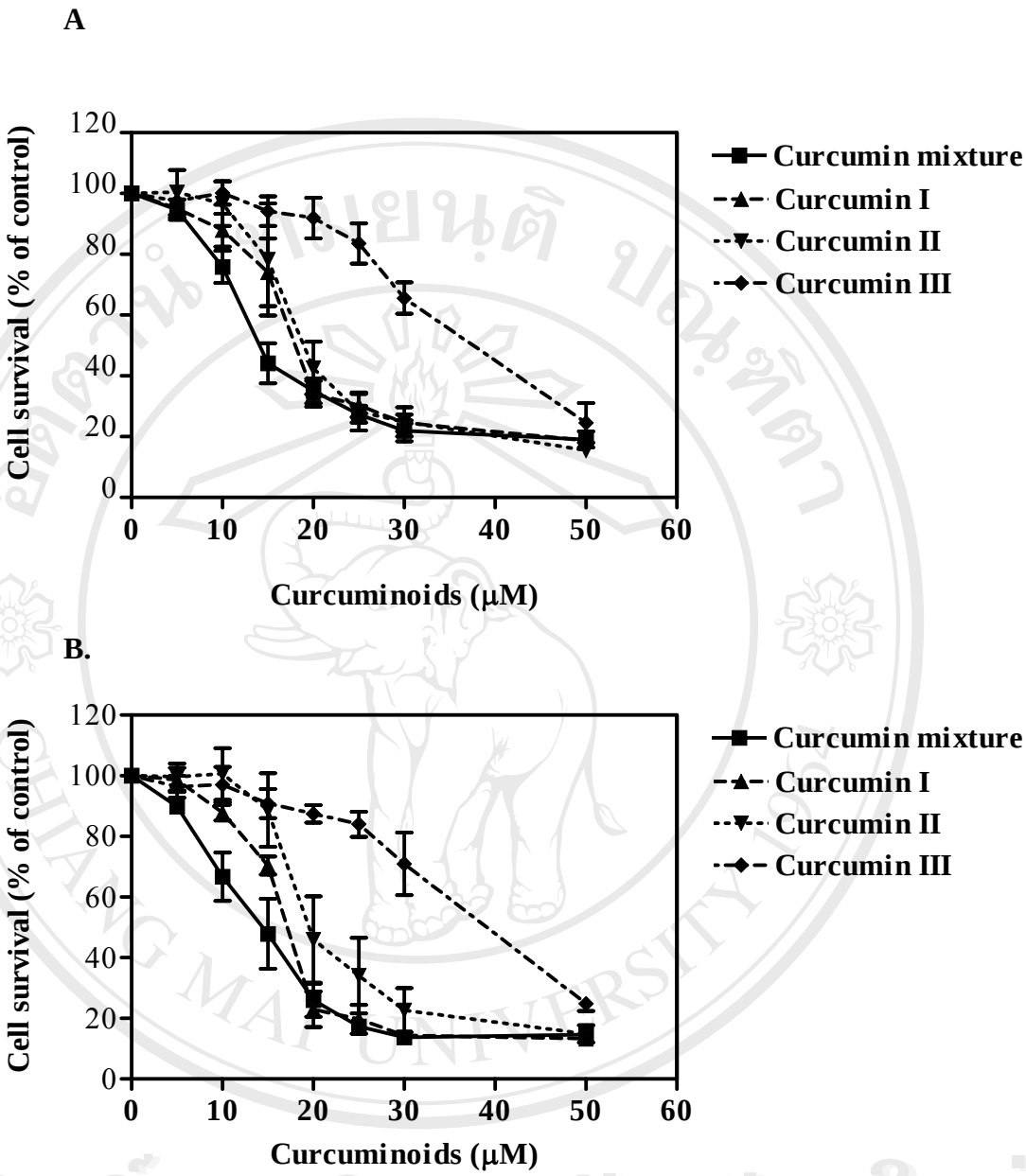


Figure 33. Cytotoxicity of curcuminoids in pcDNA3.1 and MRP1-HEK 293 cells. Standard 72 hours incubation MTT assay was used to investigate how curcuminoids affect the viability of the pcDNA3.1 control vector (A)- and MRP1-transfected HEK 293 cells (B). Various concentrations of the curcuminoids were exposed to the cells for 72 h and cytotoxicity was determined by MTT assay (Section 2.4).

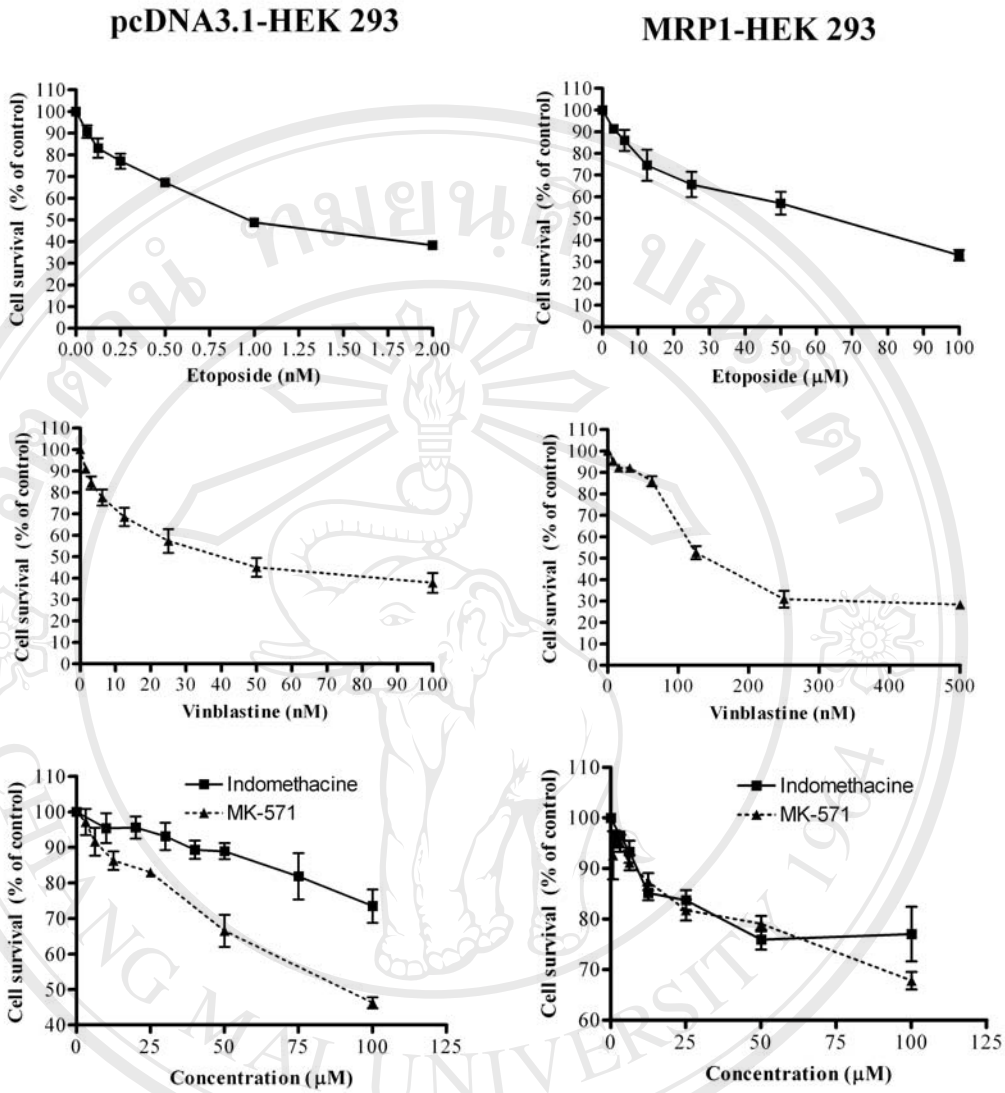


Figure34. Cytotoxicity of etoposide, vinblastine, MK-571 and indomethacinee in pcDNA3.1- and MRP1-HEK 293 cells. Various concentrations of tested compounds as indicated were exposed to pcDNA3.1- (Left Panel) and MRP1-HEK 293 cells (Right Panel) for 72 h and cytotoxicity was determined by MTT assay (Section 2.4). The mean values from three independent experiments performed in triplicate are plotted and the error bars represent standard error (SE).

Table 18. IC_{50} values of curcuminoids on cytotoxicity of pcDNA3.1- and MRP1-HEK 293 cells. The IC_{20} and IC_{50} values were calculated from dose response curves obtained from three independent experiments.

Curcuminoids	IC_{50} Values (μM) [a]		RR [b]
	pcDNA3.1-HEK 293	MRP1-HEK 293	
Curcumin M (μM)	15.4 ± 1.9	14.5 ± 1.9	1.0
Curcumin I (μM)	17.2 ± 1.1	16.7 ± 0.3	1.0
Curcumin II (μM)	17.8 ± 1.7	20.3 ± 2.8	1.1
Curcumin III (μM)	34.2 ± 2.2	39.3 ± 1.2	1.2
Etoposide (μM)	0.9 ± 0.1	72.7 ± 1.7	80.7
Vinblastine (nM)	25.0 ± 2.9	136.7 ± 8.8	25.6
MK-571 (μM)	86.6 ± 6.0	> 100	~1
Indomethacinee (μM)	> 100	> 100	~1

[a] IC_{50} values (concentration required for killing of 50% cells) were calculated from each experiment run in triplicates and the values represent the mean ± SE of three independent experiments.

[b] The relative resistance was derived from the IC_{50} ratio of the compounds in MRP1-HEK 293 cells to pcDNA3.1-HEK 293 cells indicating the degree of resistance of MRP1-HEK 293 cells towards a particular drug or compounds.

3.3.3 Modulating effect of curcuminoids on etoposide drug sensitivity in pcDNA3.1- and MRP1-HEK 293 cells

To assess the modulatory effect of curcuminoids, non-toxic concentrations of curcuminoids were chosen. As determined from the experiment shown in Figure 33, given that the concentrations lower than 10 μM produced minimal effects on cell viability of HEK293 cells. Therefore the concentrations at 5 and 10 μM of curcuminoids were selected to determine the ability of curcuminoids to sensitize MRP1 and pcDNA3.1-HEK 293 cells to the cytotoxic effects of etoposide. The cells were treated with increasing concentrations of etoposide in the presence of 5 and 10 μM of curcuminoids for three days. As positive controls, the effect of vinblastine (5 and 10 nM), MK-571 (30 and 60 μM) and indomethacinee (20 and 40 μM) on cytotoxicity of etoposide were also tested. The cytotoxicity of those modulators is shown in Figure 34, Table 18, the non toxic doses were selected to study their reversing activity to anticancer drug etoposide. The modulatory effect of curcuminoids and the positive modulators are given in Figures 35 and 36. The IC_{50} values and relative resistance factors were determined and summarized in Table 19. The results indicated that curcuminoids did not have any effect on the cytotoxicity of etoposide in control pcDNA3.1-transfected HEK293 cells. On the other hand, the MRP1-HEK293's sensitivity towards etoposide was significantly enhanced in the presence of curcuminoids; in addition, curcumin I was the most effective component among the three forms of curcuminoids. The relative resistance to etoposide was lowered by 14-to 20-fold in the presence of 10 μM curcumin mixture and curcumin I (Table 19). Both curcumin II and III were considerably less effective in reversing the resistance of MRP1-HEK293 cells to etoposide.

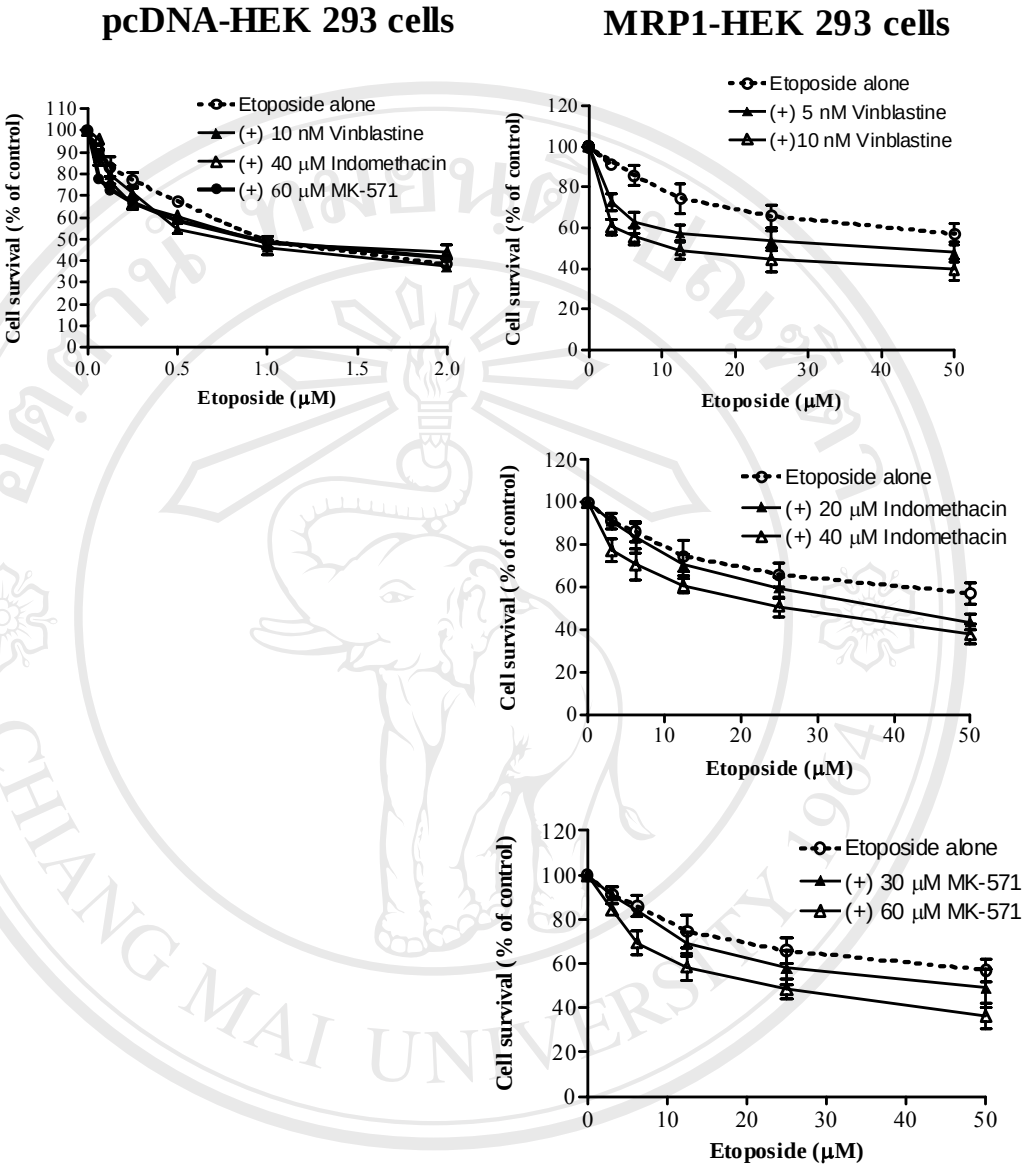


Figure 35. MDR reversing activity of vinblastine, indomethacinee and MK-571 to etoposide sensitivity in pcDNA3.1- and MRP1 transfected HEK 293 cells. The cells were grown in the presence of various concentrations of etoposide alone or with vinblastine (5,10 nM) or indomethacinee (20,40 μM) or MK-571 (30,60 μM). The number of viable cells was determined by MTT assay (Section 2.4). The experiment were conducted in triplicate and the values represented mean ± SE of three independent experiments.

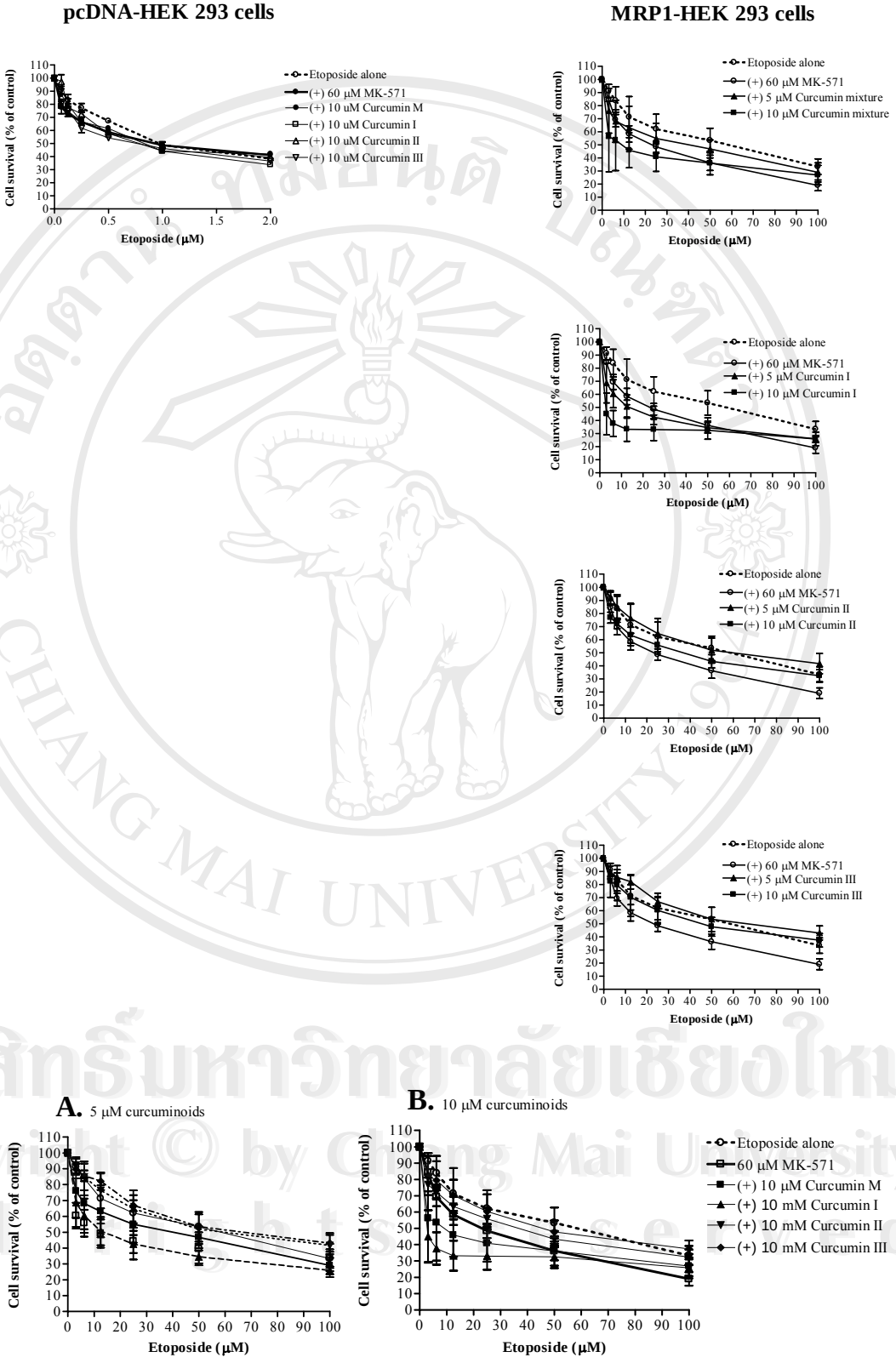


Figure 36. MDR reversing activity of curcuminoids at 5 and 10 μM to etoposide drug in pcDNA3.1- and MRP1-HEK 293 cells.

