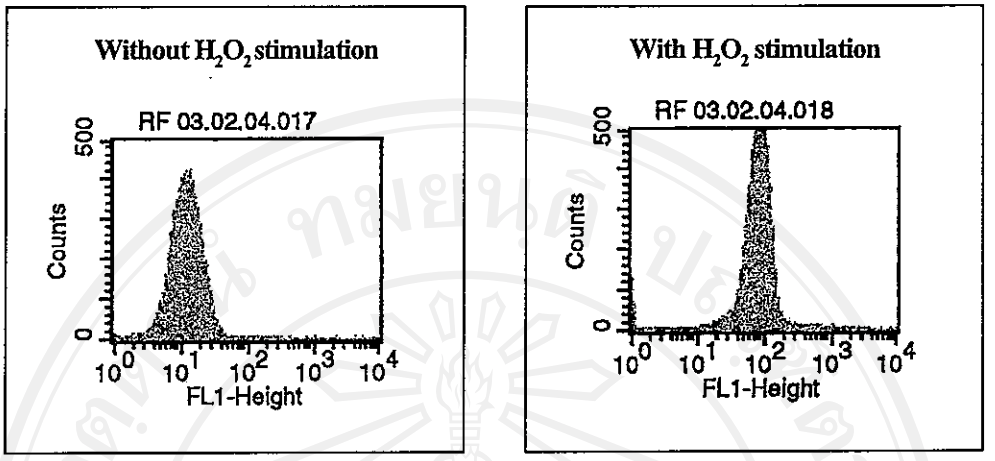
CHAPTER III

RESULTS

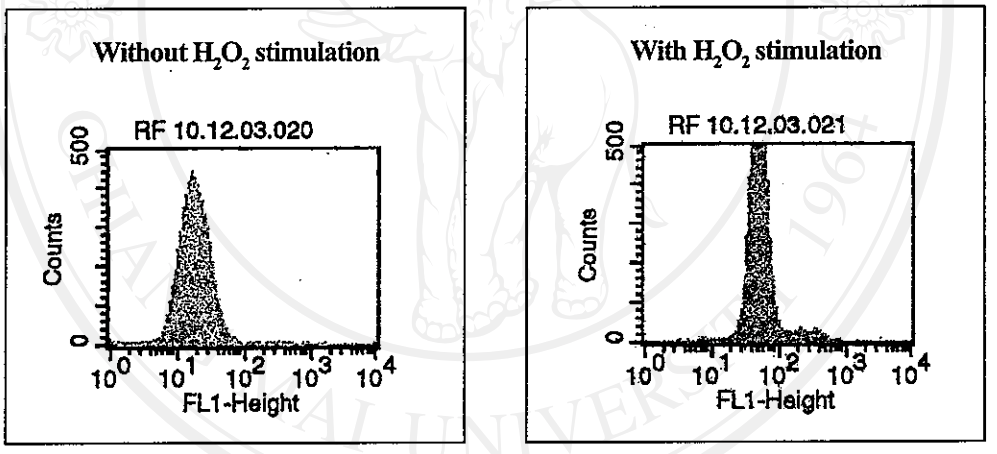
3.1 Flow cytometric analysis of free radicals in erythrocytes from end-stage renal disease patients

In this study, a very sensitive flow cytometry was used to measure the amount of ROS in the red blood cell population from normal volunteers (control) and ESRD patients. There were two main steps in this method. One was performed without hydrogen peroxide stimulation that indicated the intra-erythrocytic inherent ROS reacting with DCF and giving the green fluorescent signal. The other was done with hydrogen peroxide stimulation, which it indicated the presence of non-heme iron in red blood cells that was able to degrade hydrogen peroxide to hydroxyl radicals and subsequently reacted with DCF. The amount of intraerythrocytic reactive oxygen species (ROS) was expressed by the measured fluorescent intensity (FI). Previous study has shown that the ROS was significantly higher in stimulated and unstimulated thalassemic red blood cells than in normal red blood cells (Amer, *et al* 2003). In this study, the levels of ROS in ESRD red blood cells were varied; which some were normal, moderate and high. It was found that the histogram plot of FI seemed to be directly proportional to the ROS production in not only unstimulated but also stimulated red blood cells from normal volunteers and ESRD patients (Figure 3.1).

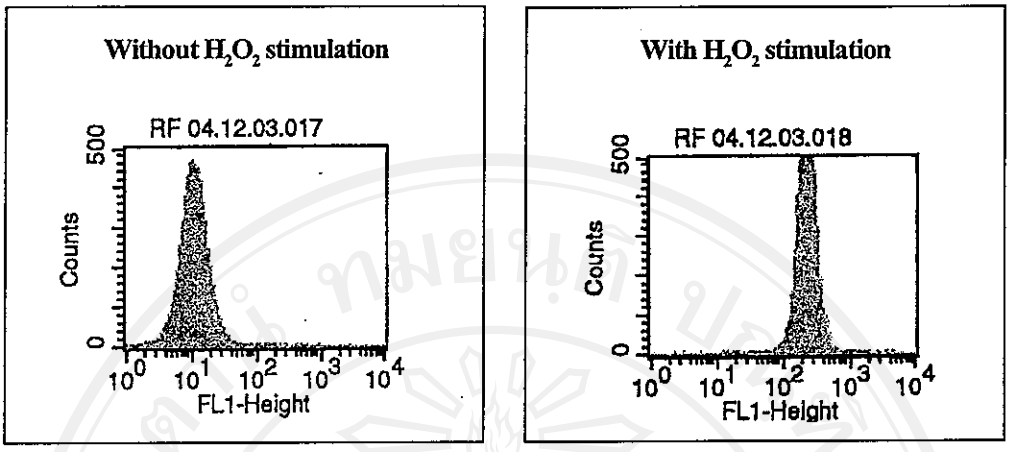
Arithmetric mean, geometric mean and median of FI were nearly the same, ROS was therefore quantified by the geometric mean of FI to illustrate the oxidative stress in red blood cells. Concerning the reliable and precise FI values, the quality control was performed during the flow cytometric assay of ROS in all ESRD patients.