Table 19. Reversal of MRP1-mediated resistance to etoposide with non-toxic concentrations of curcuminoids and positive modulators, vinblastine, MK-571, indomethacinee

Drug and/or curcuminoids		Cytotoxicity of etoposide, IC_{50} (μM)[a]		RR[b]	Degree of MDR reversal activity[c]
		pcDNA3.1-HEK 293 cells	MRP1-HEK 293 cells		
DMSO (etoposide alone)		0.9 ± 0.1	72.7 ± 1.7	80.7	1
(+) Curcumin mixture	5 μM	N.D	22.5 ± 2.5	28.8**	2.8
	10 μM	0.8 ± 0.1	4.5 ± 0.1	5.7**	14.2
(+) Curcumin I	5 μM	N.D	20.3 ± 0.2	24.6**	3.3
	10 μM	0.8 ± 0.1	3.3 ± 0.8	3.96**	20.4
(+) Curcumin II	5 μM	N.D	62.5 ± 18.9	75.3	1.0
	10 μM	0.8 ± 0.2	34.3 ± 5.5	41.4**	1.9
(+) Curcumin III	5 μM	N.D	67.0 ± 7.0	77.0	1.0
	10 μM	0.9 ± 0.2	43.9 ± 10.7	50.4**	1.6
(+) Vinblastine	5 nM	N.D	57.5 ± 6.3	53.7**	1.5
	10 nM	1.1 ± 0.3	18.3 ± 4.8	17.1**	4.7
(+) MK-571	30 μM	N.D	62.5 ± 6.1	56.8**	1.4
	60 μM	1.1 ± 0.2	23.3 ± 4.4	21.2**	3.8
(+) Indomethacinee	20 μM	N.D	57.5 ± 6.3	63.8	1.3
	40 μM	0.9 ± 0.0	18.3 ± 4.8	20.3**	4.0

[a] The data represent the mean values ± SE of three independent experiments performed in triplicate.

[b] Relative resistance values were obtained by dividing the IC_{50} value of the MRP1-HEK 293 cells by the IC_{50} value of the empty vector transfected cell line.

[c] Degree of revesal activity were obtained by dividing the relative resistance (RR) value of etoposide alone (0.5% DMSO) by the relative resistance value of etoposide in the presence of curcuminoids or positive modulators

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

N.D Not determined

3.3.4 Effect of curcuminoids on MRP1 expression

It is possible that the reversal of resistance to etoposide by curcuminoids in MRP1-HEK293 cells was caused by decrease in MRP1 protein expression in the presence of curcuminoids. To address this issue, the research was determined whether the curcuminoids are able to reduce the expression level of MRP1 in HEK293 cells, where the *MRP*1 gene is under CMV promoter instead of its native one. The MRP1-HEK293 cells were treated with 10 μM of the curcuminoids for three days (72h) and the MRP1 protein level was detected by Western blot analysis using monoclonal antibody to MRP1, MRPm6. It was found that the protein level of MRP1 in curcuminoid- treated cells was similar to the DMSO-treated control MRP1 cells (Figure 37), indicating that curcumin I, II, III as well as curcumin mixture form has no effect on the expression level of MRP1 protein. Thus, the observed effects (reversal of resistance, Table 19 and Figure 36) are indeed due to the inhibition of the function of MRP1.

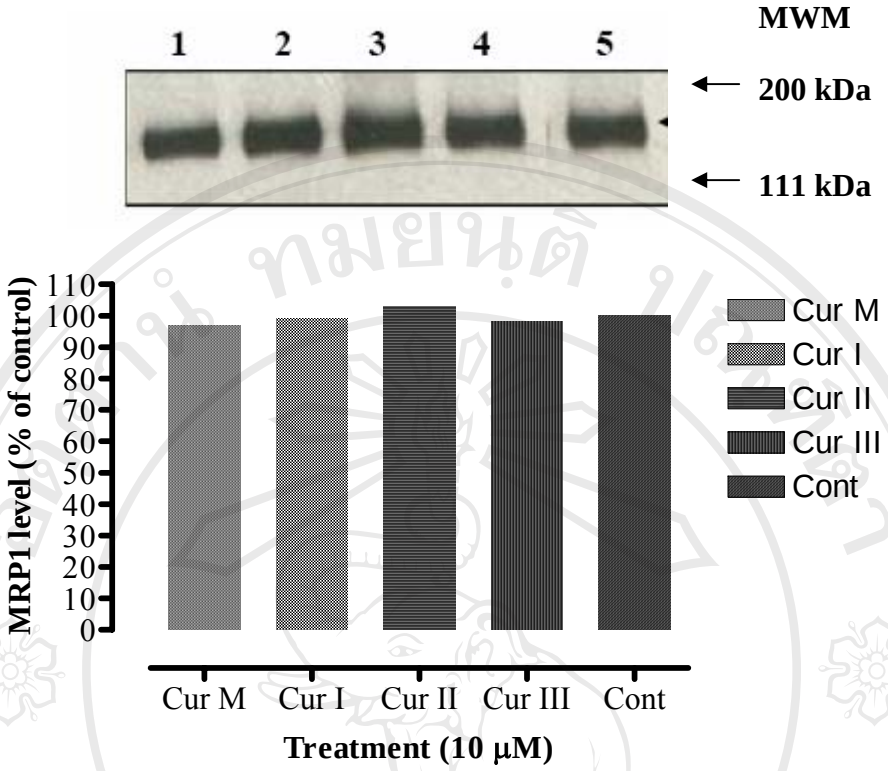
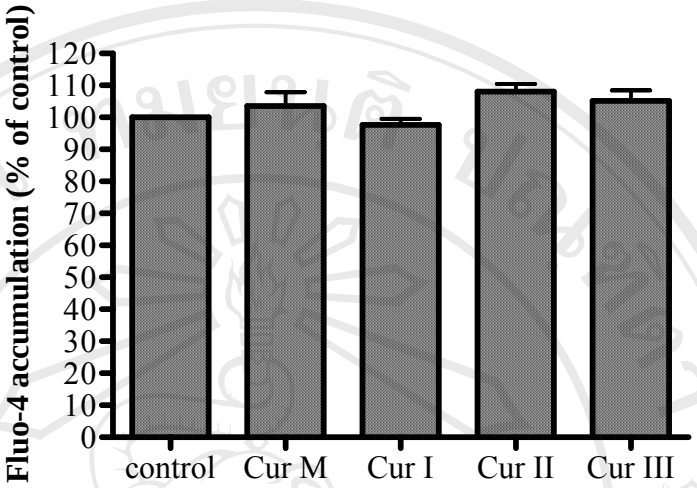


Figure 37. Curcuminoids had no effect on the MRP1 protein-expression level. MRP1-HEK 293 cells ($3x10^6$ cells) were treated for three days with DMSO (vehicle control), or 10 μM curcuminoids at 37°C then the SDS-PAGE samples were prepared as described previously [133]**.** The total cellular content were then separated by electrophoresis on NuPAGE gel (7% w/v) and the proteins were electroblotted on nitrocellulose membrane. Immunoblotting was performed using MRPm6 (1:4000) as first antibody and anti-mouse horseradish peroxidase (HRP) conjugate (1:10000) as the secondary antibody. HRP-dependent luminescence was developed with ECL reagents by following the manufacturer's protocol. The arrows show the position of molecular weight marker presenting that MRP1 had a predicted molecular weight about 180-190 kDa. Similar results were obtained when MRPr1 instead of MRPm6 was used for immunoblotting; data not shown. Lane 1, Cur M (curcumin mixture); lane 2, Cur I (curcumin I); lane 3, Cur III (curcumin II); lane 4, Cur III (curcumin III) and lane 5, Cont (control (untreated)).The bands were quantified by densitometry and show as histogram in the lower panel. One typical experiment from two independent experiments was depicted. MWM, Molecular weight marker.

3.3.5. Effect of curcuminoids on the accumulation of calcein-AM and fluo-4AM in pcDNA3.1 and MRP1-HEK 293 cells

The effect of curcuminoids on the accumulation of MRP1-fluoresscent substrates was tested to characterize the inhibitory effect on MRP1-mediated transport using flow cytometry. The observed effects in MTT assay on cell killing could be due to accumulation of metabolites of curcuminoids rather than curcuminoids themselves. To rule out this possibility, short-term (10 and 45 min) transport assays were used. The MRP1-transfected MRP1-HEK293 cells and the control pcDNA3.1-HEK293 cells ($5x10^5$ cells) were incubated with non-fluorescent precursors; fluo4-AM or calcein-AM and the intensity of fluorescence of accumulated substrate was analyzed by FACS. The results are shown in Figure. 38-40. Consistent with the MDR reversing property, curcuminoids increased the accumulation of the fluorescent substrates in MRP1-transfected cells in a concentration-dependent manner (for clarity, data with only 20 μM curcuminoids are shown in Figure 40) and among curcuminoids, curcumin I showed the greatest inhibitory effect, which was comparable to curcumin mixture. The concentrations required for 50% increase in accumulation of fluo4-AM or calcein-AM of curcumin mixture, curcumin I, II and III were 10-12.5, 10-12.5, 20-25 and 30-35 μM, respectively.

A. Effect of curcuminoids at 20 μM on fluo4 accumulation in pcDNA3.1-HEK 293



B. Effect of curcuminoids at 0-20 μM on fluo4 accumulation in MRP1-HEK 293

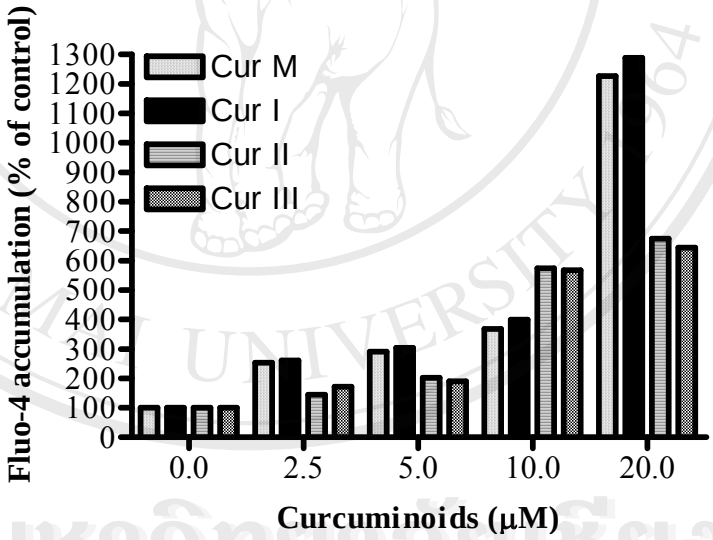
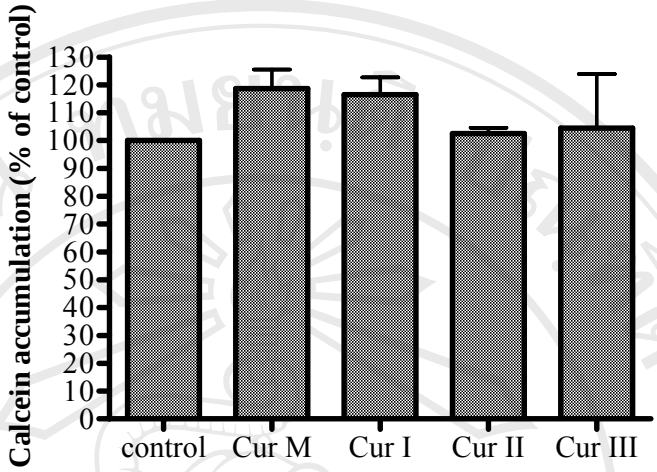


Figure 38. Curcuminoids increase fluo-4AM accumulation in MRP1-HEK 293 cells in a concentration dependent manner. Cells were incubated at 37°C in the dark for 45 min with 0.5 μM Fluo-4AM in the presence of curcuminoids (0-20 μM). The accumulation of the fluo4 was analyzed immediately by FACS. The mean fluorescence intensity values were collected and represented as histogram. Representative histogram of at least two independent experiments is shown, A. in pcDNA3.1- and B. in MRP1-HEK 293 cells.

A. Effect of curcuminoids at 20 µM on calcein accumulation in pcDNA3.1-HEK 293



B. Effect of curcuminoids at 0-20 µM on calcein accumulation in MRP1-HEK 293

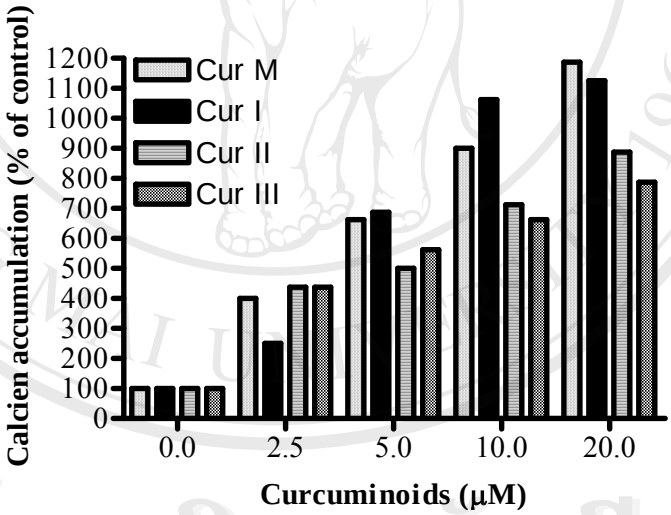
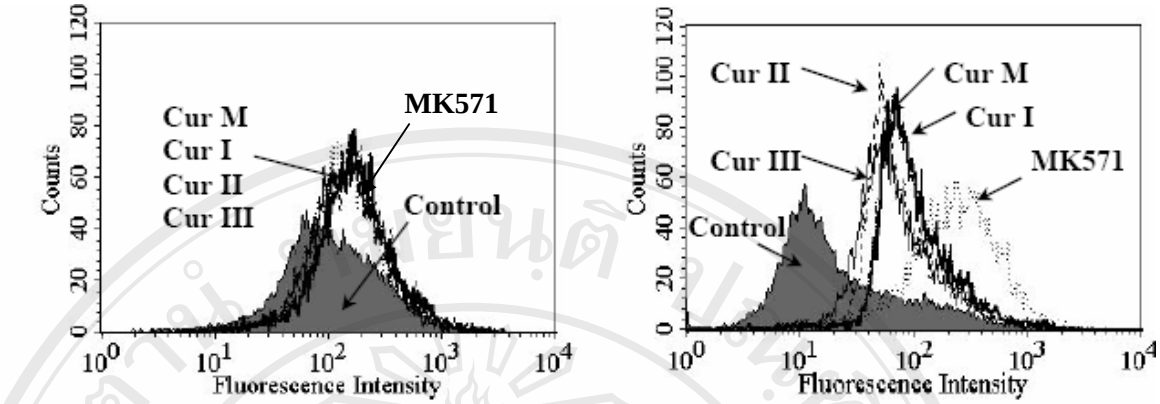


Figure 39. Curcuminoids increase calcein accumulation in MRP1-HEK 293 cells in a concentration dependent manner. Cells were incubated at 37°C in the dark for 10 min with 0.5 µM calcein-AM in the presence of curcuminoids (0-20 µM). The accumulation of the calcein was analyzed immediately by FACS. The mean fluorescence intensity values were collected and represented as histogram. Representative histogram of at least two independent experiments is shown, A. in pcDNA3.1- and B. in MRP1-HEK 293 cells.

A. Effect of curcuminoids at 20 µM on fluo-4 accumulation



B. Effect of curcuminoids at 20 µM on calcein accumulation

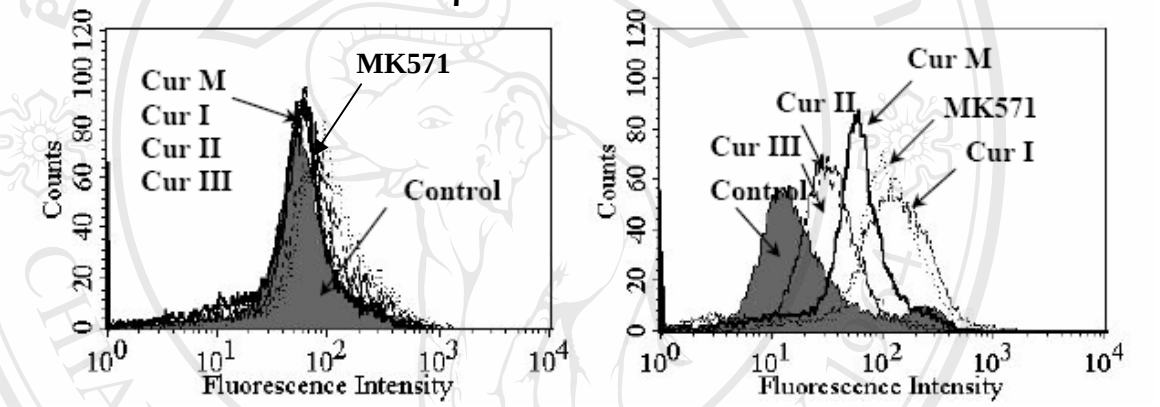


Figure 40. Curcuminoids increase calcein-AM and fluo-4AM accumulation in MRP1-HEK 293 cells but not in pcDNA3.1-HEK 293 cells. To study effect of the curcuminoids on MRP1 transport function, the activity of MRP1 was assessed by measuring the intracellular accumulation of two fluorescent MRP1 substrates: (A) Fluo-4AM and (B) calcein-AM. Cells were incubated at 37°C in the dark for 45 min with 0.5 µM Fluo-4AM or 10 min with 0.5 µM calcein-AM in the presence or absence of 20 µM MK571 or various concentrations of the curcuminoids. The reaction was stopped by centrifugation and pelleted cells were re-suspended in 300 µl of ice-cold PBS containing 0.1% BSA. The accumulation of the fluorescent substrates was analyzed immediately by FACS. Representative histogram of three independent experiments is shown. The effect of 20 µM curcuminoids in pcDNA3.1- (A and B, left panel) and MRP1-HEK 293 cells (A and B, right panel) are depicted to show in comparison among each form of curcuminoids. The traces in each histogram are labeled. Control (Gray filled); Cur M, curcumin mixture; Cur I, curcumin I; Cur II, curcumin II; and Cur III, curcumin III.

3.3.6.Effect of curcuminoids on ATPase activity of MRP1

The results described above demonstrate that curcuminoids inhibit the transport function of MRP1. To assess whether curcuminoids interact directly with the transporter, effect of curcuminoids on ATPase activity of MRP1 was investigated. The crude membranes prepared from MRP1 baculovirus-infected High Five insect cells were used in this study. As shown in Table 20, Figure 41, curcumin mixture, curcumin I, II and III had similar effects on MRP1 ATP hydrolysis as to P-gp [8]. All three forms were able to stimulate the basal ATPase activity of MRP1 at low concentrations but inhibited the activity at higher concentrations, indicating the interactions between the curcuminoids and MRP1. However, it should be noted that the extent of stimulation is considerably low 15-45%, which is consistent with the extent of stimulation by various substrates/modulators reported by other workers (see review Ref.[49]). By comparing all three curcuminoids at the same concentration (1.5 μM), curcumin I showed the highest stimulation of approximately 45% (Table 20). It is shown that bioflavonoid quercetin markedly stimulates MRP1-mediated ATP hydrolysis [155]. Curcumin mixture and curcumin I, II and III were able to inhibit quercetin-stimulated MRP1-mediated ATP hydrolysis (Figure 42) in a concentration-dependent manner.