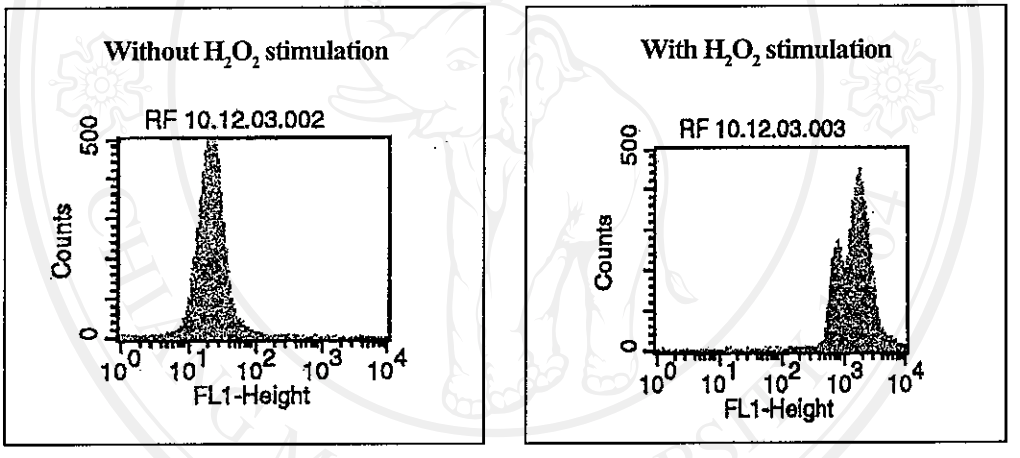
A) Erythrocytes from normal volunteers



B) Erythrocytes from ESRD patients (normal FI)



C) Erythrocytes from ESRD patients (moderate FI)



D) Erythrocytes from ESRD patients (high FI)

Figure 3.1 Representative flow cytometric patterns of oxidative stress in erythrocytes, without and with hydrogen peroxide stimulation, from A) normal volunteers B)ESRD patients normal FI. C) ESRD patients moderate FI. and D) ESRD patients high FI.

Quality control of flow cytometric measurement: Within-run assay

Since the fluorescent product is unstable, it needs to measure the FI of the compound quickly so that the repeatability assay must be performed. In our study, the FI of the same DCF-labeled ESRD red blood cell, with and without hydrogen peroxide stimulation, was repeatedly measured for eight times and determined the geometric mean±SD. The FI value of DCF-labeled cells without hydrogen peroxide stimulation was 265.3±4.5 and the FI value of DCF-labeled cells with hydrogen peroxide stimulation was 2131.2±150.8 (p<0.005) (as shown in Figure 3.2, Table 3.1). We are certain that this method is precise and reliable and can be used for measuring the amount of free radicals (ROS) in the erythrocyte from ESRD patients.

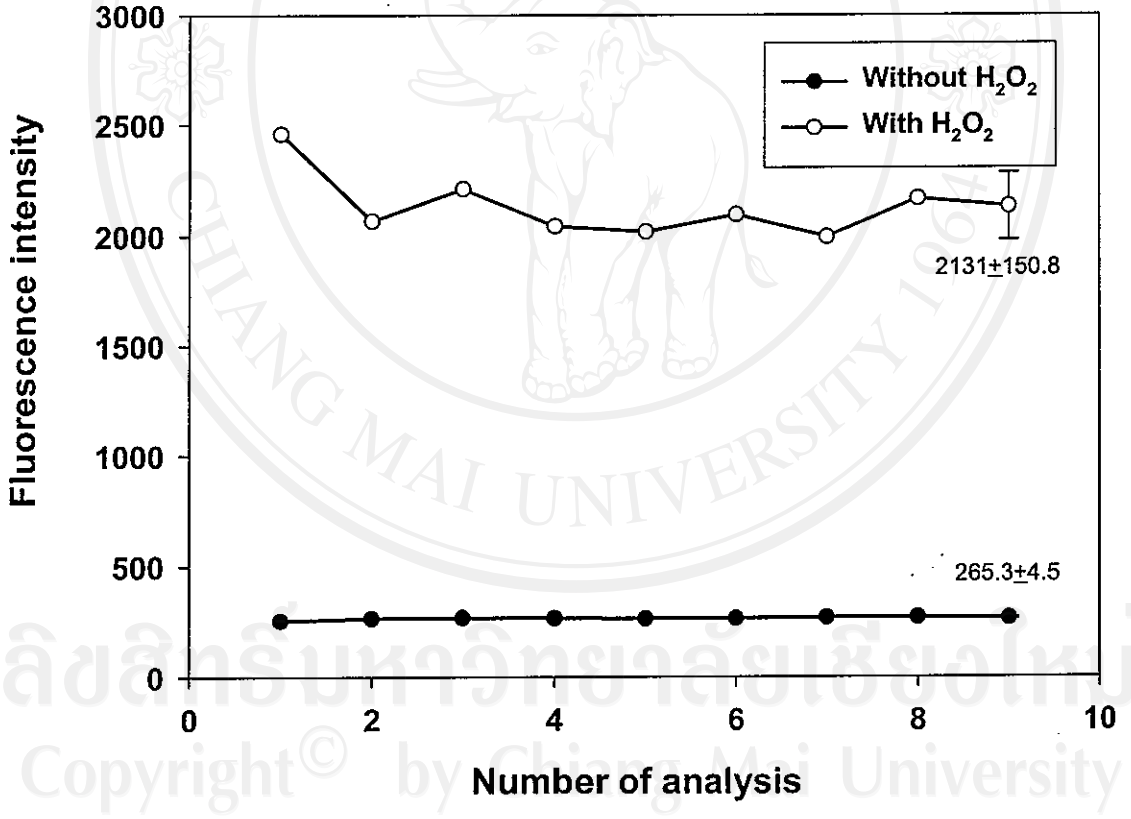


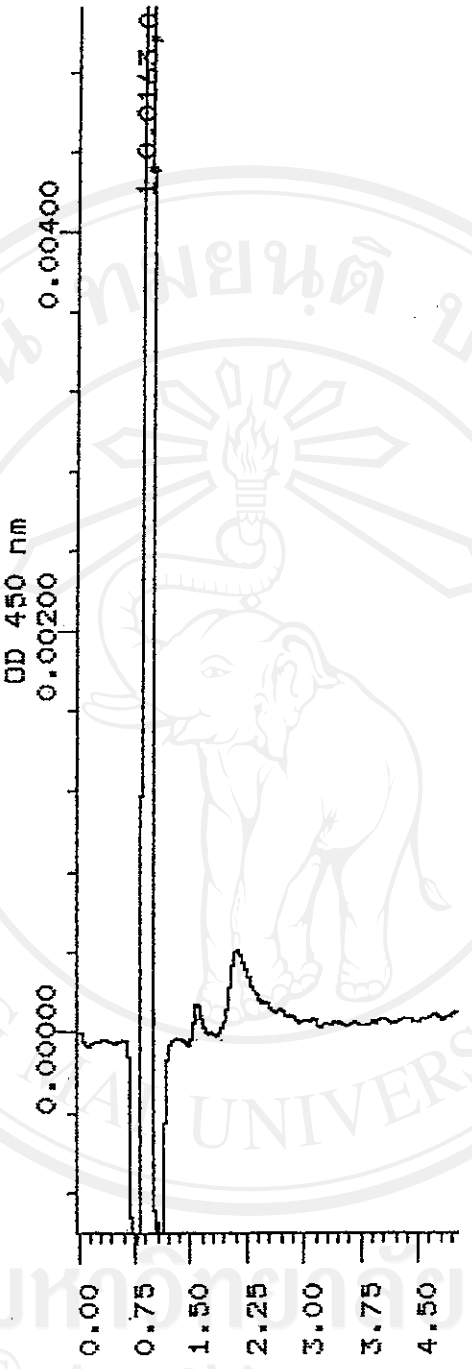
Figure 3.2 Quality control of flow cytometric measurements (n=8) of fluorescence intensity (FI) in the same DCF-labeled ESRD red blood cells without and with hydrogen peroxide.