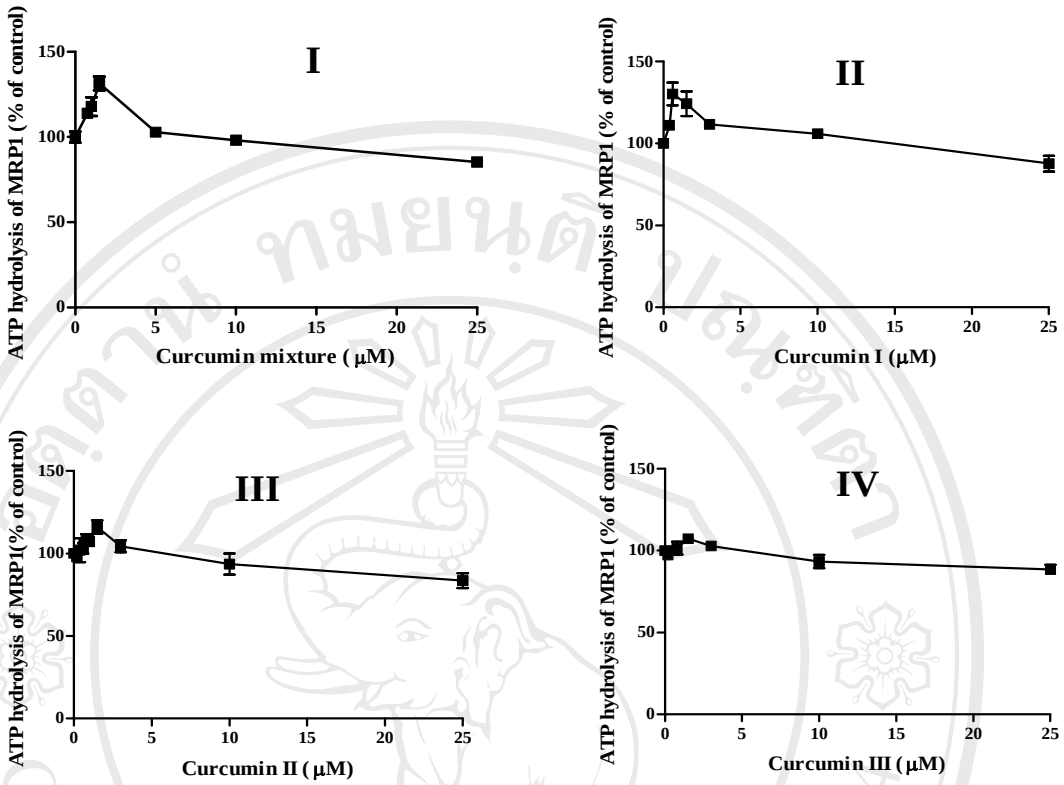


Figure 41. Curcuminoids stimulate ATP hydrolysis of MRP1. Crude membranes of (100 μg protein/ml) of MRP1 baculovirus infected HF insect cells were incubated at 37°C for 5 min with curcuminoids in the presence or absence of BeFx. The reaction was initiated by the addition of 5 mM ATP and terminated with SDS (2.5% final concentration) after 20 min incubation at 37°C; the amount of P_i released was quantitated using a colorimetric method [136]. MRP1-specific activity was recorded as the BeFx-sensitive ATPase activity. Values represent mean ± SE from at least three independent experiments. Control values show the basal activity measured in the absence of added compounds. The effect of curcumin mixture, and purified curcuminoids I, II and III on MRP1 ATPase activity are shown in panels I, II, III and IV, respectively.

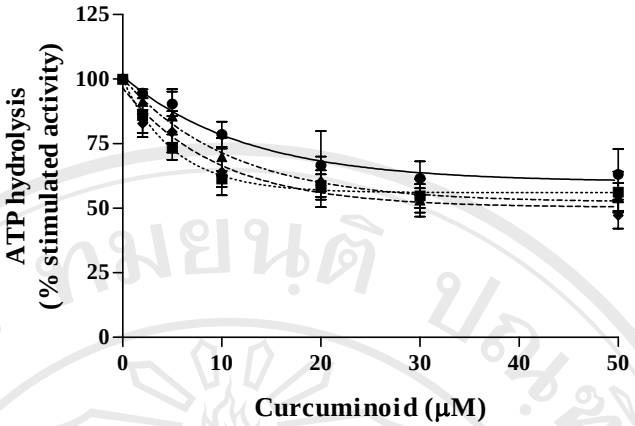


Figure 42. Effect of curcuminoids on quercetin stimulated MRP1 ATPase activity. The flavonoid quercetin at 10 μM is known to produce 2-fold increase in MRP1-mediated ATP hydrolysis. The effect of curcumin mixture (◆), curcumin I (■), curcumin II (▲) and curcumin III (●) at the indicated concentrations was tested on MRP1 ATPase activity in the presence of 10 μM quercetin. The mean values from three independent experiments are given and the error bars represent standard error (SE).

Table 20. The IC_{50} values of curcuminoids on ATPase activity of MRP1.

Compound	ATP hydrolysis (nanomoles/min/mg protein)[a]	
	Basal (Fold-stimulation)	(+) 10 μM Quercetin IC_{50} (μM)[b]
Curcumin mixture	1.4[b]	6.6
Curcumin I	1.4	3.6
Curcumin II	1.3	7.6
Curcumin III	1.2	8.6

[a] Fold stimulation was obtained by dividing the ATPase activity in the presence of the curcuminoids by the in the basal activity in the presence of DMSO. These values were obtained from the data given in Figure. 41.

[b] IC_{50} (concentration required for 50% inhibition) of quercetin (10 μM)-stimulated activity.

3.3.7 Effect of curcuminoids on photoaffinity labeling of MRP1 by 8-azido[α-^{32}P]- ATP

To determine the possible sites of interaction of curcuminoids with MRP1, photoaffinity labeling of MRP1 was performed using 8-azido [α-^{32}P]-ATP. Crude membranes of MRP1 baculovirus infected HF insect cells were incubated on ice with 8-Azido [α-^{32}P]-ATP in the presence of DMSO or curcuminoids (see Materials and Methods Section 2.15 for details). Curcumin mixture, curcumin I, II and III had no effect on 8-azido [α-^{32}P]-ATP labelling of MRP1 at 20 μM at a concentration where they affected both basal and quercetin-stimulated ATPase activity (Figure 42). This lack of effect on nucleotide binding suggests that curcuminoids produce their effects most likely by interacting at the substrates binding site(s) rather than at the nucleotide-binding sites. The photoaffinity labeling with 8-azido [α-^{32}P]-ATP was inhibited by the presence of excess (10 mM) ATP as was to be expected for nucleotide binding to ATP sites.

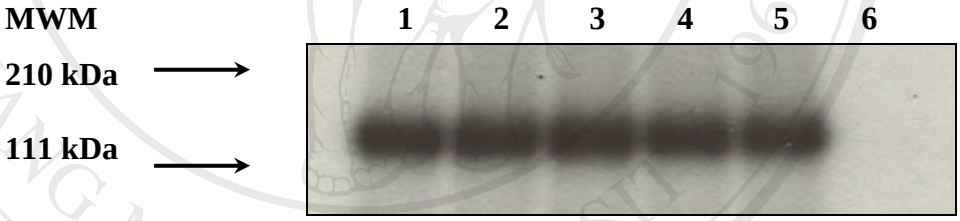


Figure 43. Curcuminoids do not inhibit photoaffinity labeling of MRP1 with 8-azido [α-^{32}P]-ATP. Crude membranes (50-75 μg protein) of MRP1 baculovirus infected HF insect cells were incubated at 4^{o}C for 5 min with 10 μM [α-^{32}P]-8-AzidoATP (10 μCi/nano mole) in the presence and absence of 20 μM curcumin mixture or purified curcuminoid. The photocross linking with 366 nm UV light was carried out on ice for 10 min as described previously [138, 151]. The incorporation of 8-azido [α -^{32}P]-ATP detected by phosphorimaging after gel electrophoresis. Lane 1, 8-azido[α -^{32}P]-ATP alone, lane 2, (+) 20 μM curcumin I; lane 3, (+) 20 μM curcumin II; lane 4, (+) 20 μM curcumin III, lane 5, (+) 20 μM curcumin mixture and lane 6, (+) 10 mM ATP. The results from a representative experiment are shown similar results were obtained in two additional experiments. MWM, Molecular weight marker.

Modulation of human mitoxantrone resistance protein (MXR, ABCG2) function by curcuminoids

3.4.1 Modulation of MXR wild type, 482R by curcuminoids

ABCG2 or MXR (mitoxantrone resistance protein) is a newly described ABC transporter that is thought to dimerize to function and has been shown to confer resistance to commonly used chemotherapeutic drugs such as mitoxantrone, anthracyclines, camptothecin, topotecan and SN-38 [11, 83, 156]. Recently [82], MXR expression was reported in relapse or refractory hematological malignancies and some showed the association of MXR expression with poor responses to chemotherapy [156, 157]. Make it possible to postulate that MXR expression is responsible, at least in part, for clinical drug resistance, and overcoming MXR-mediated drug resistance would contribute to cancer chemotherapy.

In this part of the study, the research was considered the effects of the curcuminoids on the efflux function of MXR by using human embryonic kidney cells (HEK 293) stably transfected with MXR wild type(482R), amino acid at position 482 is Arg and pcDNA3.1 vector alone. As a preliminary study, the expression and transport activity of MXR in these cell lines were determined. The immunoblot presented in the Figure 44 demonstrated the level of MXR recognized by the MXR-specific antibody, BXP-21. The MXR protein was detected at high expression level (with a predicted molecular weight about 72 kDa) only in MXR-HEK 293 not in pcDNA3.1- HEK 293 cells. Efficient bodipy-prazosin efflux (low intracellular accumulation of fluorescence intensity) was detected in MXR-HEK 293 cells, and fumitremorgin C (FTC), a specific inhibitor of MXR, effective inhibited the efflux thereby increasing the accumulation of bodipy-prazosin in this cell line (Figure 44). Plus, slightly (not significant) effect of FTC was observed in empty vector control cell line.

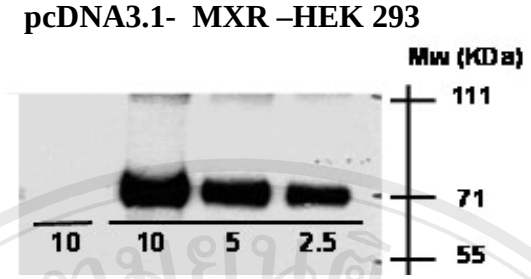


Figure 44. Expression level of MXR in pcDNA3.1- and MXR-HEK 293 cells. The proteins of pcDNA3.1-and MXR-HEK 293 cells were subjected to electrophoresis on 7% NuPAGE gels, blotted to nitrocellulose membrane, followed by immunodetection with anti-MXR, BXP-21. The blots were incubated with peroxidase conjugated antimouse antibody and visualized by using ECL (Section 2.11). MXR reveal the molecular weight about 72 kDa. The values under the bands represented the microgram proteins loaded in each well.

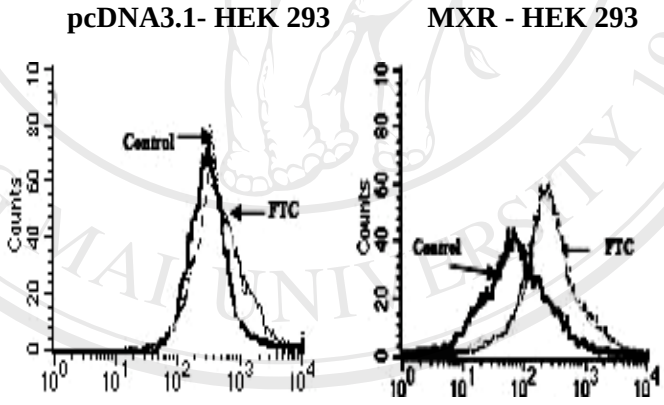


Figure 45. Transport of bodipy-prazosin by pcDNA3.1-HEK 293 and MXR-HEK 293 cells. The cells (5 x 10^5 cells) were incubated at 37°C for 45 min in dark with bodipy-prazosin in the presence of 0.1% DMSO control (solid line) or 10 μM FTC (dash line) and then analyzed on a flow cytometer as described in Section 2.8.

3.4.1.1 Cytotoxicity of curcuminoids in pcDNA3.1-and MXR-HEK 293 cells

The cytotoxicity of curcuminoids in drug sensitive (pcDNA3.1-HEK 293) and drug resistant cells lines (MXR-HEK293 cells) was determined by MTT assay (Section 2.4). Dose-response cytoxicity profiles for curcuminoids were established for both drug resistant and drug sensitive cell lines (Figure 46). The IC_{50} values of curcuminoids in pcDNA3.1- and MXR-HEK 293 cells were in the range of 19-29 μM and they are in the vicinity response to the drug sensitive and the resistance cells; suggesting that curcuminoids may not be substrate for MXR; which is similar with the case of P-gp and MRP1. The IC_{50} values were summarized in Table 21.

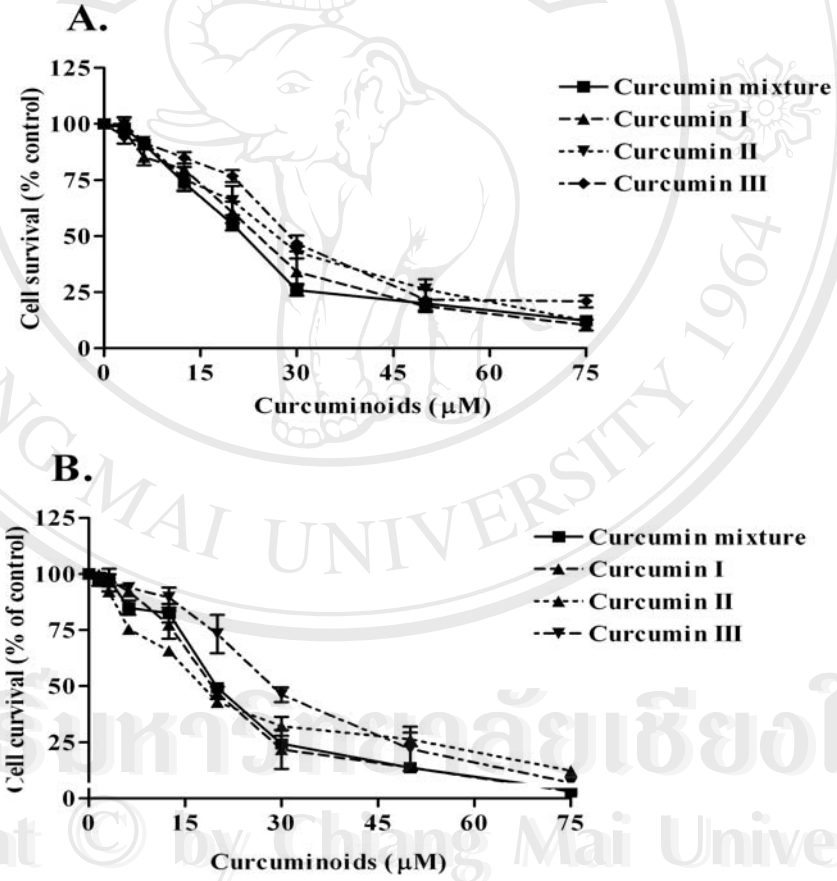


Figure 46. Cytoxicity of curcuminiods in pcDNA3.1- and MXR-HEK 293 cells. Three days of cytotoxicity assays of curcuminoids were conducted on pcDNA3.1HEK 293 cells (A) and MXR-transfected HEK 293 cells; (B) The cells were exposed to various concentrations of the curcuminoids for 72 h and cytotoxicity was determined by MTT assay as described in Section 2.4.

Table 21. IC_{50} values of curcuminoids in pcDNA3.1- and MXR-HEK 293(wild type, 482R) cells. The data represent the mean values ± SE of three independent experiment perform in triplicate.

Curcuminoids	IC_{50} Values (μM)		RR [a]
	pcDNA3.1-HEK 293	MXR-HEK 293 (wild type 482R)	
Curcumin mixture	21.5 ± 0.8	19.7 ± 0.6	0.9
Curcumin I	22.2 ± 0.5	19.8 ± 0.4	0.9
Curcumin II	26.3 ± 0.9	27.2 ± 0.1	1.0
Curcumin III	28.7 ± 1.2	29.5 ± 1.9	1.0

[a] Relative resistance values were obtained by dividing the IC_{50} value of curcuminoids in the KB-V-1 cells by the IC_{50} value of curcuminoids in the KB-3-1 cells.

3.4.1.2 Effect of curcuminoids on MXR mediated MDR phenotype in pcDNA3.1- and MXR transfected HEK 293 cells

Effects of curcuminoids in combination with increasing concentrations of anticancer agents were evaluated. Cellular resistance profiles of pcDNA3.1-HEK 293 cells and MXR-HEK293 cells to three anticancer drugs, mitoxantrone, topotecan and SN-38 in the presence and absence of curcuminoids are shown in Figure 47, 48 and 49 respectively. The result showed that at non toxic doses 3, 5 and 10 μM, curcuminoids enhanced the sensitivity of MXR-HEK 293 cells to mitoxantrone, topotecan and SN-38 in a concentration dependent manner. Where as in pcDNA3.1-HEK293 cells, curcuminiods had slightly effect even at 10 μM, the highest concentration used in the experiment.

Different from P-gp and MRP1, the degree of reversal activity of curcuminoids to mitoxantrone (Table 22),topotecan (Table 23) and SN-38 (Table 24) are not significant difference compare among each form. Curcumin mixture and curcumin I, II and III are most likely to exhibit the similar reversal activity to MXR wild type, 482R expressed in HEK 293 cells.

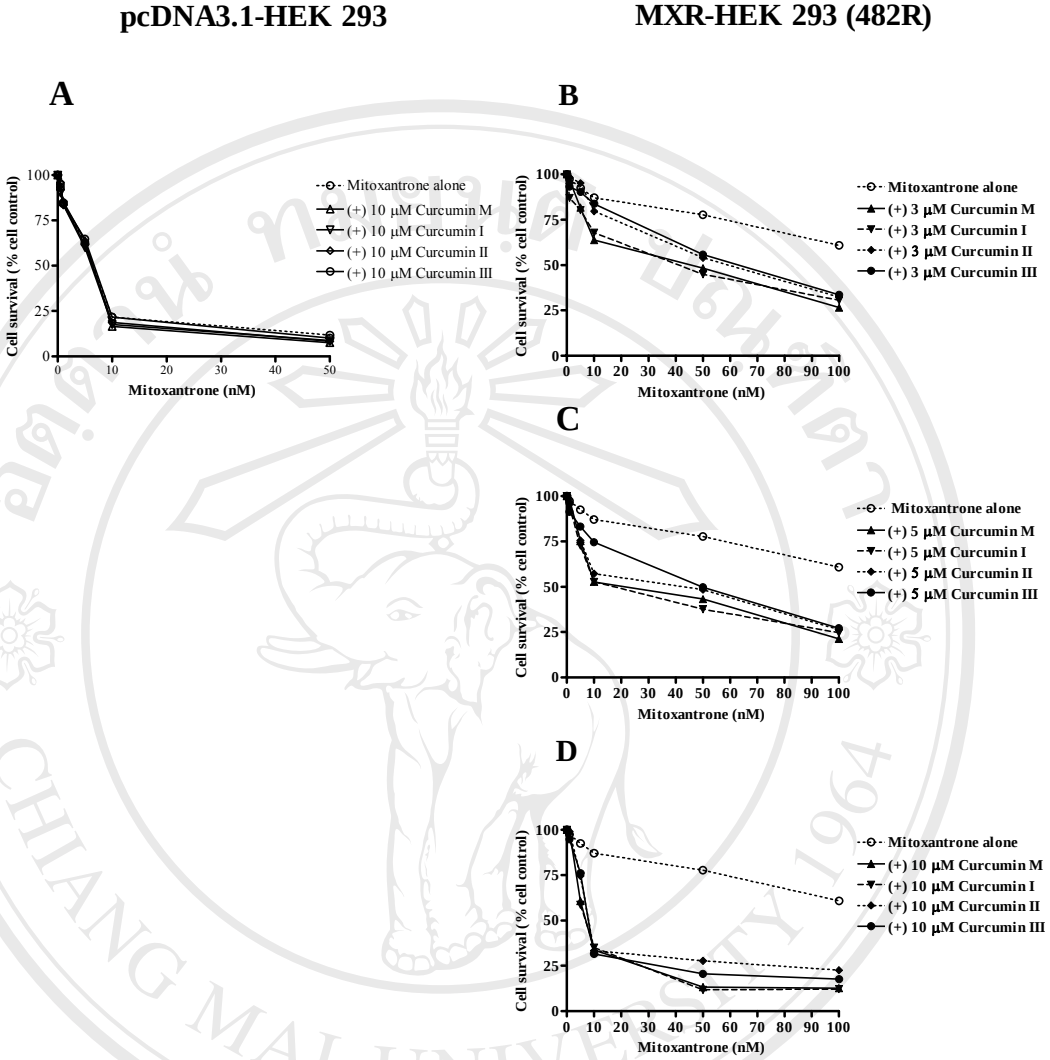


Figure 47. Effects of curcuminoids on the sensitivity of mitoxantrone in pcDNA3.1- and MXR-HEK 293 cells. The cells were incubated for three days with mitoxantrone in the presence of 3 (B),5 (C) and 10 μM (D) of curcuminoids, then the survival cells were determined by the MTT assay as described in Section 2.4. A typical result from two independent experiment was depicted . (A); pcDNA3.1-HEK 293 cells were treated with 10 μM curcuminoids, the highest concentration used in the experiment.

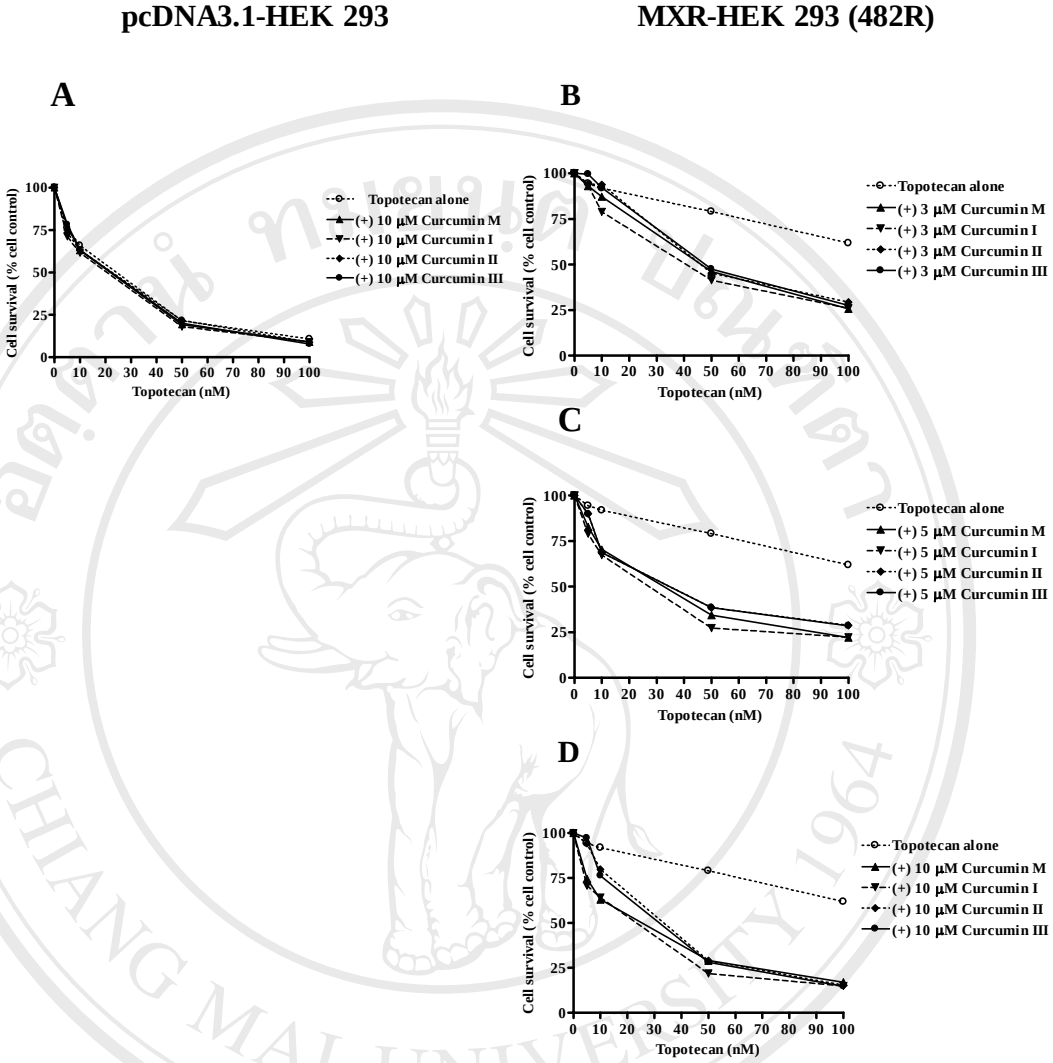


Figure 48. Effects of curcuminoids on the sensitivity of topotecan in pcDNA3.1- and MXR-HEK 293 cells. The cells were incubated for three days with topotecan in the presence of 3 (B),5 (C) and 10 μM (D) of curcuminoids, then the survival cells were determined by the MTT assay as described in Section 2.4. A typical result from two independent experiment was depicted . (A); pcDNA3.1-HEK 293 cells were treated with 10 μM curcuminoids, the highest concentration used in the experiment.

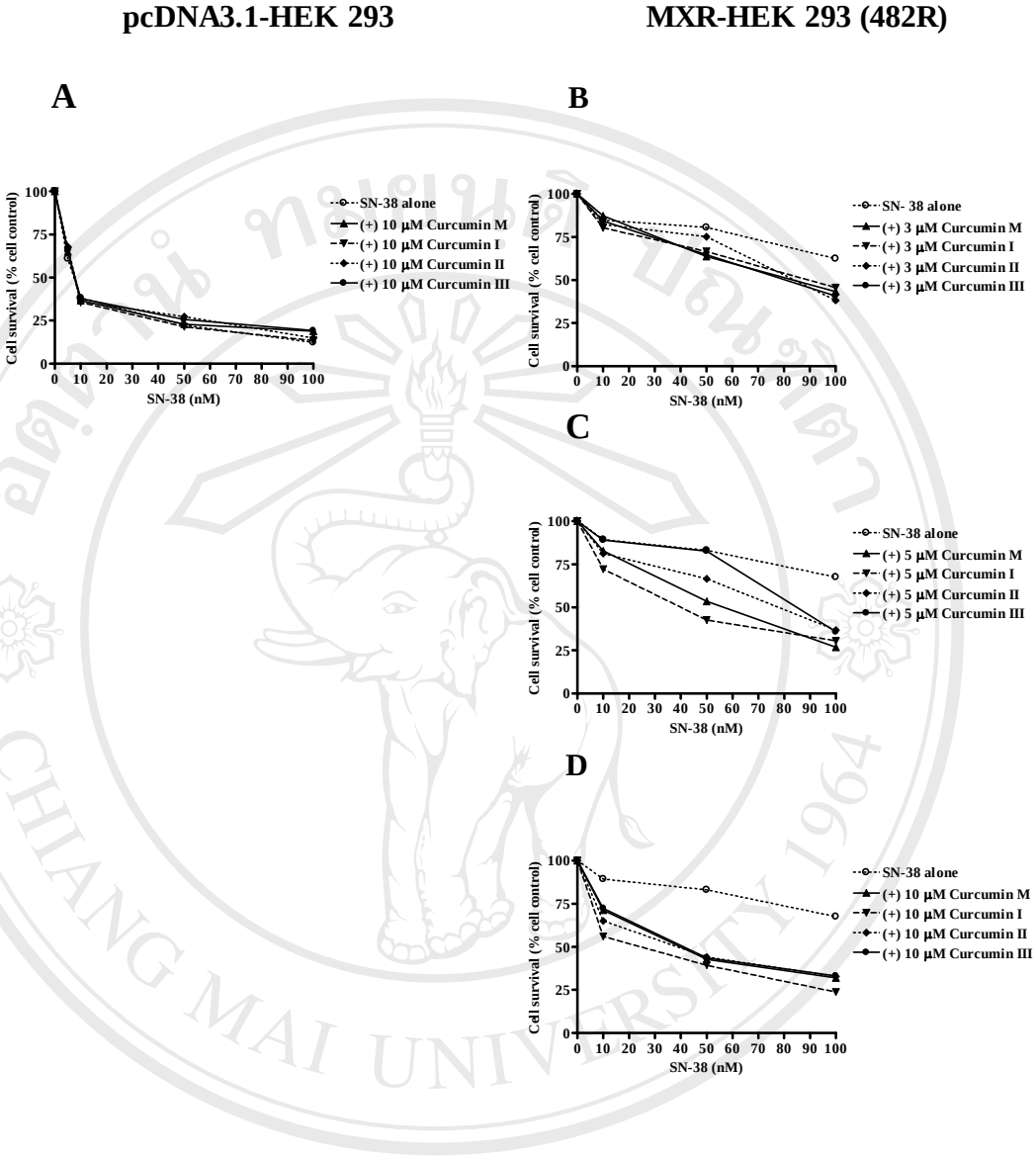


Figure 49. Effects of curcuminoids on the sensitivity of SN-38 in pcDNA3.1- and MXR-HEK 293 cells. The cells were incubated for three days with SN-38 in the presence of 3 (B),5 (C) and 10 μM (D) of curcuminoids, then the survival cells were determined by the MTT assay as described in Section 2.4. A typical result from two independent experiments was depicted . (A); pcDNA3.1-HEK 293 cells were treated with 10 μM curcuminoids, the highest concentration used in the experiment.

Table 22. Reversal of resistance to mitoxantrone with non toxic concentration of curcuminoids in pcDNA3.1- and MXR-HEK293

Drug and/or curcuminoids		Cytotoxicity of mitoxantrone IC_{50} (nM)[a]		RR[b]	Degree of MDR reversal activity[c]
		pcDNA3.1-HEK 293 cells	MXR-HEK 293 cells		
DMSO (mitoxantrone alone)		4.8	140.2	29.2	1.0
(+) Curcumin mixture	3 μM	N.D	32.8	7.3	4
	5 μM	N.D	7.9	1.7	17.7
	10 μM	4.6	5.3	1.2	24.3
(+) Curcumin I	3 μM	N.D	36.1	8.0	3.7
	5 μM	N.D	7.7	1.7	17.2
	10 μM	4.5	5.2	1.2	24.3
(+) Curcumin II	3 μM	N.D	42.4	8.8	3.3
	5 μM	N.D	24.0	5.0	5.8
	10 μM	4.8	5.5	1.1	26.5
(+) Curcumin III	3 μM	N.D	49.3	10.9	2.7
	5 μM	N.D	38.9	8.6	3.4
	10 μM	4.5	6.9	1.5	19.4

[a] The data represent the mean value from two independent experiments performed in triplicate. IC_{50} values refer to the drug concentration required for half-maximum inhibition of the growth rate.

[b] The value is calculated by the IC_{50} ratio of anticancer drug in the presence of DMSO control divided by the IC_{50} of value of the drug in the presence of curcuminoids.

[c] Degree of reversal activity values were obtained by dividing the relative resistance (RR) value of mitoxantrone alone (0.5% DMSO) by the relative resistance value of mitoxantrone in the presence of curcuminoids

N.D Not determined

Table 23. Reversal of resistance to topotecan with non toxic concentration of curcuminoids in pcDNA3.1- and MXR-HEK293

Drug and/or curcuminoids		Cytotoxicity of topotecan IC_{50} (nM)[a]		RR[b]	Degree of MDR reversal activity[c]
		pcDNA3.1-HEK 293 cells	MXR-HEK 293 cells		
DMSO (topotecan alone)		14.9	136.9	9.1	1
(+) Curcumin mixture	3 μM	N.D	39.4	2.3	3.9
	5 μM	N.D	21.5	1.5	6.0
	10 μM	14.6	18.1	1.2	7.6
(+) Curcumin I	3 μM	N.D	35.0	2.8	3.2
	5 μM	N.D	19.5	1.5	6.1
	10 μM	12.6	15.1	1.2	7.6
(+) Curcumin II	3 μM	N.D	41.7	3.0	3.0
	5 μM	N.D	18.0	1.3	7.0
	10 μM	14.1	23.4	1.6	5.7
(+) Curcumin III	3 μM	N.D	41.3	2.8	3.3
	5 μM	N.D	17.9	1.2	7.6
	10 μM	14.5	23.3	1.6	5.7

[a] The data represent the mean value from two independent experiments performed in triplicate. IC_{50} values refer to the drug concentration required for half-maximum inhibition of the growth rate.

[b] The value is calculated by the IC_{50} ratio of anticancer drug in the presence of DMSO control divided by the IC_{50} of value of the drug in the presence of curcuminoids.

[c] Degree of reversal activity were obtained by dividing the relative resistance (RR) value of topotecan alone (0.5% DMSO) by the relative resistance value of topotecan in the presence of curcuminoids

N.D Not determined

Table 24. Reversal of resistance to SN-38 with non toxic concentration of curcuminoids in pcDNA3.1- and MXR-HEK293

Drug and/or curcuminoids		Cytotoxicity of mitoxantrone IC_{50} (nM)[a]		RR[b]	Degree of MDR reversal activity[c]
		pcDNA3.1-HEK 293 cells	MXR-HEK 293 cells		
DMSO (mitoxantrone alone)		5.5	171.0	31.0	1
(+) Curcumin mixture	3 μM	N.D	66.3	12.3	2.5
	5 μM	N.D	45.4	8.4	3.7
	10 μM	5.4	39.5	7.3	4.2
(+) Curcumin I	3 μM	N.D	80.6	14.1	2.2
	5 μM	N.D	38.1	6.7	4.6
	10 μM	5.7	29.1	5.1	6.1
(+) Curcumin II	3 μM	N.D	76.9	13.7	2.3
	5 μM	N.D	61.4	10.9	2.8
	10 μM	5.6	40.4	7.2	4.3
(+) Curcumin III	3 μM	N.D	68.2	12.1	2.5
	5 μM	N.D	72.7	12.8	2.4
	10 μM	5.6	42.2	7.5	4.1

[a] The data represent the mean value from two independent experiments performed in triplicate. IC_{50} values refer to the drug concentration required for half-maximum inhibition of the growth rate.

[b] The value is calculated by the IC_{50} ratio of anticancer drug in the presence of DMSO control divided by the IC_{50} of value of the drug in the presence of curcuminoids.

[c] Degree of reversal activity were obtained by dividing the relative resistance (RR) value of SN-38 alone (0.5% DMSO) by the relative resistance value of SN-38 in the presence of curcuminoids

N.D Not determined

3.4.1.3 Effect of curcuminoids on the accumulation of bodipy-prazosin, mitoxantrone and rhodamine123 in HEK 293 cells transfected with pcDNA3.1 and MXR wild type 482R.

In this study, to confirm the inhibitory effect of curcuminoids on the efflux function of MXR wild type, 482R, the accumulation assay was evaluated by using three fluorescence compounds; bodipy-prazosin, mitoxantrone and rhodamine123. pcDNA3.1- and MXR-HEK 293 cells were incubated with a fixed concentration of the fluorescent substrate(s) in combination with increasing concentrations of curcuminoids, the accumulation of the fluorescent substrate inside the cells was analyzed by flow cytometer.

Figures 50-52 showed that curcuminoids increased the accumulation of bodipy-prazosin and mitoxantrone in MXR-HEK 293 cell in a concentration-dependent pattern. Plus, there was no effect to the drug sensitive cell lines (pcDNA3.1-HEK293) up to 20 μM. For bodipy-prazosin accumulation (Figure 50 and 51), when compare at 1 μM, curcumin mixture and curcumin I exhibited the highest activity whereas curcumin II and III were less effective. However at higher concentrations (2.5, 5 and 10 μM) of curcuminoids, they all reversed activity of MXR with similar pattern and comparable with 10 μM of FTC. No significant effect was observed in pcDNA3.1-HEK 293 cells even at 20 μM curcuminoids. The similar effect was observed in the case of mitoxantrone (Figure 52), curcuminoids exert their inhibitory effect in similar manner. The result in Figure 53, the fluorescence intensity of rhodamine123 was not significant different compared between pcDNA3.1-HEK 293 cells and MXR-HEK 293 cells, demonstrating that rhodamine123 was not transported by MXR wild type, 482R, and curcuminoids even at 20 μM had no effect on rhodamine123 accumulation in either HEK 293 cells transfected with pcDNA3.1 vector alone or MXR wild type 482R.

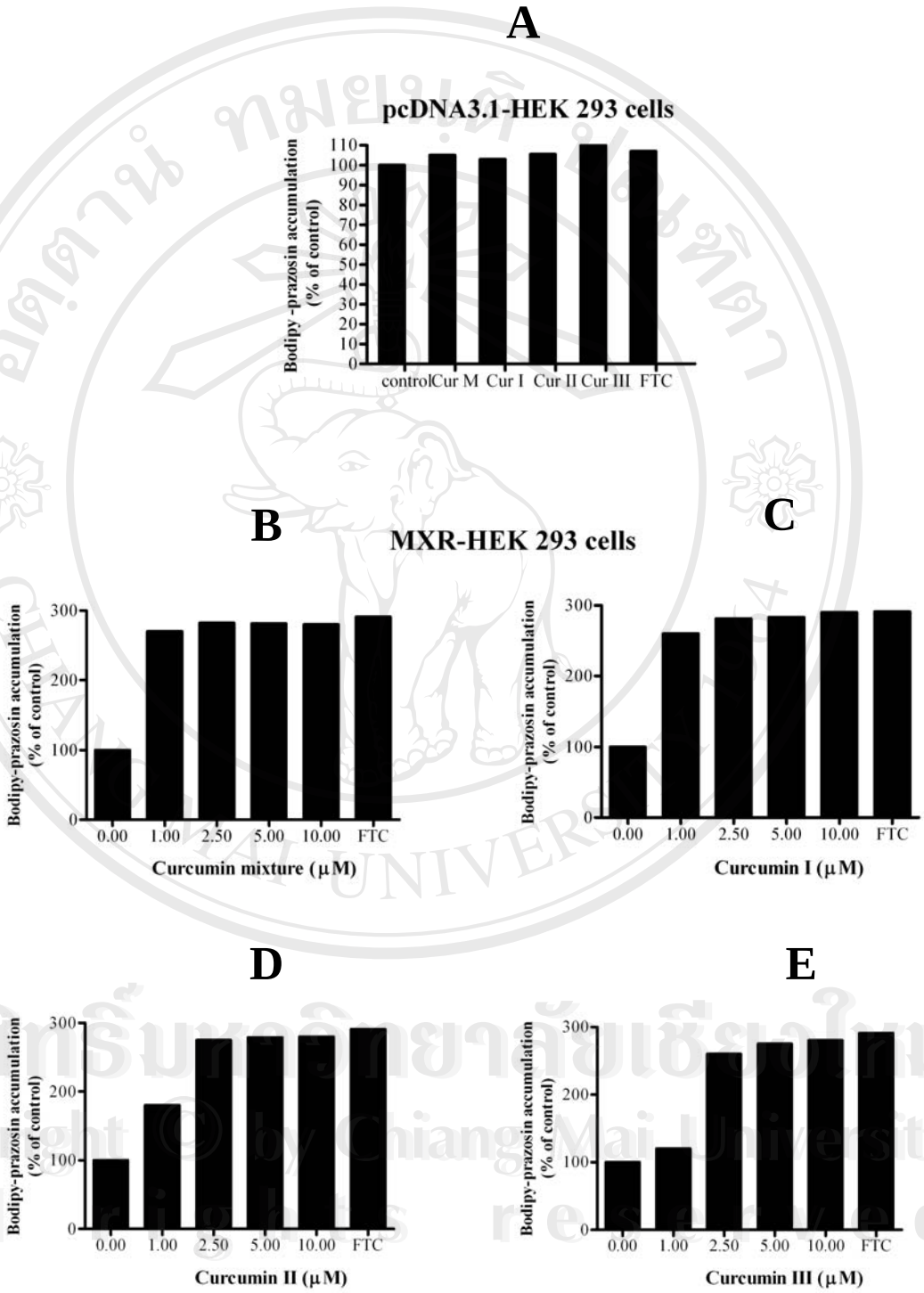


Figure 50. Effect of curcuminoids on the accumulation of bodipy-prazosin in pcDNA3.1-and MXR-HEK 293 cells. Cells were incubated at 37^{o}C for 45 min in dark condition with 250 nM bodipy-prazosin in the presence of curcuminoids (1, 2.5,