Table 3.1 Quality control of flow cytometric measurements (n=8) of fluorescent intensity in the same DCF-labeled ESRD red blood cells without and with hydrogen peroxide.

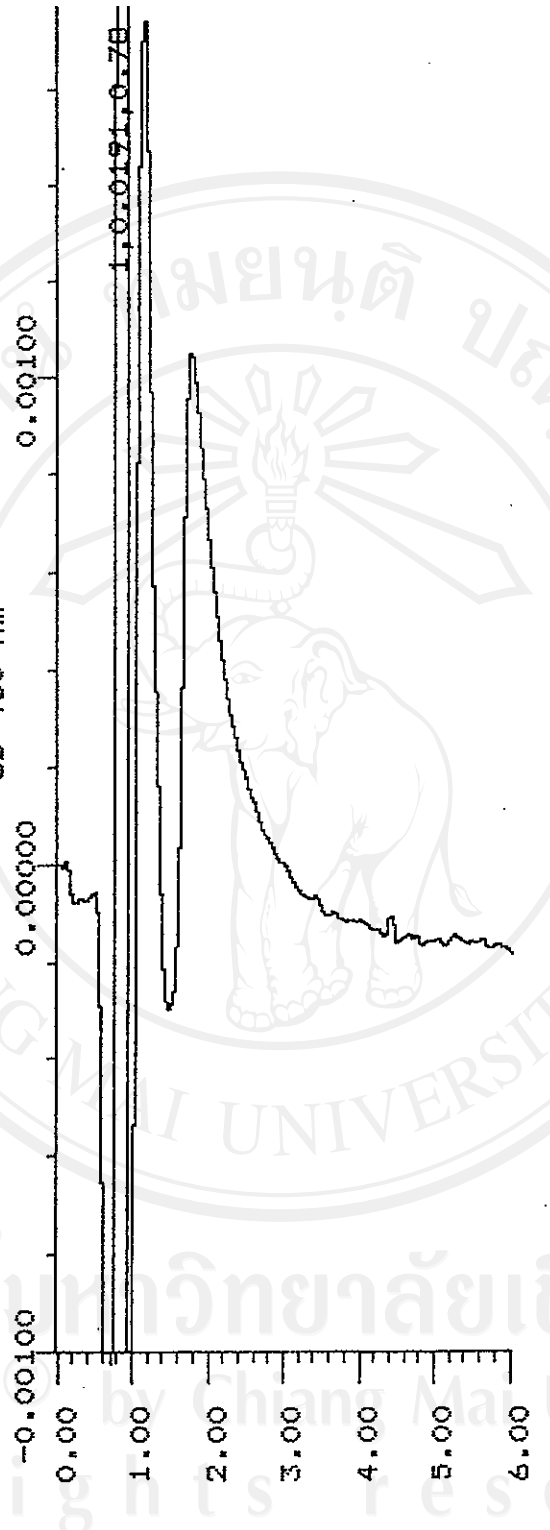
Condition	Number of analysis								Mean±SD
	1	2	3	4	5	6	7	8	
Without H_2O_2	255	265	269	266	264	264	268	270	265±5
With H_2O_2	2458	2067	2210	2043	2017	2094	1994	2167	2131±151

3.2 Measurement of NTBI using the NTA/HPLC method

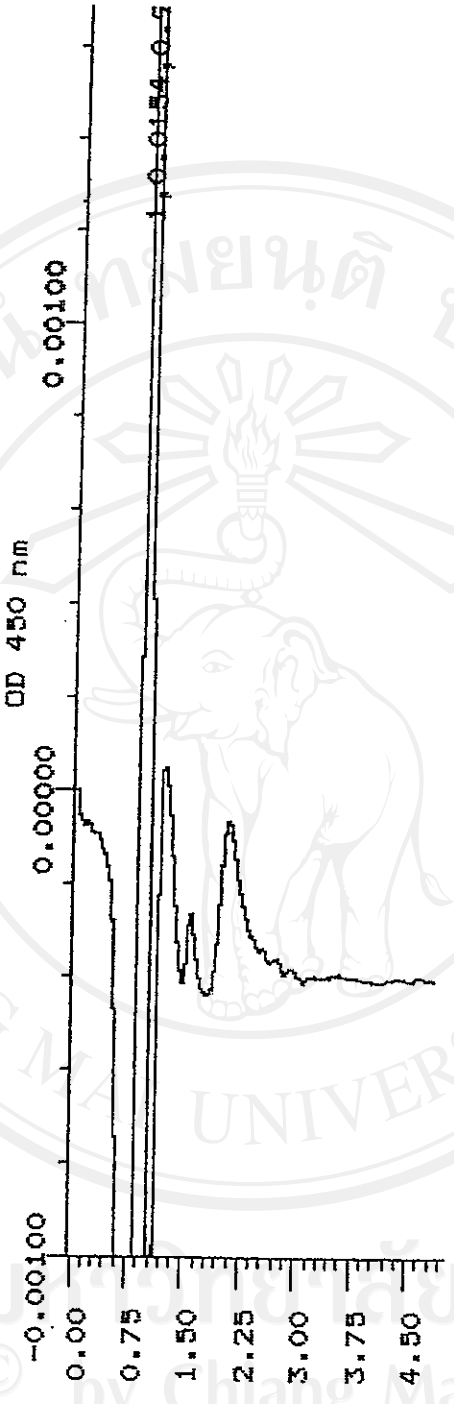
As mentioned before, NTBI is detectable when plasma transferrin is fully or nearly saturated (Durken, *et al* 1997, Porter, *et al* 1996). It was found in this study that NTBI was detectable in β-thalassemia plasma (Figure 3.3B) but not in normal volunteer plasma (Figure 3.3A). However, NTBI was not present in the plasma of some ESRD patients (Figure 3.3C) and it was detectable in the plasma of other ESRD patients (Figure 3.3D). The NTBI peak came out from the column and appeared at the retention time of 2.30 minutes (Figure 3.3C). It is probable that their plasma transferrin of NTBI-positive patients could not be available to bind the released or absorbed iron. Hence, unbound iron will bind to other ligands such as citrate, phosphate, some amino acids and plasma proteins especially albumin.



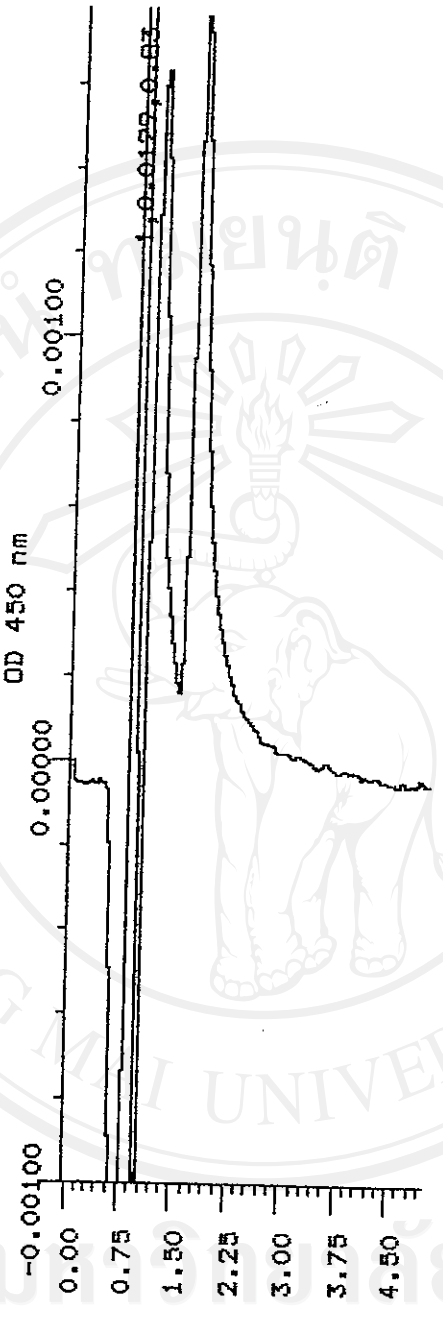
A) HPLC chromatograms of NTBI in plasma from normal volunteers



B) HPLC chromatograms of NTBI in plasma from β-thalassemia patients



C) HPLC chromatograms of NTBI in plasma from ESRD patients (low level)



D) HPLC chromatograms of NTBI in plasma from ESRD patients (high level)

Figure 3.3 HPLC chromatograms of NTBI in plasma from A) normal volunteers, B) β-thalassemia patients and C, D) ESRD patients.

We here have validated the method for measuring NTBI by using the NTA/HPLC technique (al-Refaie, *et al* 1992). The obtained calibration curve of NTBI was directly proportional to iron (as ferric nitrate) concentration and linear in the range of 0-16 μM with a correlation coefficient (R^2) of 0.98 (Figure 3.4). We are quite sure that the NTBI assay was reliable and accurate enough to measure the concentrations of NTBI in ESRD patients who had received regular hemodialysis.

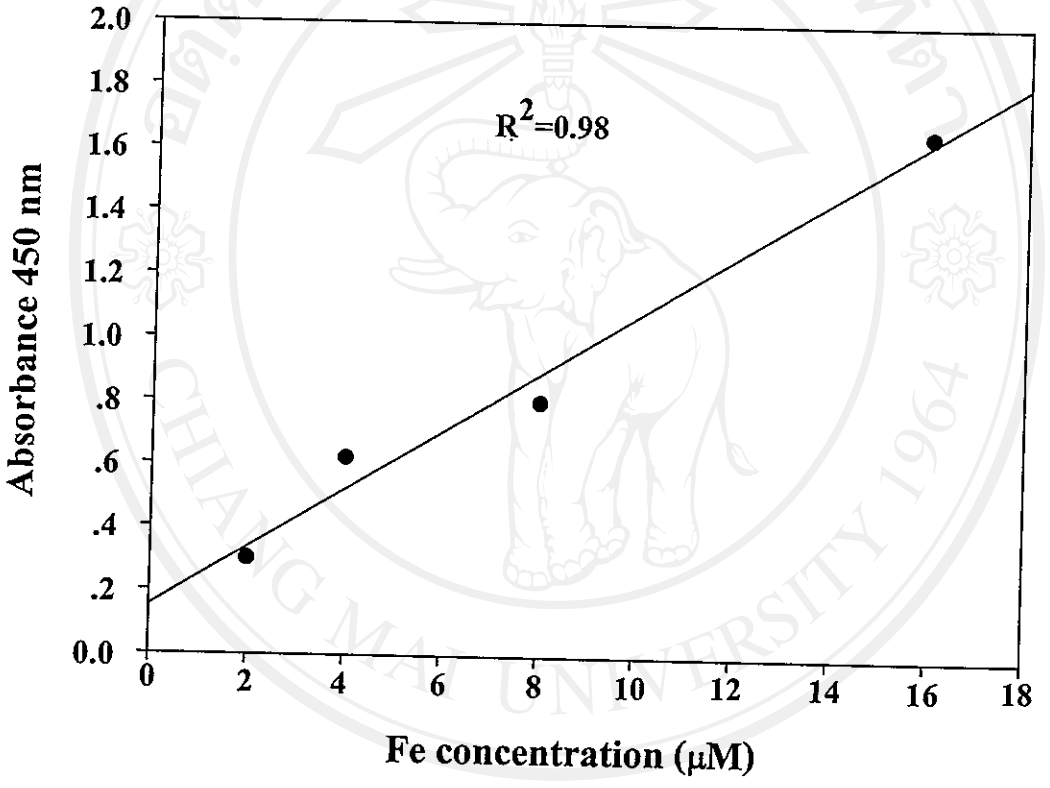


Figure 3.4 Representative calibration curve of NTBI assayed using the NTA chelation/HPLC technique.