5, 10 μM). The accumulation of the bodipy-prazosin was analyzed immediately by FACS (Section 2.8). The mean of fluorescence intensity values were collected and represented as histogram (percentage of control). **(A);** The effect of curcuminoids at 20 uM on bodipy-prazosin accumulation in pcDNA3.1-HEK 293 cells. **(B-E);** The effect of curcuminoids at various concentrations as indicated on bodipy-prazosin accumulation in MXR-HEK 293 cells

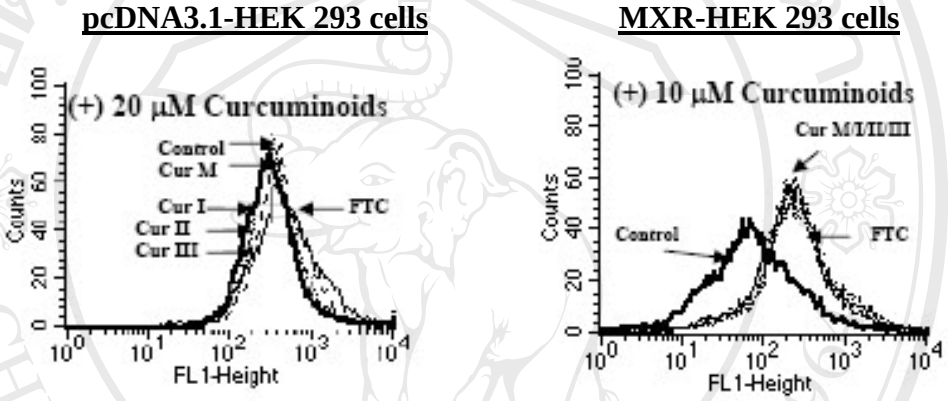


Figure 51. Effect of curcuminoids (in comparison among each form) on the accumulation of bodipy-prazosin in pcDNA3.1-and MXR-HEK 293 cells. Representative histogram results from flow cytometry analysis of curcuminoids at 10 μM was depicted to show their inhibitory effect in comparison among each form, also to compare with 10 μM of a positive control FTC. The cells were incubated with 250 nM bodipy-prazosin in the presence of 10 μM curcuminoids then the accumulation of bodipy-prazosin was analyzed immediately by FACS (See Section of 2.8 for details). Cur M; curcumin mixture, Cur I, II, III; curcumin I, II, III.Control (□), FTC (–), Cur M (....), Cur I (….), Cur II (---), Cur III (-.-)

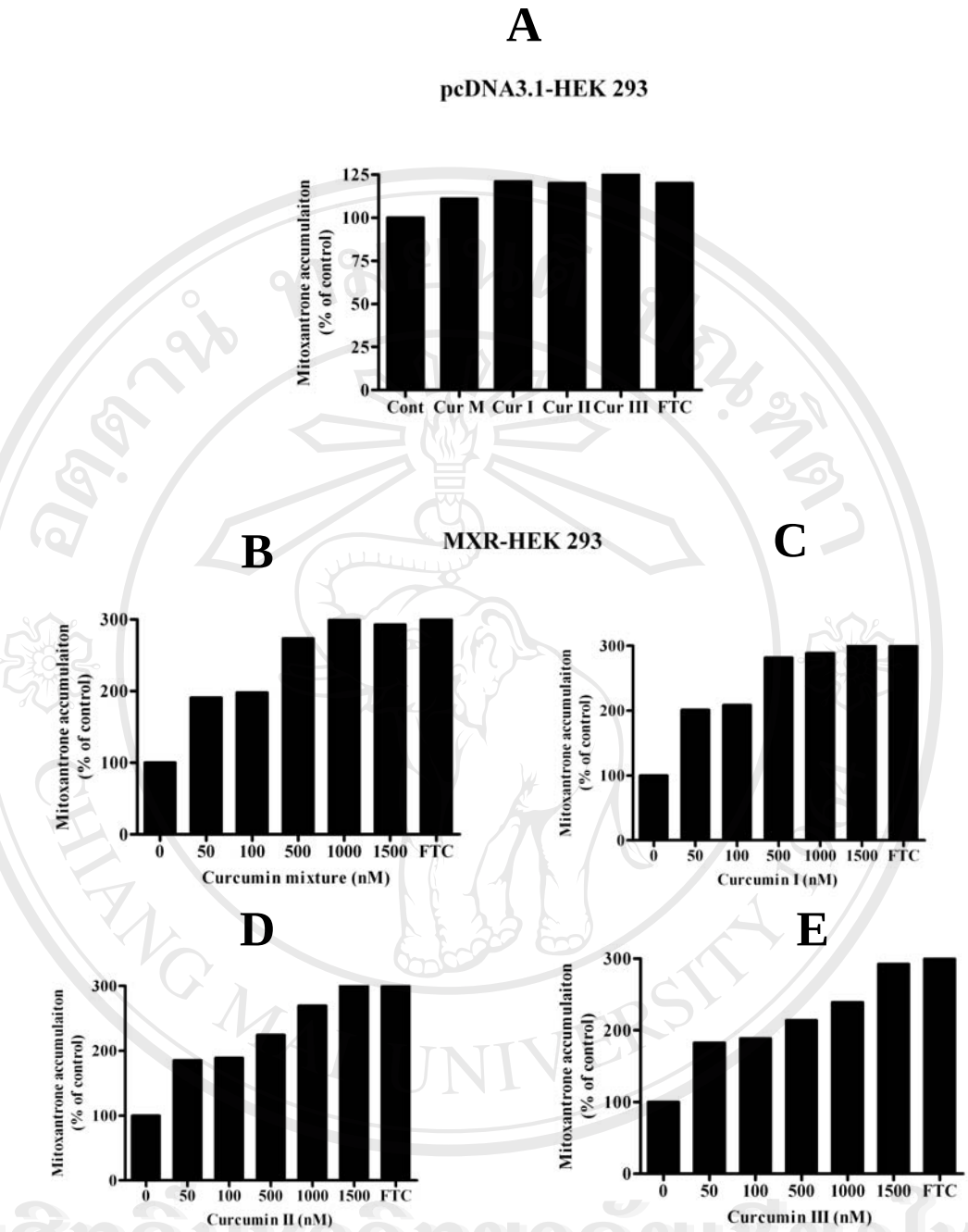


Figure 52. Effect of curcuminoids on the accumulation of mitoxantrone in pcDNA3.1-and MXR-HEK 293 cells. Cells were incubated at 37°C for 45 min in dark condition with 20 μM mitoxantrone in the presence of various concentrations of curcuminoids (0-1500 nM) or 10 μM FTC. The accumulation of the mitoxantrone was analyzed immediately by FACS. The mean of fluorescence intensity values were collected and represented as histogram (percentage of control). **(A);** The effect of curcuminoids at 20 uM on mitoxantrone accumulation in pcDNA3.1-HEK 293 cells.

(B-E); The effect of curcuminoids at 0-1500 nM on mitoxantrone accumulation in MXR-HEK 293 cells

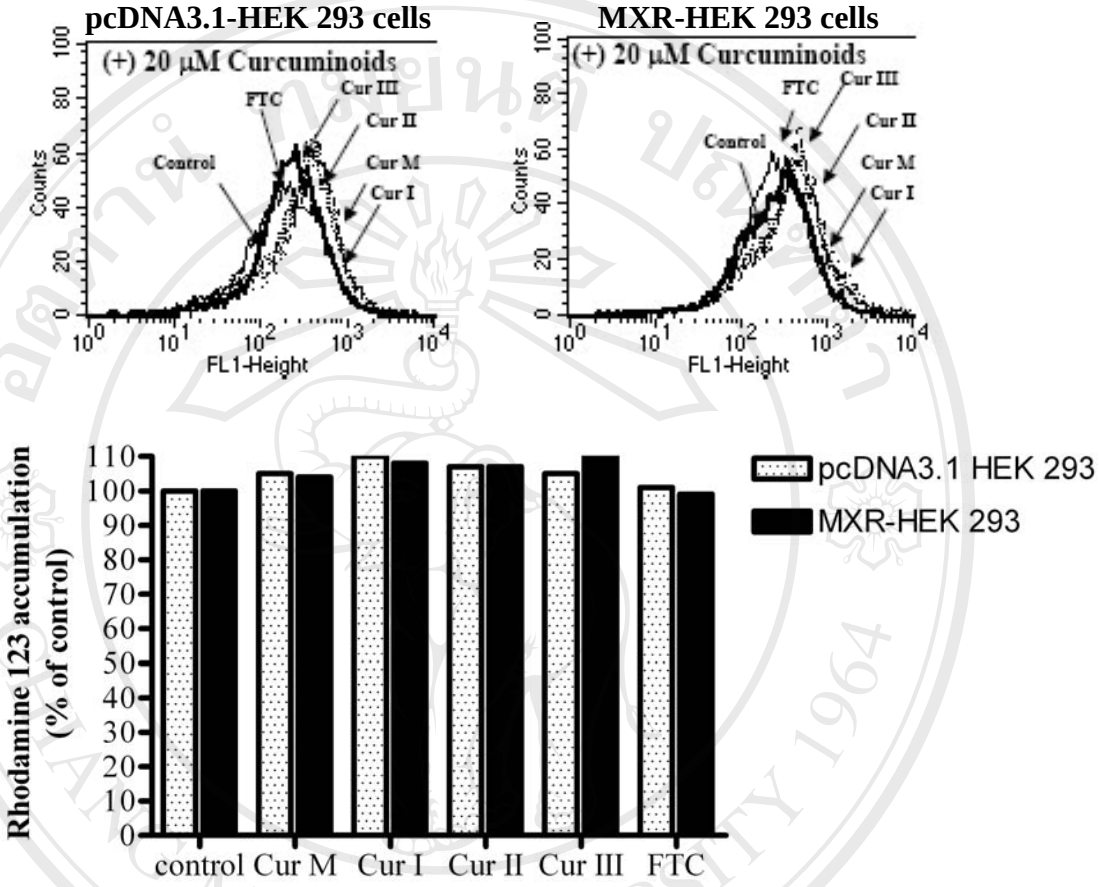


Figure 53. Effect of curcuminoids on rhodamine123 accumulation in pcDNA3.1- and MXR-HEK 293 cells. The cells were incubated with 0.5 μg/ml of rhodamine123 in the presence of 20 μM curcuminoids or 10 μM FTC then analysed on a flow cytometer (Section 2.8). ***Upper panel***, Representative histogram results from flow cytometry analysis was depicted to show the inhibitory effect of curcuminoids in comparison among each form, also with 10 μM of a positive control FTC. Cur M; curcumin mixture, Cur I, II, III; curcumin I, II, III. Control (□), FTC (–), Cur M (....), Cur I (….), Cur II (---), Cur III (-.-.). ***Lower panel***: the mean fluorescence intensity values were collected and represented as histogram (percentage of control).

3.4.2 Modulation of MXR mutant, R482T function by curcuminoids

It has been reported previously [64, 65, 158, 159] that the amino acid of MXR at position 482 has a crucial role in the substrate and inhibitor specificity of MXR [19-21]. The mutation of MXR at this position from arginine (called wild type, 482R) to threonine (called mutant, R482T) has altered drug resistance profiles and substrate specificity of MXR [19-21]. The results mentioned in Section 3.4.1 are the effects of curcuminoids on the function of MXR wild type 482R. They clearly show that curcuminoids are potent modulators of MXR wild type. In this part of the experiment, the effect of curcuminoids on MXR mutant (R482T) were determined. MCF-7AdrVp and its parental cell line, MCF-7 were used as a model of the study.

MCF-7/AdrVp was obtained upon long-term selection of the MCF-7 human breast cancer cell line in doxorubicin in the presence of P-gp inhibitor verapamil [160]. MCF-7AdrVp cells express only MXR and do not express P-gp or MRP1 [160]; however, display typical multidrug resistance phenotypes against doxorubicin with cross-resistance to daunorubicin and mitoxantrone but remain sensitive to vinca alkaloids, paclitaxel and cisplatin. Some of these agents are either P-gp or MRP1 substrates. In addition, MCF-7/AdrVp cells displayed ATP-dependent and reduced intracellular accumulation of daunorubicin and the fluorescent compound rhodamine123 compared with the parental MCF-7 cells [160]. In 2001, Honjo et al [67] reported that amino acid of MXR at position 482 expressed in MCF-7AdrVp was theronine (T). Therefore, MCF-7AdrVp and its parental cell line MCF-7 were used as models of drug resistant and drug sensitive cell lines to study effects of curcuminoids on MXR mutant 482T function.

Consistent with the previous study [161], a high expression of MXR was noticed in MCF-7AdrVp and not in its parental MCF-7 (Figure 54). As shown in Figure 55, the transport ability of MXR in this cell line was assessed by rhodamine123 accumulation assay. FTC, a potent modulator of MXR strongly increased the rhodamine123 accumulation in MCF-7AdrVp and did not cause significant effect to MCF-7 (Figure 53).

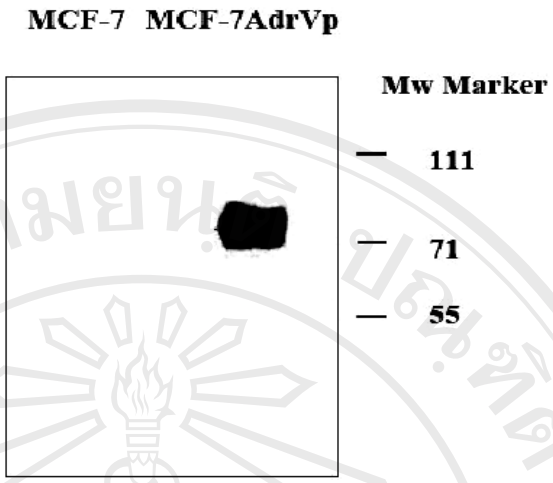


Figure 54. Expression level of MXR in MCF-7 and MCF-7AdrVp cells. Different concentrations of protein samples (15 µg) were subjected to SDS-PAGE and transferred onto nitrocellulose membrane, followed by immunodetection with anti-MXR, BXP-21(1:500). The blots were incubated with peroxidase conjugated antimouse antibody (1:10,000) and visualized using ECL detection kit. The arrows indicate the position of molecular weight marker, revealing that MXR had a predicted molecular weight around 72 kDa.

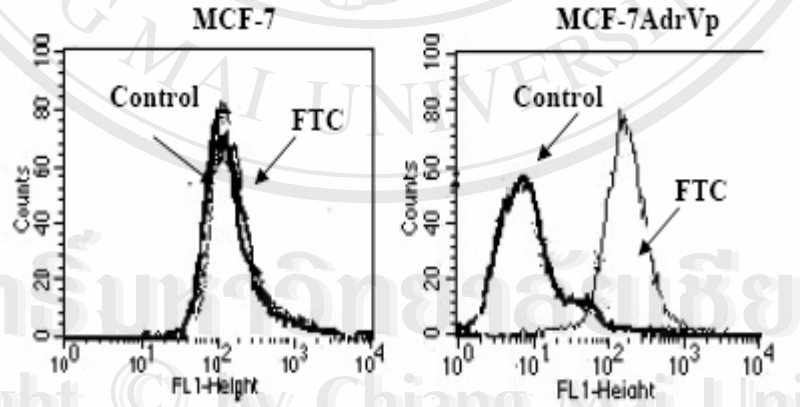


Figure 55. Rhodamine123 accumulation in MCF-7 and MCF-7AdrVp. The cells (5 x 10^5 cells) were incubated at 37°C for 45 min with 0.5 µg/ml of rhodamine123 in the presence of 0.1% DMSO control or 10 µM FTC and then analyzed on a flow cytometer as described in Section 2.8.

3.4.2.1 Cytotoxicity of curcuminoids in MCF-7 and MCF-7AdrVp

To examine the cytotoxicity of curcuminoids in MCF-7 and MCF-7AdrVp, the cells were exposed to various concentrations of curcuminoids (0-60μM) for 72 h and cell viability was determined by MTT assay as described in Section 2.4. The results are shown in Figure 56, the IC_{50} values of curcuminoids in these cell lines are in the range of 25-50 μM (Table 25). The non cytotoxic concentrations of curcuminoids (10 μM) were used for MDR phenotype study.

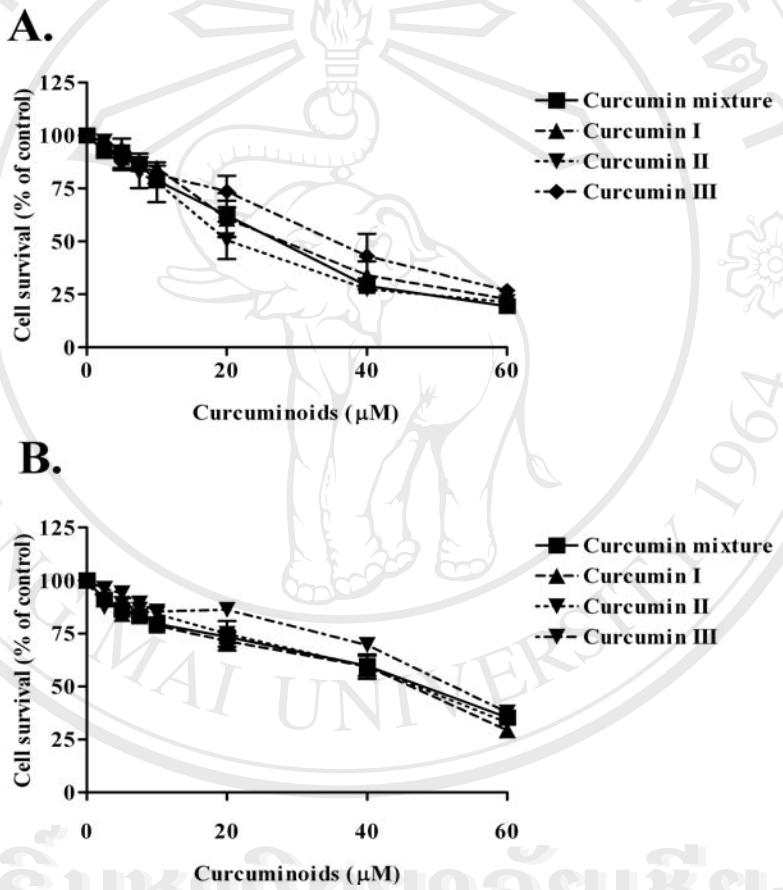


Figure 56. Cytotoxicity of curcuminoids in MCF-7 and MCF-7AdrVp. The cells (3x103 cells/well) were grown in the presence of curcuminoids (0-60 μM) for 72 h then the cell viability was determined by MTT assay (Section 2.4). A; cytoxicity of curcuminoids in MCF-7, and B; cytoxicity of curcuminoids in MCF-7 AdrVp

Table 25. IC_{50} values of curcuminoids in MCF-7 and MCF-7AdrVp cells.

Curcuminoids	IC_{50} Values (μM) [a]		Relative resistance [b]
	MCF-7	MCF-7AdrVp	
Curcumin M	30.5 ± 1.3	25.0 ± 3.6	0.8
Curcumin I	28.1 ± 5.5	25.0 ± 3.6	0.9
Curcumin II	30.0 ± 4.1	37.0 ± 10.6	1.2
Curcumin III	52.5 ± 6.4	50.7 ± 8.8	1.0

[a] The data represent the mean value ± SE of at least two independent experiments performed in triplicate.

[b] Relative resistance values was calculated by the IC_{50} ratio of resistant cells divided by that of the parental cells.

3.4.2.2 Effects of curcuminoids on anticancer drug sensitivity in MCF-7 and MCF-7AdrVp

The effect of curcuminoids on MXR mutant, R482T mediated MDR phenotype was evaluated by MTT assay. Two anticancer drugs, doxorubicin and mitoxantrone were used in this part of the study. The result in Figure 57 showed that curcuminoids increased the sensitivity of doxorubicin and mitoxantrone significantly.

The IC_{50} values of doxorubicin in drug sensitive (MCF-7) and drug resistant cell lines (MCF-7AdrVp) were 101.0 nM and 24.10 μM, respectively. It is indicated that MCF-7AdrVp was 241 times more resistant to doxorubicin than the parental MCF-7 cells (Table 26). When 10 μM curcuminoids was added, the IC_{50} value of doxorubicin in MCF-7AdrVp strongly decreased from 24.1 μM to 6.7, 6.2, 6.2 and 10.2 for curcumin mixture, curcumin I, II and III respectively, suggesting curcuminoids significantly increased the sensitivity of doxorubicin in MCF-7AdrVp. The inhibitory effect was not observed in MCF-7.

Mitoxantrone was used as a positive modulator, a non toxic concentration of mitoxantrone at 10 nM was drastically decreased the IC_{50} value of doxorubicin from 24.1 to 3.9 μM in MCF-7AdrVp. Similar manner was noticed when combined curcuminoids with mitoxantrone. Curcuminoids at 10 μM significantly decreased the

IC_{50} of mitoxantrone from > 100 μM to 7.8, 4.6,13.3 and 28 μM for curcumin mixture, curcumin I, II and III respectively. Therefore, they are implying that curcuminoids were able to reverse MXR mutant, 482T-mediated MDR phenotype.

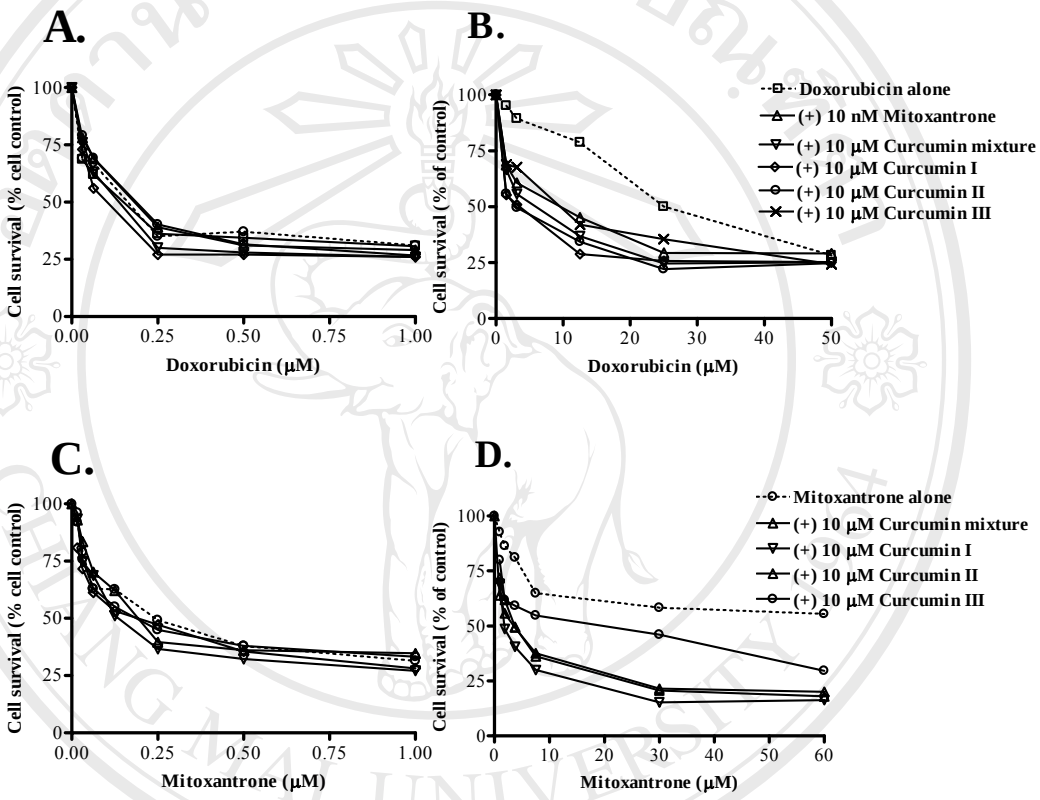


Figure 57. Effect of curcuminoids on anticancer drug (s) sensitivity in MCF-7 and MCF-7AdrVp. Three days cytotoxicity assays of doxorubicin and mitoxantrone were conducted in the presence of 0.5% DMSO or 10 μM curcuminoids. The cell viability was determined by MTT assay (Section 2.4). The effect of curcuminoids on doxorubicin and mitoxantrone sensitivity are shown in A-B and C-D respectively. A-C; in MCF-7 and B-D; in MCF-7AdrVp.

Table 26. Reversal of resistance to doxorubicin and mitoxantrone with non toxic concentration of curcuminoids in MCF-7 and MCF-7AdrVp

Drug and/or curcuminoids	IC_{50} (nM)[a]		RR[b]	Degree of MDR reversal activity[c]
	MCF-7	MCF-7AdrVp		
DMSO (doxorubicin alone)	0.1	24.1	241	1.0
(+) Mitoxantrone	0.1	0.5	5.0	48.2
(+) Curcumin mixture	0.1	6.7	67	3.6
(+) Curcumin I	0.1	6.2	62.0	3.9
(+) Curcumin II	0.1	6.2	62.0	3.9
(+) Curcumin III	0.1	10.2	102.0	2.4
DMSO (mitoxantrone alone)	0.2	60.0	300.0	1.0
(+) Curcumin mixture	0.2	7.8	39.0	7.7
(+) Curcumin I	0.2	4.6	23.0	13.0
(+) Curcumin II	0.2	13.3	66.5	4.5
(+) Curcumin III	0.2	28.0	140.0	2.1

[a] The data represent the mean value from two independent experiments performed in triplicate. IC_{50} values refer to the drug concentration required for half-maximum inhibition of the growth rate.

[b] The value is calculated by the IC_{50} ratio of anticancer drug in the presence of DMSO control divided by the IC_{50} of value of the drug in the presence of curcuminoids.

[c] Degree of reversal activity were obtained by dividing the relative resistance (RR) value of doxorubicin or mitoxantrone (0.5% DMSO) by the relative resistance value of doxorubicin or mitoxantrone in the presence of curcuminoids.

3.4.2.3 Effect of curcuminoids on the accumulation of bodipy-prazosin and rhodamine123 in MCF-7 and MCF-7AdrVp

To study the effect of curcuminoids on MXR mutant R482T function, the activity of MXR was assessed by measuring the intracellular accumulation of bodipy-prazosin and rhodamine123 in drug resistant MCF-7AdrVp compared with drug sensitive MCF-7 cells. The amount of intracellular bodipy-prazosin and rhodamine123 were investigated by using flow cytometer. As shown in Figures 58-61, curcuminoids substantially increased the accumulation of bodipy-prazosin and rhodamine123 in a concentration dependent manner. Compared at 10 μM, curcumin mixture and curcumin I showed the most two active forms to increased the accumulation of bodipy-prazosin, whereas curcumin II and III were less active (Figure 59). Similar manner was observed with the effect of 5 μM curcuminoids on rhodamine123 accumulation (Figure 61). However at higher concentration (15 μM), curcumin mixture and curcumin I, II and III increased the accumulation of rhodamine123 in a similar activity and in comparable with positive modulator FTC at 10 μM (Figure 61).

There was no significant effect of curcuminoids and FTC were found either on bodipy-prazosin or rhodamine123 accumulation in parental MCF-7 cells.

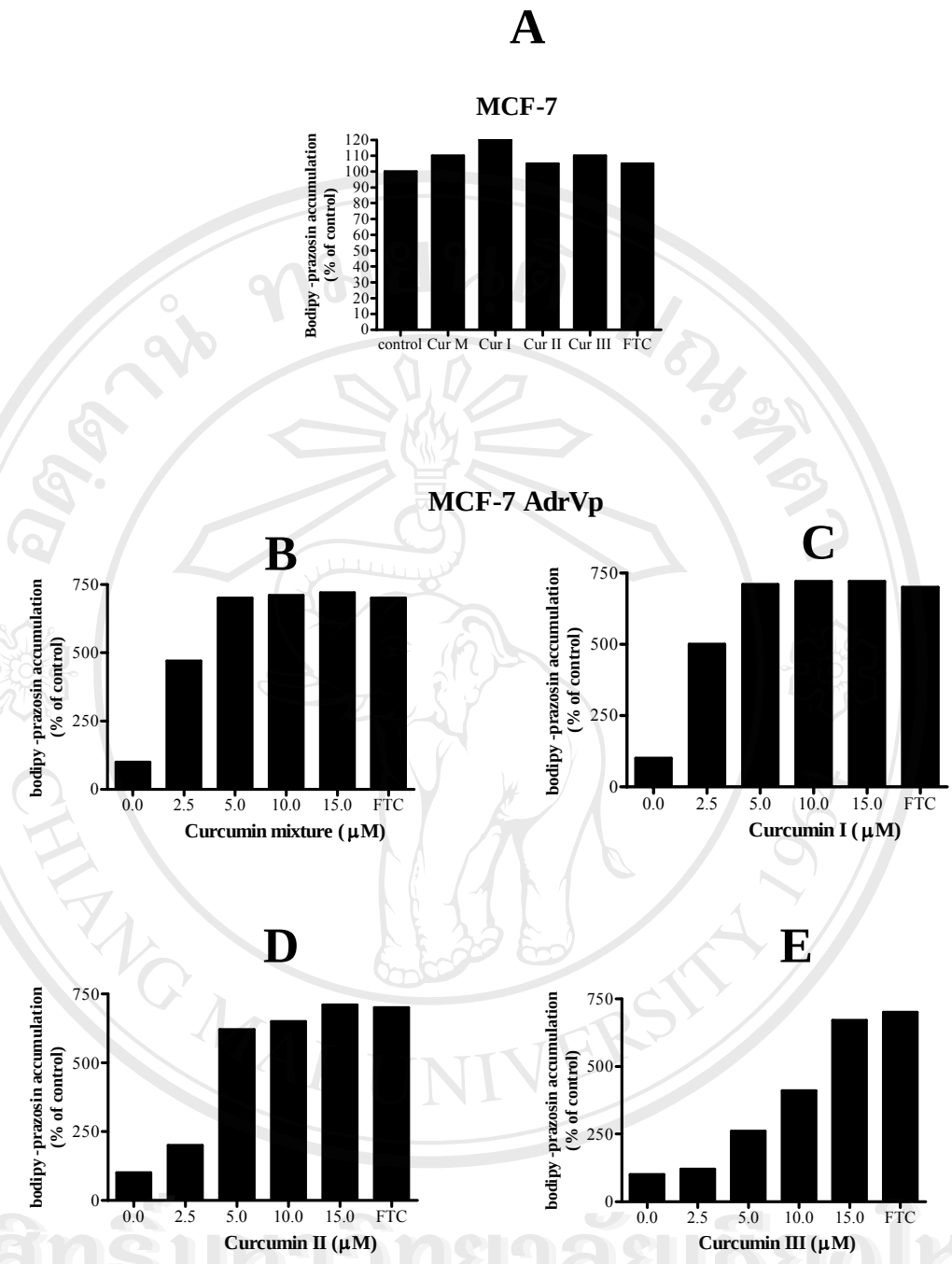


Figure 58. Effect of curcuminoids on the accumulation of bodipy-prazosin in MCF-7 and MCF-7AdrVp cells. The amount of intracellular bodipy-prazosin fluorescence was determined by flow cytometry. The mean of fluorescence intensity values were collected and represented as histogram (% of control). **(A);** The effect of curcuminoids at 20 uM on bodipy-prazosin accumulation in MCF-7. **(B-E);** The effect of curcuminoids at 0-15 μM on bodipy-prazosin accumulation in MXR-HEK 293 cells.

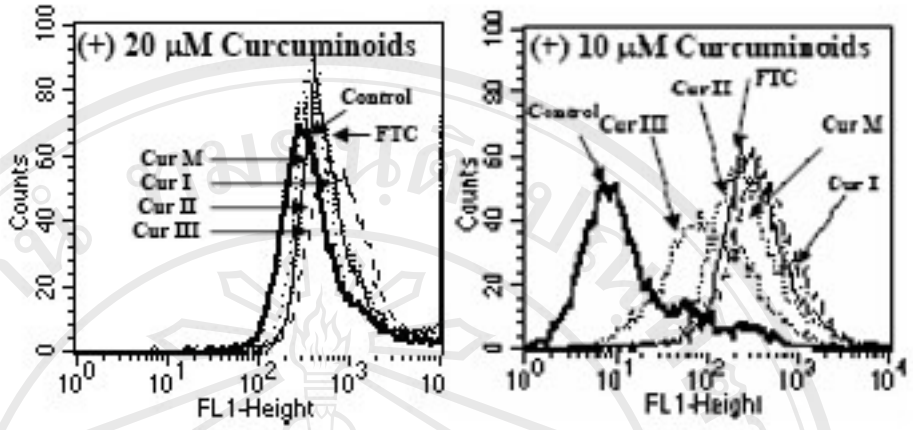


Figure 59. Effect of curcuminoids at 10 μM on the accumulation of bodipy prazosin in MCF-7 and MCF-7AdrVp. To determine the effect of curcuminoids on the transport bodipyprazin by pcDNA3.1- and MXR-HEK 293 cells, the cells were incubated for 45 min at 37 ^{o}C in dark with 250 nM bodipy prazosin in the presence of various concentration of curcuminoids or 10 μM FTC. The cells were then washed and analysed on a flow cytometer. Curcuminoids increase the accumulation of the bodipy prazosin in MCF-7 and MCF-7AdrVp. Cur M; curcumin mixture, Cur I, II, III; curcumin I, II, III. Control (□), FTC (–), Cur M (....), Cur I (….), Cur II (---), Cur III (-.-.).

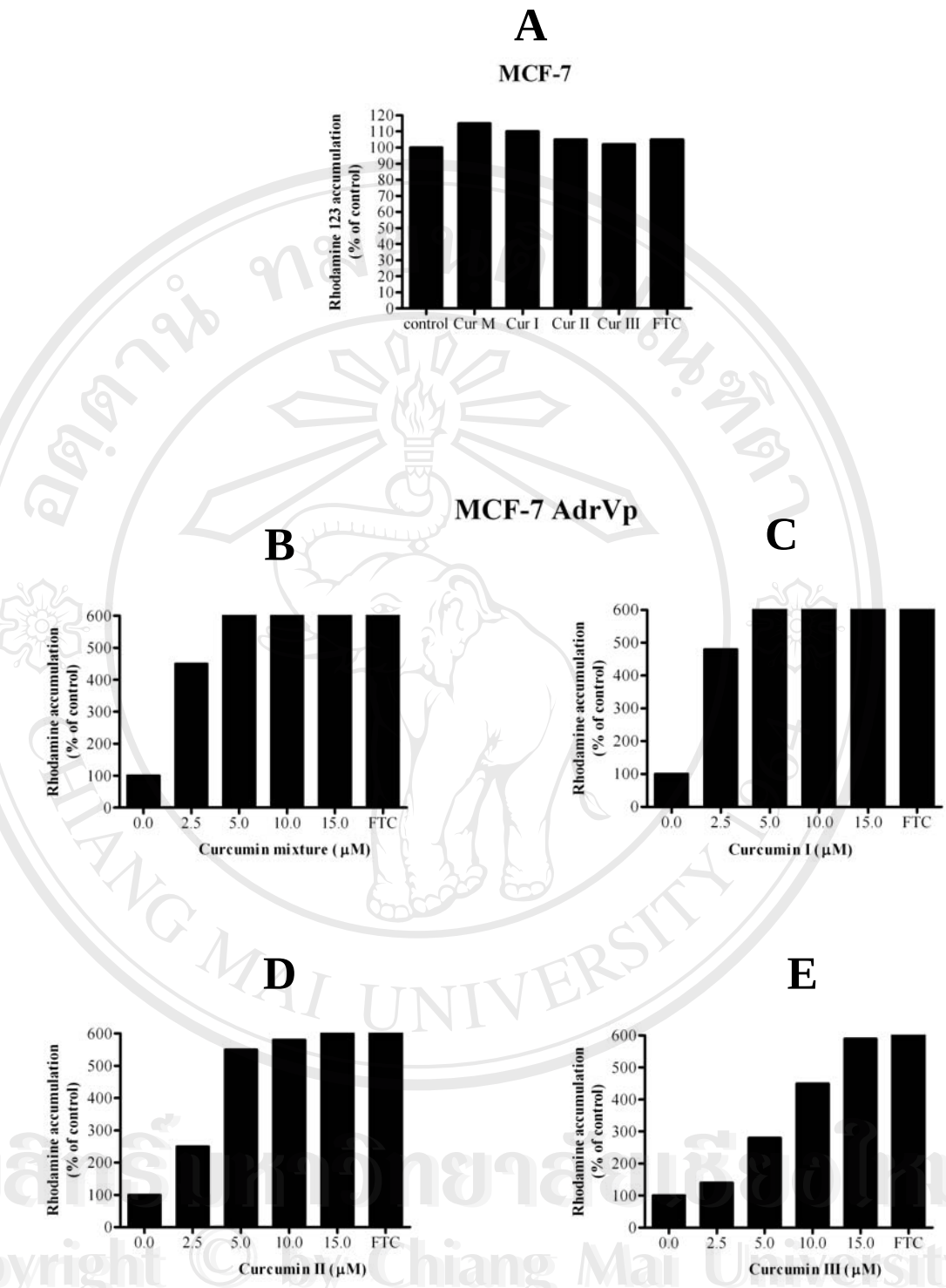


Figure 60. Effect of curcuminoids on the accumulation of rhodamine 123 in MCF-7 and MCF-7AdrVp cells. The amount of intracellular rhodamine123 fluorescence was determined by flow cytometry. The mean of fluorescence intensity values were collected and represented as histogram (% of control). **(A);** The effect of curcuminoids at 20 μM on rhodamine123 accumulation in MCF-7. **(B-E);** The effect of curcuminoids at 0- 15μM on rhodamine123 accumulatin in MCF-7AdrVp.

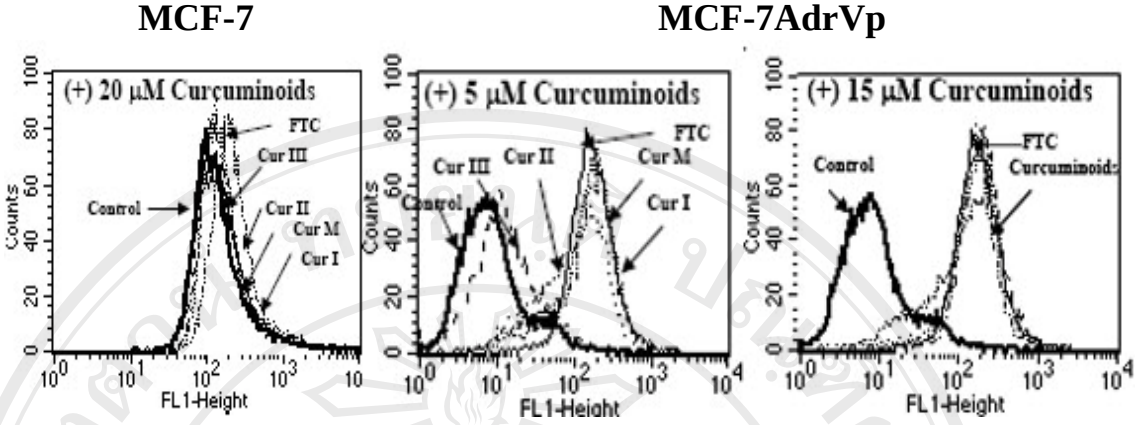


Figure 61. Efffect of curcuminoids on rhodamine123 accumulation in MCF-7 and MCF-7AdrVp. To determine the effect of curcuminoids on the accumulation of rhodamine123 in MCF-7 and MCF-7AdrVp, the cells were incubated with 0.5 μg/ml rhodamine123, in the presence of 3 or 10 μM curcuminoids or 10 μM FTC then analysed by flow cytometer. Cur M; curcumin mixture, Cur I, II, III; curcumin I, II, III. Control (□), FTC (─), Cur M (....), Cur I (….), Cur II (---), Cur III (-.-.).