3.3 Characteristics of ESRD patients

Patient history

One-hundred and ten ESRD patients on regular hemodialysis at the Kawila hospital, Nakomping hospital and Chankpeuk hospital in Chiang Mai were recruited in this study. One-hundred and three ESRD patients (55 male and 48 female) with an average age of 68±10 years (a range of 18-85 years) were received regular hemodialysis and subcutaneous injections of recombinant human erythropoietin (rHuEPO). Their mean frequency of hemodialysis was 150±129 (a range of 10-738). The rest seven ESRD patients with an average age of 58±12 years (a range of 39-68 years) had received regular hemodialysis (115±50) and intermittent blood transfusion (5±6 transfusions). Six patients (4 male and 2 female) were from Nakomping hospital, one male patient was from Changpeuk hospital (Table3.2). Eleven normal volunteers (4 male, 7 female) were healthy graduate students in Chiang Mai University and have never been received any hemodialysis, blood transfusion and subcutaneous erythropoietin injection. Their average age was 23±2 years (a range of 21-24 years).

Table 3.2 Clinical history of normal volunteers and ESRD patients on regular hemodialysis. These ESRD patients had received intermittent blood transfusion (ESRD-T) or subcutaneous rHuEPO injection (ESRD-E). The numbers of hemodialysis and blood transfusions are expressed as mean±SD.

Subjects	No	Gender	Age (years)	Hemodialysis (times)	Blood transfusion (times)	rHuEPO (times)
Normal volunteers	11	4M, 7F	23±2	No	No	No
ESRD-T patients	7	4M, 3F	58±12	120±47	6.4±6.5	No
ESRD-E patients	103	55M, 48F	68±10	150±129	No	Yes

Because the ESRD patients are usually insufficient erythropoietin production by themselves and sometimes are anemic, effective regimens are used to raise their blood hemoglobin concentration. The ESRD-T patients had received blood transfusions to maintain the normal hemoglobin level and the ESRD-E patients had been administered subcutaneous rHuEPO injection to stimulate the hemoglobin synthesis. However, all of them also had oral iron tablets (e.g. ferrous sulfate, FBC, Ferli-6) daily to supply for hemoglobin synthesis. ESRD-T patients (58±12 years) were younger than ESRD-E patients (68±10 years), but it was not significantly different.

Hematological parameters

Previous evidence has shown that most of ESRD patients who are treated with regular hemodialysis were hypochromic and few ESRD patients were normochromic (Maiz, *et al* 2002). In Table 3.3, it was found that the average hemoglobin concentration of ESRD patients (ESRD-T= 7.8±2.2 g/dl, ESRD-E=9.4±1.8 g/dl) was clearly lower than that of normal volunteers (14.2±1.5 g/dl). The average hematocrit value of ESRD-T patients (26.7±6.1%) and ESRD-E patients (32.5±5.2%) was also lower than that of normal volunteers (43.1±5.2%). The data indicated that they were anemic. Injection of erythropoietin could be potential to stimulate the hemopoiesis; therefore, hemoglobin and hematocrit level in ESRD-E patients was higher than those in ESRD-T patients. The persisting ROS (FI without hydrogen peroxide stimulation) in red blood cells of ESRD-T patients (22.3±38.8) and in red blood cells of ESRD-E patients (25.3±22.2) was not different from that of normal volunteers (30.4±19.7). Anyway, the amount of ROS (FI* with hydrogen peroxide stimulation) was higher in ESRD-E red blood cells (422.9±510.4) than in ESRD-T red blood cells (177±45.4) and normal volunteer red blood cells (227.1±119.1).

Table 3.3 Hematological parameters of normal volunteers and ESRD patients on regular hemodialysis. The ESRD patients have received intermittent blood transfusion (ESRD-T) or subcutaneous erythropoietin injection (ESRD-E). The values of hemoglobin concentration, hematocrit and ROS (expressed as fluorescence intensity, FI) are expressed as mean±SD.

Subjects	No	Hemoglobin (g/dl)	Hematocrit (%)	FI	FI*
Normal volunteers	11	14.3±1.5	43.1±5.2	30.4±19.7	227.1±119.1
ESRD-T patients	7	7.8±2	26.7±6.1	22.3±38.8	177±45.4
ESRD-E patients	103	9.4±1.8	32.5±5.2	25.3±22.2	422.9±510.4

Abbreviation: FI= fluorescence intensity, FI*=fluorescence intensity with hydrogen peroxide stimulation.

Biochemical parameters

The concentrations of BUN were measured to confirm the renal function of study population and the results showed BUN concentrations were 11.9±4.0, 28.2±8 and 50.2+19.2 mg/dl in normal volunteers, ESRD-T patients and ESRD-E patients respectively (Table 3.4). This meant that BUN levels were higher in ESRD patients than in normal volunteers. Indirect measures were recommended by International Committee for Standardization in Hematatology (ICSH, 1978) and used for evaluating iron status including transferrin saturation, plasma iron and total iron-binding capacity (TIBC) of transferrin. These parameters usually correlate with hemoglobin levels during iron therapy and are useful for determining iron status and monitoring the effectiveness of iron supplementation. This study showed that all ESRD patients who had been treated with regular hemodialysis demonstrated a variety of iron levels including iron deficiency, normal iron level and iron overload. PI and TIBC levels in ESRD-T patients were 15.6±5.7 μM and 28.2±9.1 μM respectively, and the PI and TIBC levels in ESRD-E patients were 17.1±8.8 μM and 35.1±18 μM respectively. Expectedly, the levels of PI and TIBC in both ESRD-T and ESRD-E patients were insignificantly lower than those in normal volunteers which their PI was 20.9±8.6 μM and TIBC was 54.7±14.4 μM respectively. It was found that transferrin saturation was 71.7±42.4% in ESRD-T patients and 57.7±35.5% in ESRD-E patients as compare to, 38.7±14.9 % in normal volunteers. Because the transferrin in plasma of normal volunteers was one-third saturated, plasma NTBI therefore was below zero

or undetectable (-0.69±1.29 μM), consistent to previous report (Porter et al., 1996). In this study, NTBI levels were 6.1±4 μM in plasma of ESRD-T patients and 1.76±2.2 μM in plasma of ESRD-E patients. Considerably, NTBI values in both ESRD patients were very significantly higher than those in normal volunteers (p<0.0001). Previous evidence has shown that oxidative stress could occur following regular hemodialysis, iron administration and blood transfusion. Malondialdehyde (MDA), one of important lipid peroxidation indicators, was measured in the plasma of ESRD patients to investigate whether they were under oxidative stress or not. It was shown that MDA level was the highest in ESRD-T patients (8.6±6.0 μM) (a range of 1.5-16.9 μM) and it was comparable in between ESRD-E (6.8±4 μM) (a range of 1.9-26.7 μM) and normal volunteers (7±2.2 μM) (a range of 5.3-13.1 μM) (Table 3.4).

Table 3.4 Biochemical parameters of normal volunteers and ESRD patients on regular hemodialysis. The ESRD patients received intermittent blood transfusion (ESRD-T) or subcutaneous erythropoietin injection (ESRD-E).