3.4.3 Effect of curcuminoids on MXR expression

Effect of curcuminoids on MXR protein level in MCF-7AdrVp was studied to determine whether the curcuminoids reduced the protein level of MXR thereby the efflux function of MXR was decreased. The cells were incubated with 3 and 10 μM curcuminoids for three days. Then membrane protein was prepared and separated on a 7.5% SDS-PAGE and analyzed by Western blot. The concentration of curcuminoids used in this experiment did not affect the cell viability and morphology when observed under phase microscope. The result in Figure 62 showed clearly that the treatment the cells with 5 and 10 μM of curcuminoids, no significant difference of MXR protein level was observed when compared with the treatment with DMSO control.

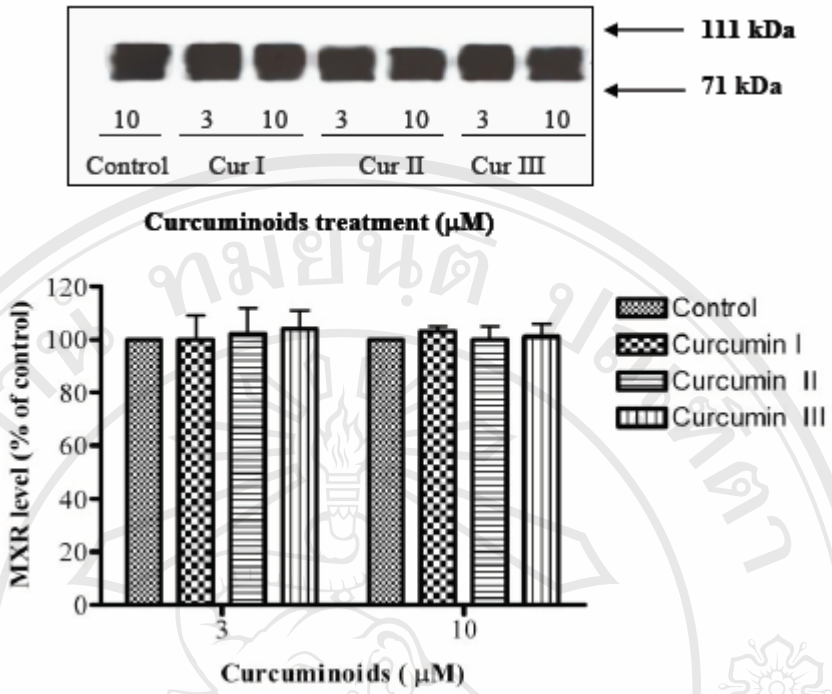


Figure 62. Effect of curcuminoids on MXR expression in MXR overexpressing cells, MCF-7AdrVp. To investigate the effect of curcuminoids on MXR expression, MCF-7AdrVp cells were treated with 3 and 10 μM curcuminoids for three days. Membrane protein (15 μg) was separated by SDS-PAGE and Western blot analysis. Immunoblotting was performed using BXP-21 (1:500) as first antibody and anti-mouse horseradish peroxidase (HRP) conjugate (1:10000) as the secondary antibody. The arrow shows the position of molecular weight marker indicating that MXR had a predicted molecular weight ~ 72 kDa. The data represent the mean values of two independent experiments.

3.4.4 Effect of Mitoxantrone, prazosin and FTC on ATPase activity of MXR

Similar to other ABC transporters, MXR utilizes the energy of ATP for its drug transport activity [21, 22]. In this part of the experiment, membrane of MXR was prepaed from HEK transfected with MXR. Then the basal ATPase activity of MXR was studied in the presence of known MXR substrates (mitoxantrone, prazosin) and modulator (FTC). The results were consistent with the previous study [162, 163], mitoxantrone and prazosin (PZN) significantly stimulated the ATPase activity of MXR whereas FTC showed substantially inhibited (Figure 63). It is also suggested

that the MXR membrane used in this part of the experiment was appropriated model to study the effect of curcuminoids on the ATPase activity of MXR.

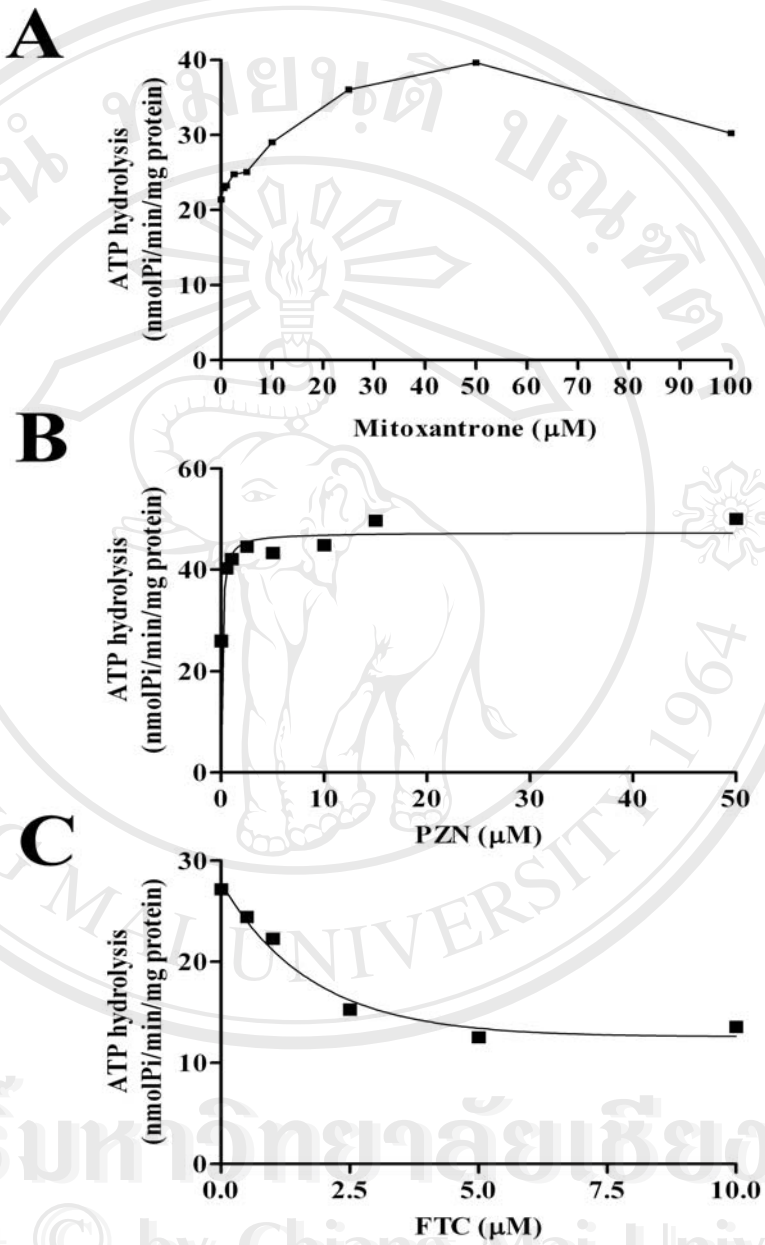


Figure 63. Effect of Mitoxantrone, prazosin and FTC on ATP hydrolysis of MXR. Crude membrane of MXR (100 μg protein/ml) was incubated with various concentrations of mitxantone (A) or prazosin (PZN) (B) or FTC (C) in the presence and absence of BeFx. The MXR-specific activity was recorded as the BeFx-sensitive ATPase activity as described in Section 2.13.

3.4.5 Effect of curcuminoids on ATPase activity of MXR

In order to characterize the effect of curcuminoids on the ATP hydrolysis of MXR, the ATPase assay in the presence of curcuminoids was carried out. As shown in Figure 64, curcuminoids stimulated the MXR- ATPase activity (in both wild type 482R and mutant R482T) in a concentration dependent manner. No effect of curcuminoids was observed in membranes prepared from pcDNA3.1-HEK293 cells (data not shown). This data implied the actual binding of curcuminoids on the MXR molecule. Curcumin mixture and curcumin I showed the most potent forms to stimulate the ATPase activity of MXR. Curcumin II showed similar activity in wild type, 482R when compared with curcumin mixture and curcumin I however slightly less active in mutant, 482T (Table 27), whereas; curcumin III showed less effective in both wild type 482R and mutant 482T.

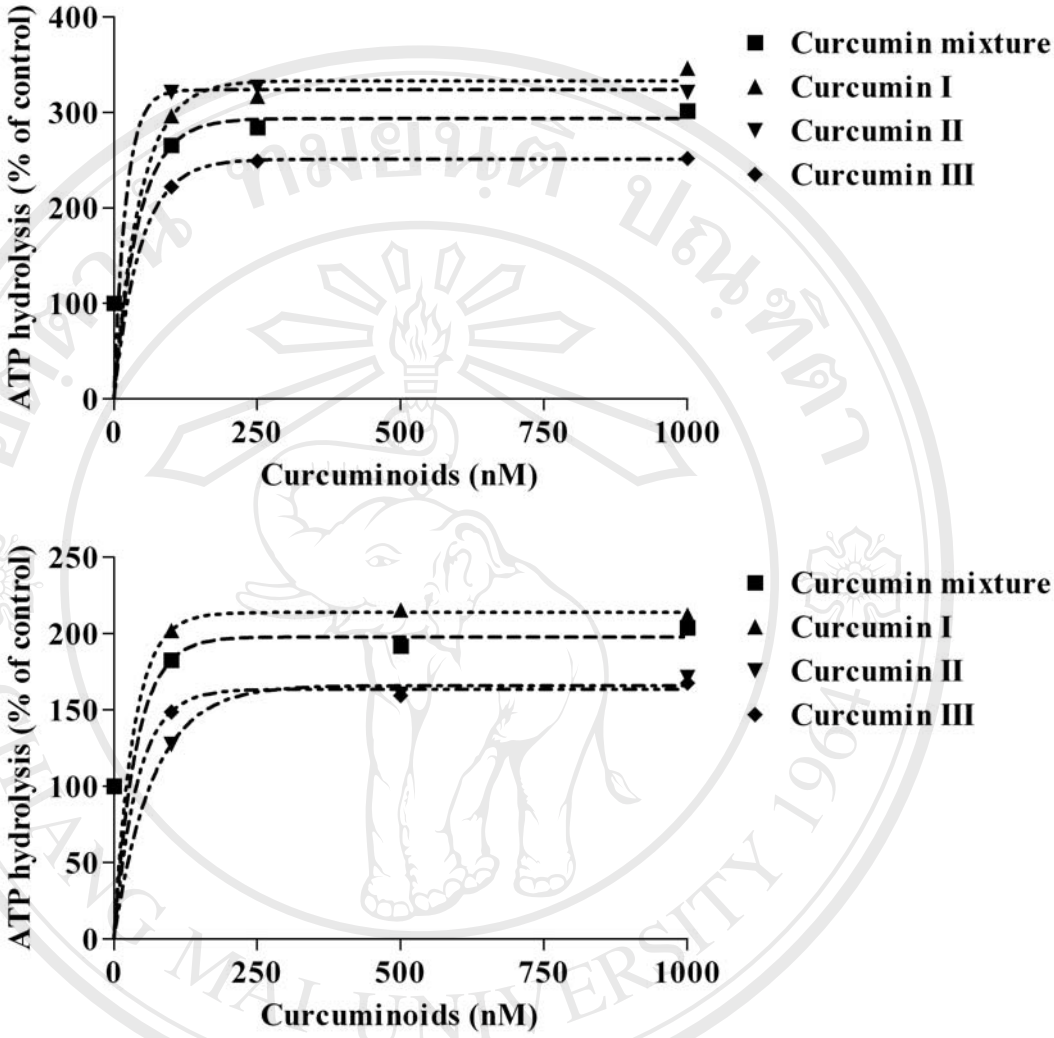


Figure 64. Effect of crucuminoids on ATP hydrolysis of MXR. Crude membranes of MXR-HEK 293 cells both wild type, 482R and mutant,R482T (100 μg protein/ml) were incubated with increasing concentrations of curcuminoids (0-1000 nM). MXR-specific activity was recorded as the BeFx-sensitive ATPase activity. The stimulatory effect of curcuminoids was observed in both wild type 482R **(*Upper panel*)** and mutant R482T **(*Lower Panel*)**.

Table 27. The IC_{50} values of curcuminoids on ATPase activity of MXR.

Compound	ATP hydrolysis (nanomoles P_i/min/mg protein) Basal (Fold- stimulation) [a]	
	MXR-wild type (482R)	MXR-mutant(R482T)
Curcumin mixture	3.0	2.0
Curcumin I	3.5	2.1
Curcumin II	3.2	1.7
Curcumin III	2.5	1.7

[a] Fold stimulation was obtained by dividing the ATPase activity in the presence of the curcuminoids by the in the basal activity in the presence of DMSO. These values were representative of at least three independent experiments, one typical experiment is depicted.

Modulation of P-gp, MRP1 and MXR function by tetrahydrocurcumin (THC)

Tetrahydrocurcumin is valued as the ultimate metabolite of the curcuminoids in vivo [121, 164-166]. As shown in Figure 9, it has been proposed by Pan et al [164] that after absorption most curcumin would be reduced to dihydrocurcumin and tetrahydrocurcumin by endogenous reductase system, and subsequently were converted to monoglucoronoside conjugates by UDP-glucuronosyl transferase. To date curcumin-glucuronide, dihydrocurcumin-glucuronoside, THC-glucuronoside, and THC has been demonstrated as the major curcumin metabolites in vivo [121, 164, 166, 167]. The research was revealed previously that curcumin I, II and III which are major curcuminoids isolated from turmeric are able to inhibit function of P-gp, MRP1 and MXR, the ABC transporters that have been claimed so far to confer multidrug resistance in cancer cells. Plus the research was suggested that among three major forms, curcumin I are the most active form to modulate the function of P-gp, MRP1 and MXR. This study, is an additional brief study to show that the major metabolite form, tetrahycrocurcumin, inhibit the function of P-gp, MRP1 and MXR, implying the extended effects of curcumin in vivo. This finding provides a further support evidence to develop curcumin I or curcumin mixture (major component is curcumin I) as MDR modulator by using in combination with conventional chemotherapy, however; bioavailability as well as in vivo study of curcuminoids are still necessary.

3.5.1 Modulation of the P-gp function by tetrahydrocurcumin

3.5.1.1 Effect of tetrahydrocurcumin on the accumulation of calcein AM in KB-3-1 and KB-V-1 cells

The research had first evaluated the inhibitory effect of tetrahydrocurcumin on P-gp function by measuring the intracellular accumulation of calcein which is the cleavage product of calcein-AM by cellular esterase. The result in Figure 65 showed that tetrahydrocurcumin increased the accumulation of calcein in KB-V-1 cells in a concentration dependent pattern (Figure 65). Also, cyclosporin A (CsA) at 10 μM was strongly increased the accumulation of calcein in KB-V-1. The inhibitory effect was observed only in P-gp overexpressing cells, KB-V-1 (B, C) not in KB-3-1 (A).

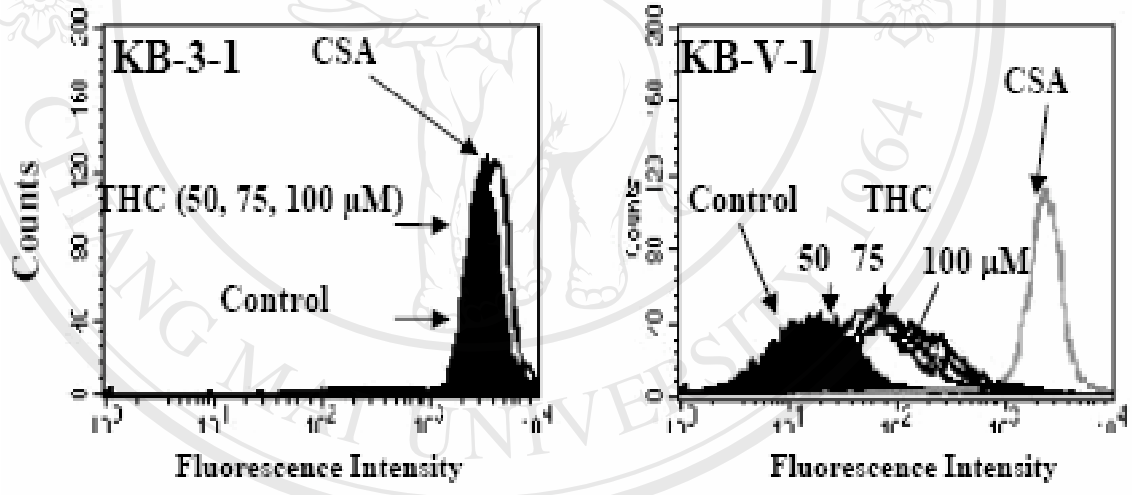


Figure 65. Effect of tetrahydrocurcumin on calcein-AM in KB-3-1 and KBV-1 cells. Cells (5×10^5) were incubated with calcein-AM (0.25 μM) in the presence of DMSO or CsA (10 μM) or tetrahydrocurcumin (50, 75, 100 μM). After 10 min incubation, the cells were washed and analyzed as described in Section 2.8. Each histogram shows the overlayer of DMSO control or in the presence of tetrahydrocurcumin or positive modulator, CsA. The results are representative of at least two independent experiments, one typical experiment is depicted.

3.5.1.2 Effect of tetrahydrocurcumin on the accumulation and efflux of ^{3}[H]-vinblastine in MCF-7 and MCF-7MDR cells

To confirm the effect of tetrahydrocurcumin on the P-gp function, the activity of P-gp was assessed by determining the intracellular retention of radiolabeled drug, ^{3}[H]-vinblastine in drug resistant MCF-7MDR and drug sensitive MCF-7 cells. This method had been claimed to be the appropriate model to test the actual vinblastine accumulation in the intact cell. The effect of various concentrations of tetrahydrocurcumin on the P-gp mediated accumulation and efflux of ^{3}[H]-vinblastine revealed that tetrahydrocurcumin increased ^{3}[H]-vinblastine uptake in MCF-7AdrVp in concentration dependent manner (Figure 66A).

For the efflux study of ^{3}[H]-vinblastine (Figure 66B), similar effect was found, tetrahydrocurcumin caused an increase in the amount of ^{3}[H]-vinblastine retention compared with vehicle control in a dose dependent pattern. Verapamil at 25 μM was used as a positive control in this study and the data showed that it strongly increased ^{3}[H]-vinblastine accumulation and retention in MCF-7MDR.