Subjects	No	BUN (mg/dl)	PI (μM)	TIBC (μM)	Transferrin Saturation (%)	NTBI (μM)	MDA (μM)
Normal volunteer	11	12±4.2	21.1±8.9	56±14.4	38±15.5	-0.78±1.31	7.2±2.2
ESRD-T patients	7	31±10	17.3±6.8	29.7±9.2	71.6±38.7	6.3±3.7	8.6±6
ESRD-E patients	103	50.2±19.2	17.1±8.8	35.1±18	57.7±35.5	1.76±2.2	6.8±4

3.4 Oxidative stress in ESRD'S RBCs

The result shown in Figure 3.5 A and Figure 3.5 B compared the ROS production in the red blood cells of ESRD patients (both ESRD-T and ESRD-E) with that in normal volunteers by using scatter plot. In the condition without H_2O_2 stimulation, the fluorescence intensity in red blood cells from normal volunteers (30.4±19.7), from ESRD-T (22.3±33.8) and from ESRD-E (25.3±22.2) were similar. However, in the presence of H_2O_2, the fluorescence intensity of ESRD-E patients (422.9±510.4) was the higher than that of ESRD-T patients (177±45.4) and normal volunteers (227.1±119.1) (Figure 3.3A, 3.3B). Regarding to red blood cells of normal volunteers, FI was approximately 7-fold higher in H_2O_2-stimulated cells than in unstimulated cells. Considering red blood cells of ESRD patients, FI was approximately 8-fold higher in H_2O_2-stimulated ESRD-T cells and 17-fold higher in H_2O_2-stimulated ESRD-E cells than in unstimulated cells respectively.

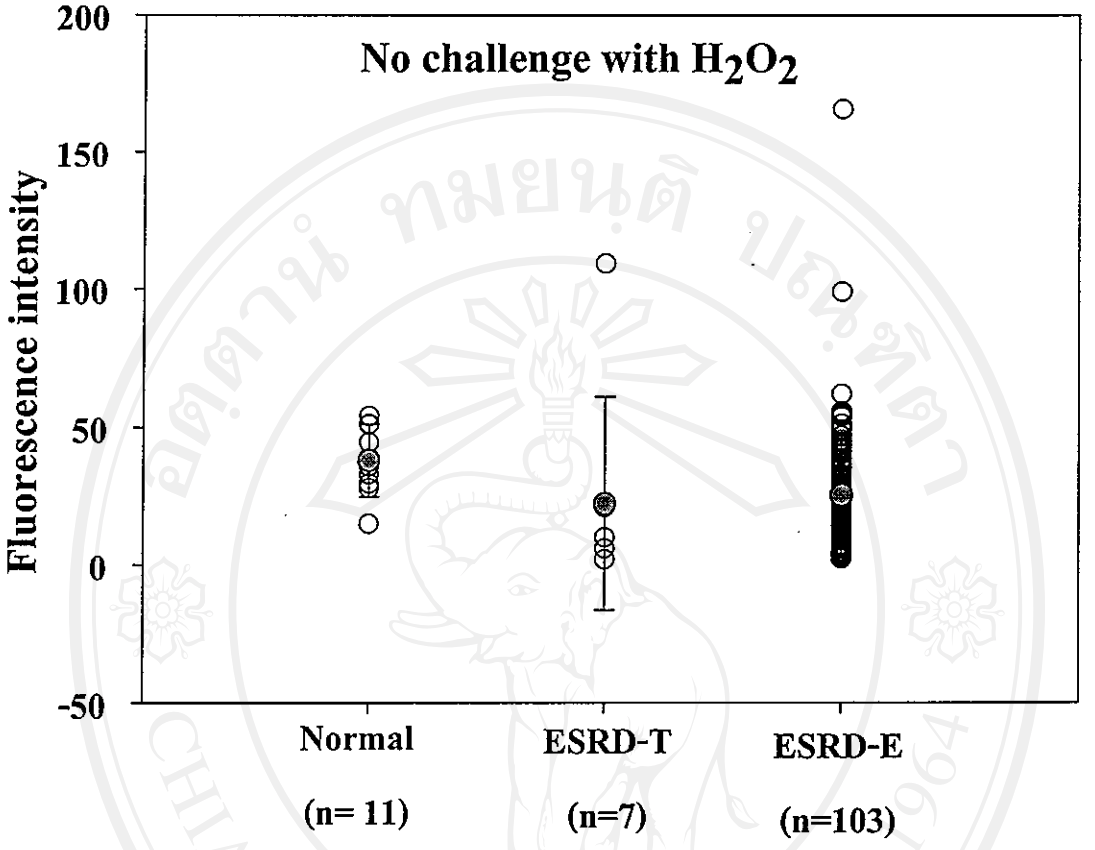


Figure 3.5A Distribution of oxidative stress expressed as fluorescence intensity in erythrocytes from normal volunteers, and end-stage renal disease patients on hemodialysis who received blood transfusion (ESRD-T) and erythropoietin injection (ESRD-E).