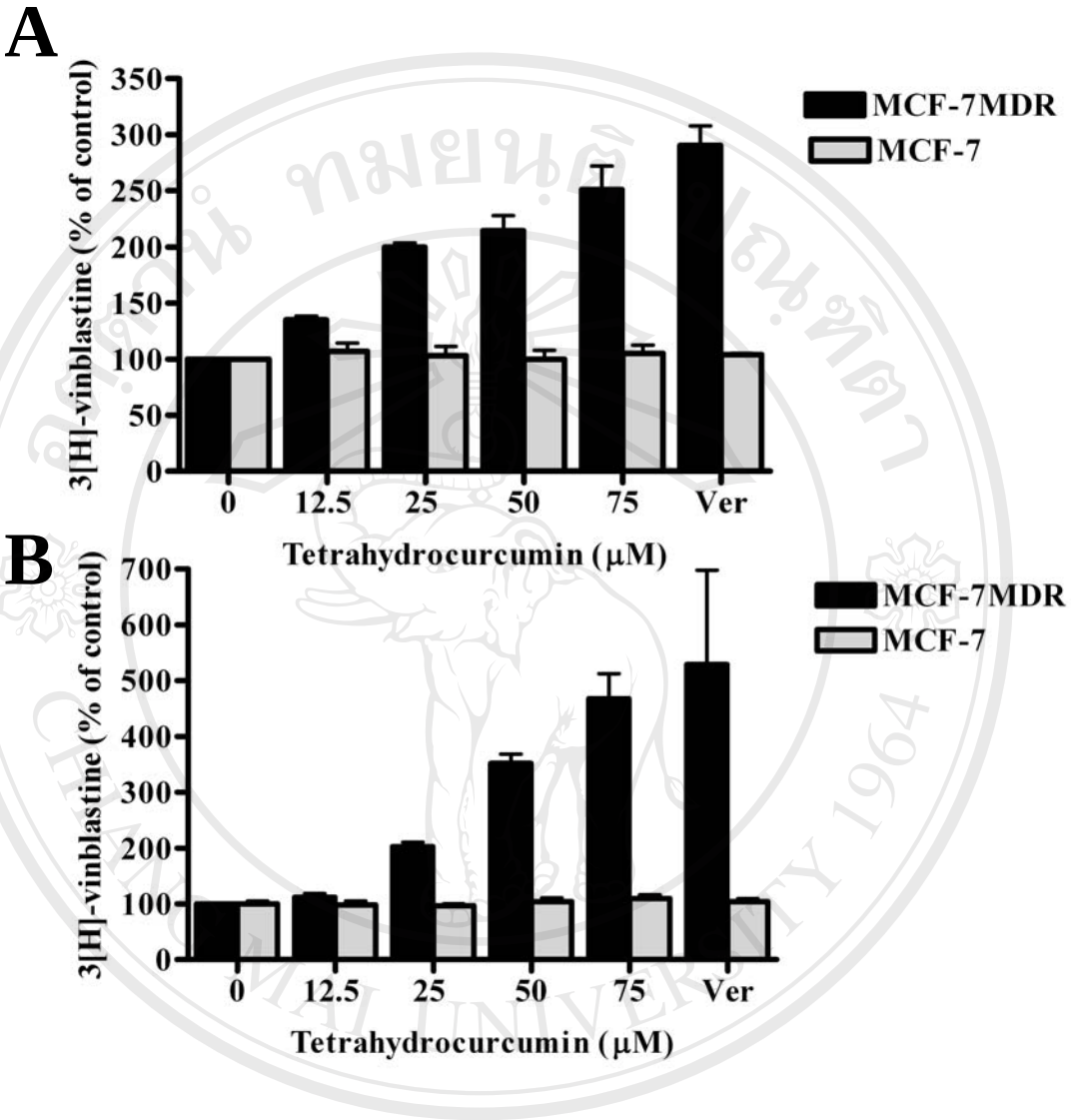


Figure 66. Effect of tetrahydrocurcumin on ^{3}[H]-vinblastine accumulation and efflux. The amount of intracellular radioactivity was determined by β-counter (Section 2.20.3). The data are represented as mean values ± SE of three-independent experiments. **A.** The effect of curcuminoids on ^{3}[H]-vinblastine accumulation and **B.** The effect of curcuminoids on ^{3}[H]-vinblastine efflux.

3.5.1.3. Effect of tetrahydrocurumin on photoaffinity labeling of P-gp with IAAP

Direct interaction of THC on P-gp was confirmed by IAAP photoaffinity labeling assay. Figure 67 showed that tetrahydrocurcumin inhibited the incorporation of [^{125}I] IAAP into P-gp in a concentration dependent pattern, suggesting that, similar with curcuminoids, tetrahydrocurcumin binds directly to the substrate binding site of P-gp, probably at the same binding sites with prazosin.

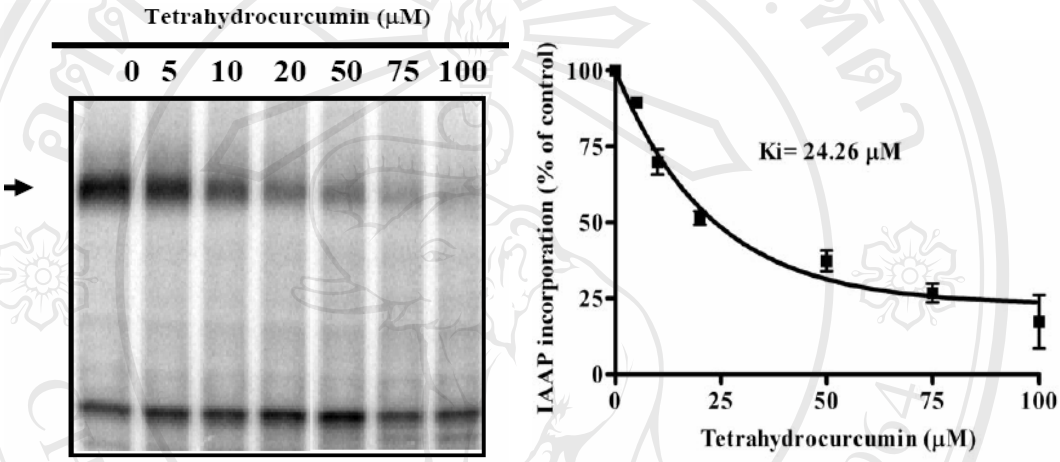


Figure 67. Effect of various concentrations of tetrahydrocurcumin on photoaffinity labeling of P-gp with IAAP. The crude membranes of High Five insect cells were incubated with increasing concentrations of tetrahydrocurcumin (0-100 μM) in the presence IAAP following SDS-PAGE. Then the gels were dried and exposed to Bio-Max MR film at –70 ^{o}C for 12-24 hr. The radioactivity incorporated into the P-gp band was quantified using the STORM 860 phosphorimager system. ***Left Panel***: the autoradiograms show incorporation of IAAP into the P-gp band in the presence of various concentration of tetrahydrocurcumin. The arrow indicated the band occurring from the incorporation of [^{125}I] IAAP into P-gp. ***Right Panel***: the data were fitted by non-linear least squares regression analysis using the software GraphPad Prism are from three independent experiments.

3.5.2 Effect of tetrahydrocurcumin on the MDR phenotype mediated by MRP1

3.5.2.1 Cytotoxicity of tetrahydrocurcumin in pcDNA3.1- and MRP1 transfected HEK 293 cells

To investigate the cytotoxicity of tetrahydrocurcumin in pcDNA3.1- and MRP1 transfected HEK 293 cells, both cell lines were incubated with various concentrations of tetrahydrocurcumin (0-100 μM) for 72 h. Then the survival cells were detected by MTT assay as described in section 2.4. As shown in Table 28 and Figure 67. The IC_{20} and IC_{50} of tetrahydrocurcumin in pcDNA3.1- and MRP1-HEK 293 cells were in the range of 21-38 μM. The minimal low toxicities (10, 15, 25 μM) were chosen for the experiment in Section 3.5.2.2

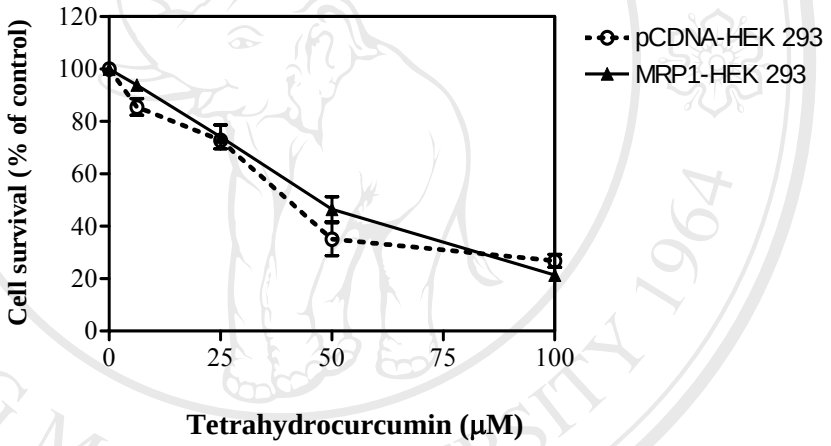


Figure 68. Cytotoxicity of tetrahydrocurcumin in pcDNA3.1 and MRP1-HEK 293 cells. The survival cells were determined by MTT assay as described in Section 2.4. Each point represents the mean value from three-independent experiments performed in triplicate.

Table 28. IC_{20} and IC_{50} values of tetrahydrocurcumin in pcDNA3.1 and MRP1-HEK 293 cells. The data represented the mean values ± SE of three-independent experiments performed in triplicate.

Cell lines	IC_{20} value	IC_{50} value
pcDNA3.1-HEK 293	21.0 ± 3.4	33.7 ± 0.7
MRP1-HEK 293	23.0 ± 1.2	38.7 ± 1.8

3.5.2.2 Effect of tetrahydrocurcumin on etoposide (VP-16) sensitivity in pcDNA3.1 and MRP1-HEK 293 cells

The effect of tetrahydrocurcumin on the etoposide sensitivity was examined, pcDNA3.1- and MRP1 transfected HEK 293 cells were seeded into 96-well plate and incubated for 24 h. Afterthat fresh medium containing tetrahydrocurcumin (10, 15 and 25 μM) and positive inhibitors (indomethacinee, MK-571 and vinblastine) was added in the presence of various concentrations of etoposide and incubated for additional 72 h. The survival cells were detected by MTT assay (Section 2.4). The result showed that the tetrahydrocurcumin and all the three positive inhibitors (indomethacinee, MK-571 and vinblastine) did not affect the etoposide sensitivity in pcDNA3.1-HEK 293 cells, whereas significantly increased the sensitivity in MRP1-HEK 293 cells (Figure 69).

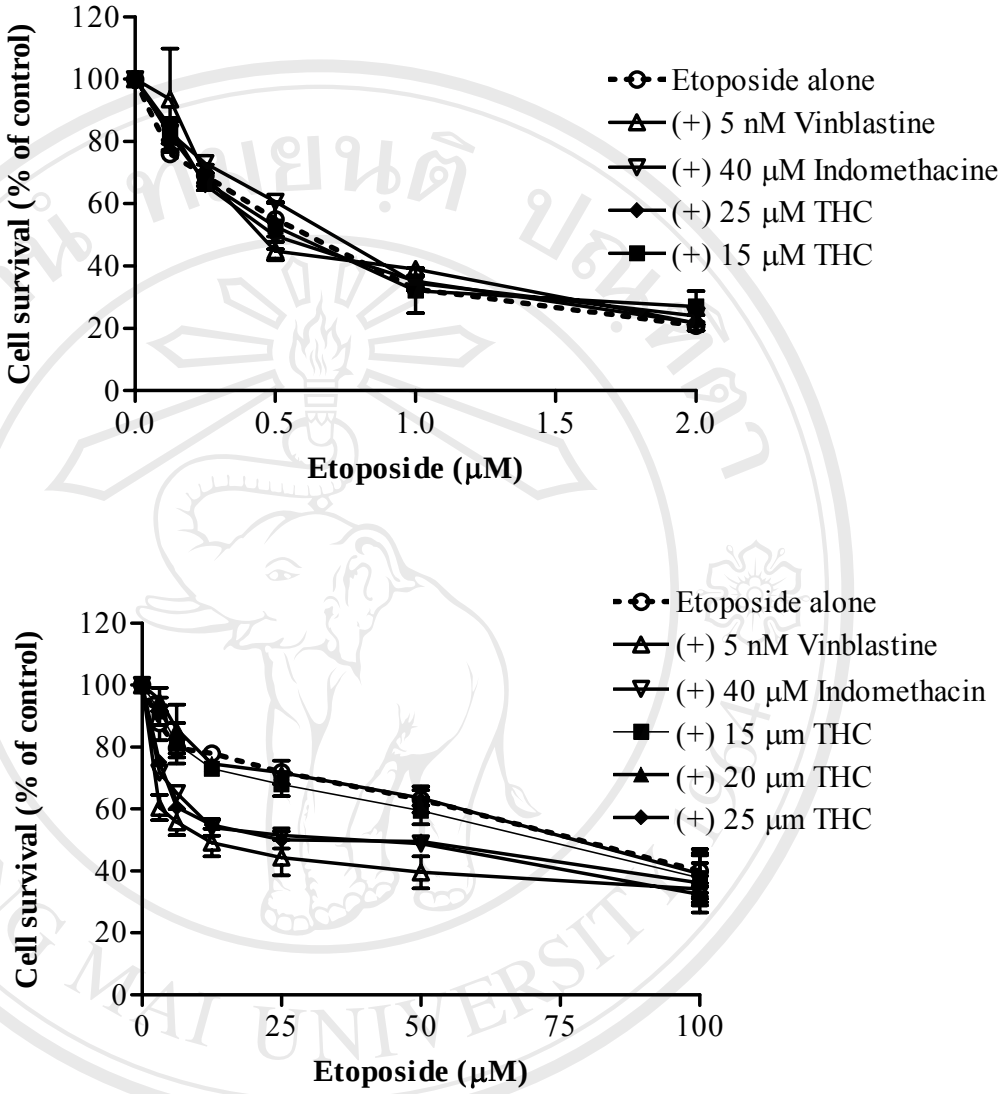


Figure 69. Effect of tetrahydrocurcumin on etoposide drug sensitivity in pcDNA3.1- and MRP1 transfected HEK 293 cells. The cells were grown in the presence of various concentrations of etoposide alone or with 15, 20, 25 μM tetrahydrocurcumin and 10 nM vinblastine or 40 μM indomethacine. The number of viable cells was determined by MTT assay (Section 2.4). The experiment was conducted in triplicate and the values represented mean ± SE of three independent experiments.

3.5.3 Modulation of MXR by tetrahydrocurcumin

To study the effect of tetrahydrocurcumin on MXR mediated MDR phenotype, MXR overexpressing cells, MCF-7AdrVp and its parental cells were used as a model system.

3.5.3.1 Cytotoxicity of tetrahydrocurcumin in MCF-7 and MCF-7AdrVp cells

MTT assay was assessed to determine cytotoxicity profile of tetrahydrocurcumin in MCF-7 and MCF-7AdrVp. The IC_{20} and IC_{50} values were calculated and shown in Table 29, The minimal low toxicities (10, 25, 30 μM) were chosen for the MDR phenotype experiment (Section 3.7.2)

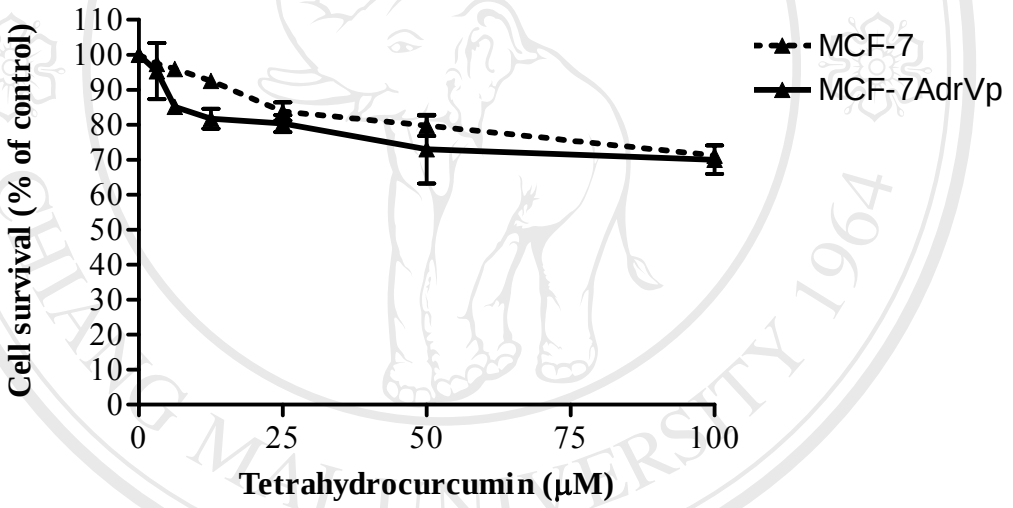


Figure 70. Cytotoxicity of tetrahydrocurcumin in MCF-7 and MCF-7AdrVp. MTT assay was used to investigate how tetrahydrocurcumin affect the viability of MCF-7 and MCF-7AdrVp. The cells were exposed to various concentrations of tetrahydrocurcumin (0-100 μM) for 72 h then the viability of cells were determined by MTT as described in Section 2.4. Each point represents the mean value for three-independent experiments performed in triplicate.

Table 29. IC_{20} and IC_{50} values of tetrahydrocurcumin in MCF-7 and MCF-7AdrVp cells. The data represented the mean values ± SE of three-independent experiments performed in triplicate.

Cell lines	IC_{20} value (μM)	IC_{50} value (μM)
MCF-7	30 ± 5.0	> 100
MCF-7AdrVp	27.5 ± 2.5	> 100

3.5.3.2 Effect of tetrahydrocurcumin on mitoxantrone sensitivity in MCF-7 and MCF-7AdrVp cells

To explore the reversal potential of tetrahydrocurcumin on MXR mediated MDR phenotype, MCF-7 and MCF-7AdrVp were exposed to increasing concentrations of mitoxantrone in the presence tetrahydrocurcumin (10, 25, 30 μM). The viability of the cells were examined by MTT assay (see Section 2.4 for the details). As seen in Figure 71, tetrahydrocurcumin was able to sensitize MXR expressing cells, MCF-7AdrVp to mitoxantrone anticancer drug in a concentration dependent manner, suggesting the reversal activity of tetrahydrocurcumin on MXR mediated MDR phenotype.

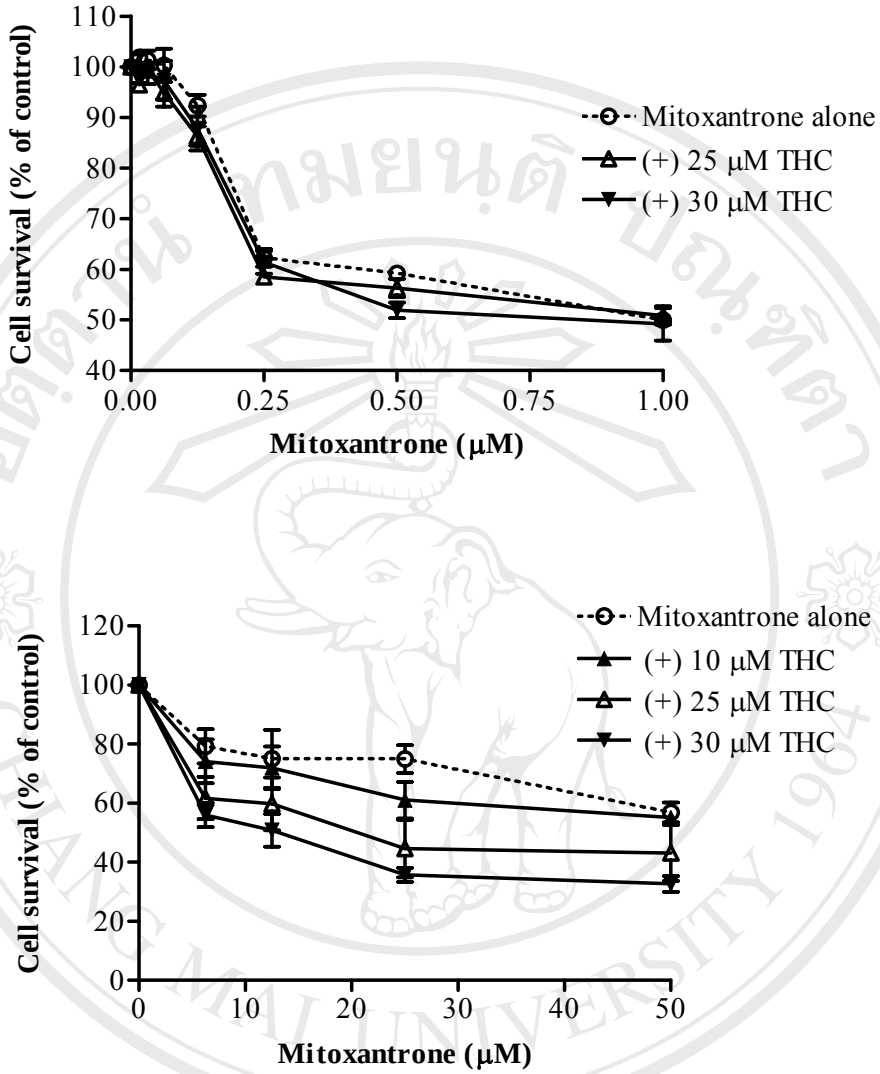


Figure 71. Effect of tetrahydrocurcumin on mitoxantrone sensitivity in MCF-7 and MCF-7AdrVp. The cells were grown in the presence of various concentrations of mitoxantrone alone (0.5% DMSO) or with 10, 25, 30 μM tetrahydrocurcumin. The number of viable cells was determined by MTT assay (Section 2.4). The experiment was conducted in triplicate and the values represented mean ± SE of three independent experiments.