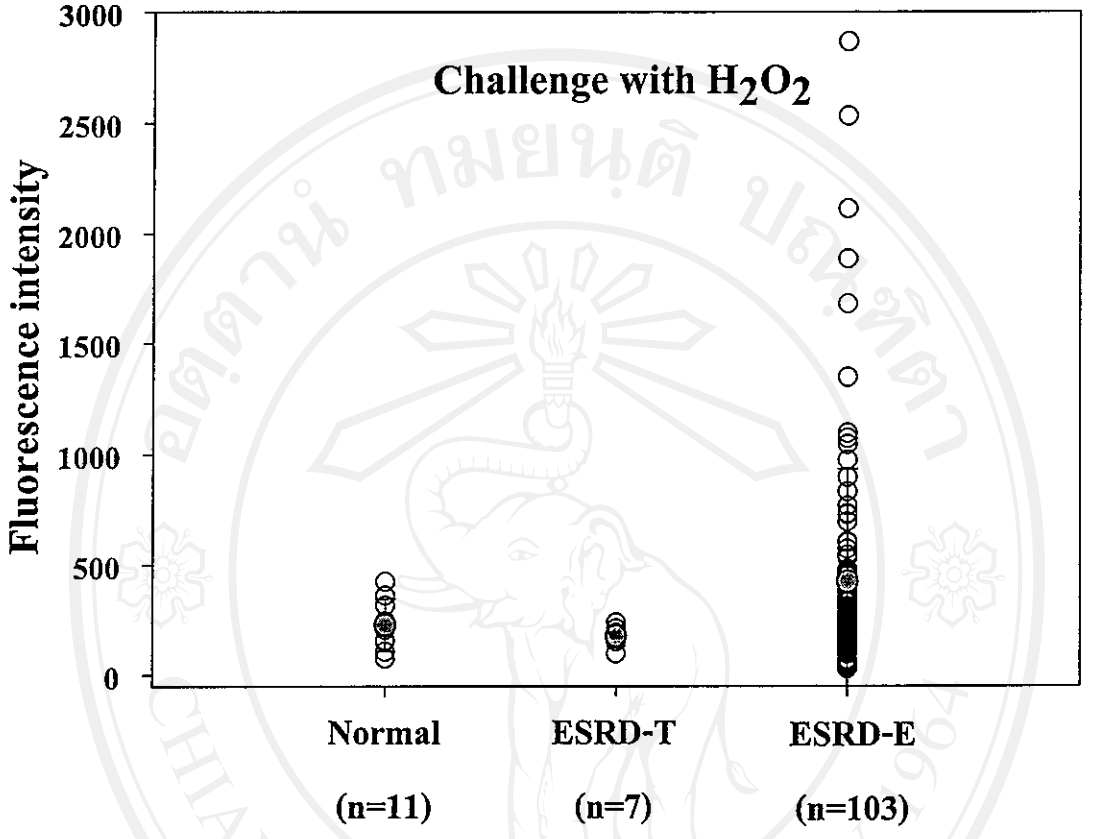


Figure 3.5B Distribution of oxidative stress expressed as fluorescence intensity with H_2O_2 stimulation in erythrocytes from normal volunteers, and end-stage renal disease patients on hemodialysis who received blood transfusion (ESRD-T) and erythropoietin injection (ESRD-E).

3.5 Correlation between parameters of iron overload and oxidative stress in blood of ESRD patients

We determined the correlation between iron status (PI concentration, transferrin saturation and plasma NTBI concentration) and degree of oxidative stress (red blood cell ROS and plasma MDA). It was found that PI level was not correlated with the amount of ROS in ESRD red blood cells without and with hydrogen peroxide stimulation. Similarly, plasma NTBI concentration was not correlated with the amount of ROS in ESRD red blood cells. In addition, the concentration of plasma MDA was not correlated with red blood cell ROS level (data not shown).

3.6 Effect of curcumin on oxidative stress in whole blood

To study the effect of curcumin on ROS scavenging, the oxidative stress in whole blood of seven ESRD patients was determined by using the flow cytometric technique. In stead of using red cell suspension, whole blood was preincubated with curcumin at room temperature for 1 hour. Afterwards, blood sample was duplicated; one was added Fe-EDTA, the other one was added Fe-EDTA and hydrogen peroxide. Finally, red blood cell ROS level was measured and the result was shown in Table 3.5, Figure 3.6A, 3.6B, 3.7A and 3.7B. Without challenge with hydrogen peroxide, curcumin at 20 and 100 μM did not affect or decrease the ROS level in ESRD red blood cells (FI = 35±6, and 34±7 respectively) when compared with control PBS-treated red blood cells (FI =38±10). Similarly, 20 and100 μM curcumin, in the presence of 300 μM Fe-EDTA, did not decrease or affect the ROS levels in ESRD red blood cells (FI = 41±14 and 40±18 respectively) when compared with control cells. With hydrogen peroxide challenge (Figure 3.7A and 3.7B), curcumin at 20 μM did not decrease the ROS level in ESRD red blood cells (FI =41±15); in contrast, curcumin at 100 μM decreased the ROS level in ESRD red blood cells (FI = 27±6) when compared with control PBS-treated red blood cells (FI = 41±15). In the presence of Fe-EDTA, curcumin at 20, 100 μM slightly decreased the ROS level in ESRD red blood cells (FI = 36±14 and 32±11 respectively) when compared with control cells. Nevertheless, Fe-EDTA in the presence and absence of hydrogen peroxide, did not enhance the ROS formation in red blood cells from whole blood (Figure 3.6A and 3.7A). As a result, Fe-EDTA and hydrogen peroxide treatment did not stimulate or increase the ROS formation in whole blood markedly, probably there may be powerful antioxidants such as albumin, uric acid, creatinine and bilirubin in plasma compartment.

Table 3.5 Determination of oxidative stress in whole blood of seven ESRD patients were treated in the presence and absence of hydrogen peroxide with PBS (as a control), curcumin, Fe^{2+}-EDTA and curcumin coincubated with Fe^{2+}-EDTA. Data are shown as mean±SEM of and % of control of FI.

Effectors	No challenge with H_2O_2		Challenge with H_2O_2	
	Fluorescence intensity	% of control	Fluorescence intensity	% of control
PBS	38±10	100	41±15	100
20 μM Curcumin	35±6	77±9	41±15	102±15
100 μM Curcumin	34±7	103±14	27±6	112±27
PBS+300 μM Fe-EDTA	33±7	111±33	37±10	111±17
20 μM Curcumin+300 μM Fe-EDTA	41±14	135±55	36±14	81±12
100 μM Curcumin +300 μM Fe-EDTA	40±18	95±16	32±11	92±20

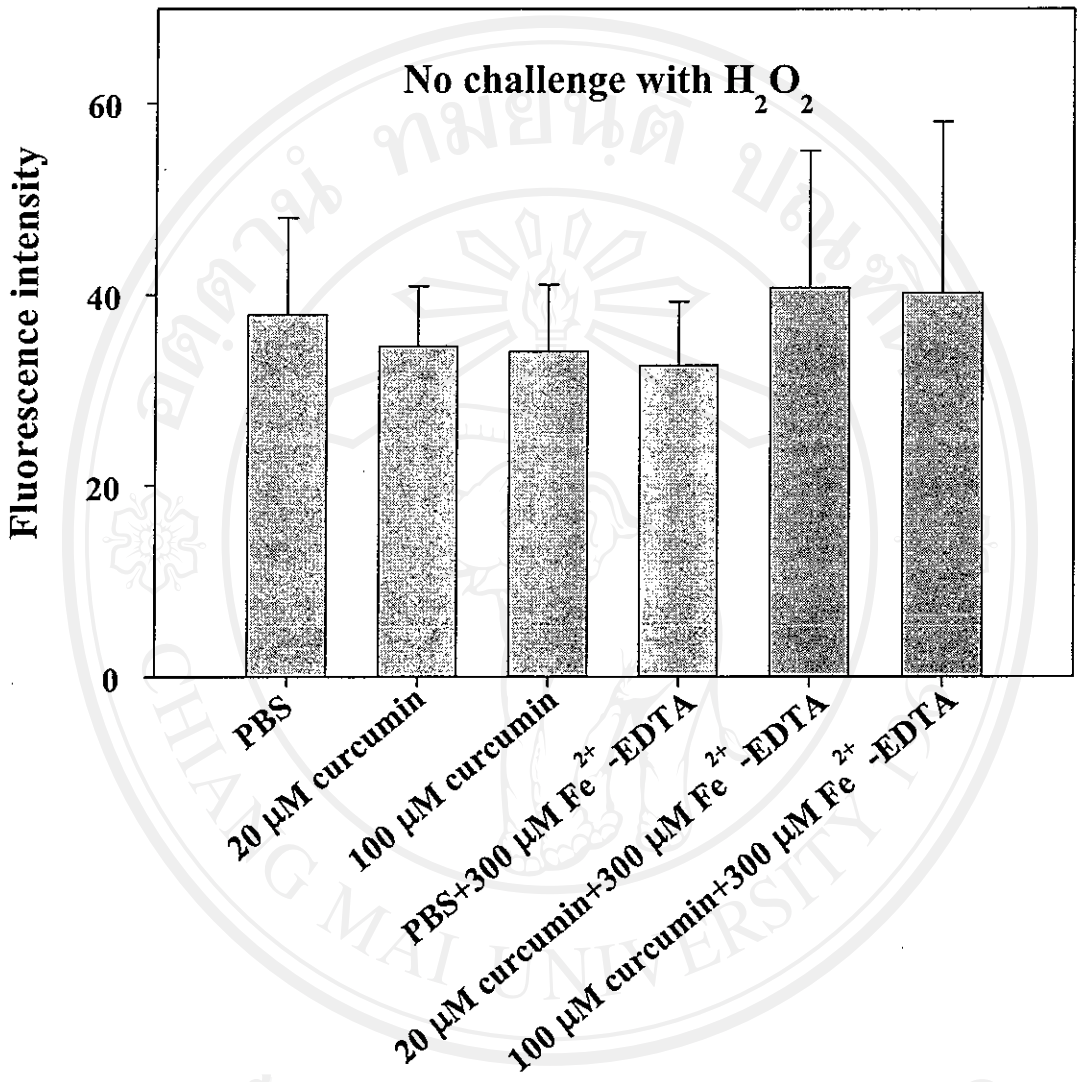


Figure 3.6A Determination of oxidative stress (represented as fluorescence intensity, FI) in whole blood of seven ESRD patients were treated with PBS (as a control), curcumin, Fe^{2+}-EDTA and curcumin coincubated with Fe^{2+}-EDTA. Data are shown as mean±SEM of FI.

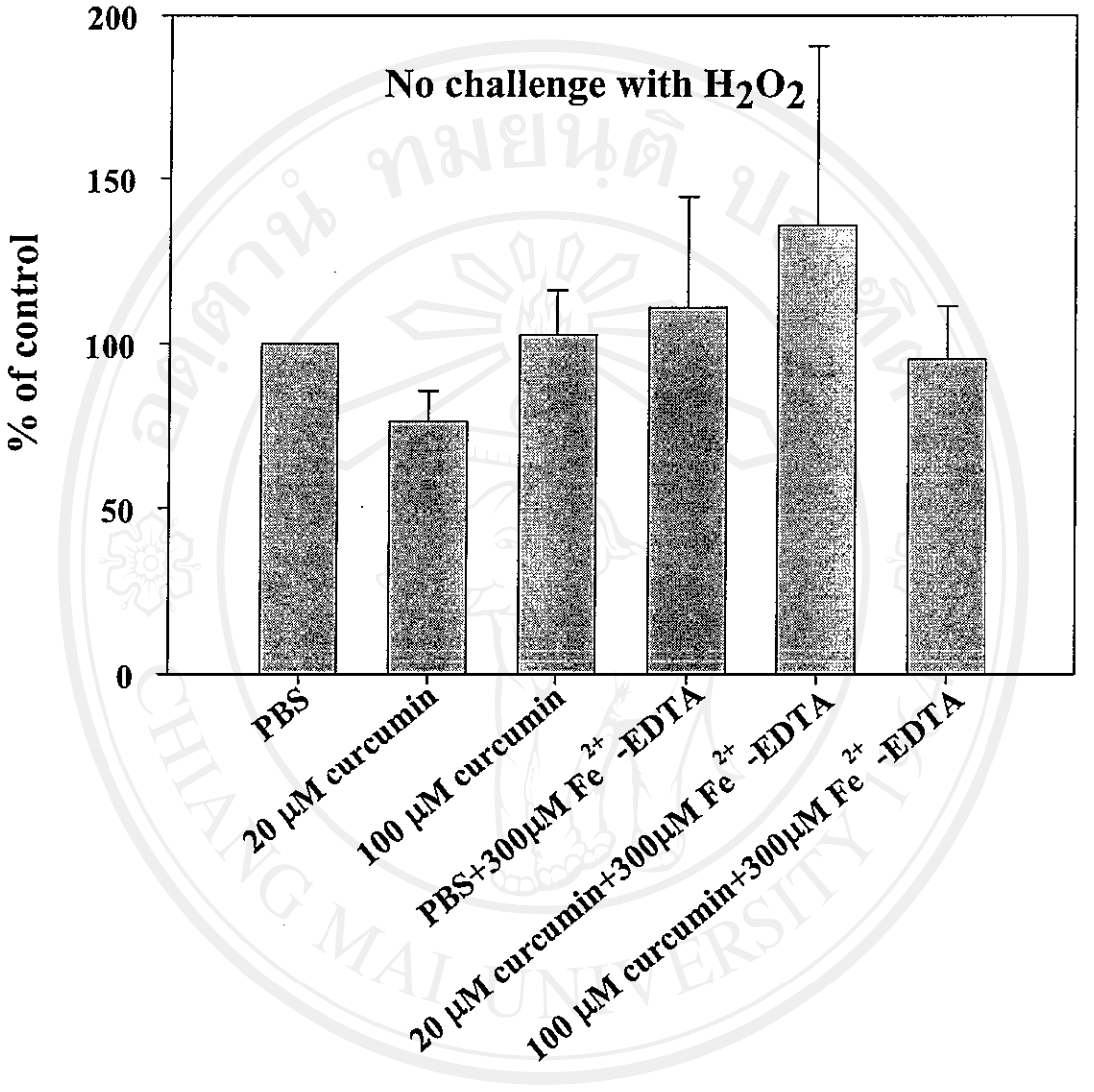


Figure 3.6B Determination of oxidative stress (represented as fluorescence intensity, FI) in whole blood of seven ESRD patients treated with PBS (as a control), curcumin, Fe^{2+}-EDTA and curcumin coincubated with Fe^{2+}-EDTA. Data are shown as %control of FI.

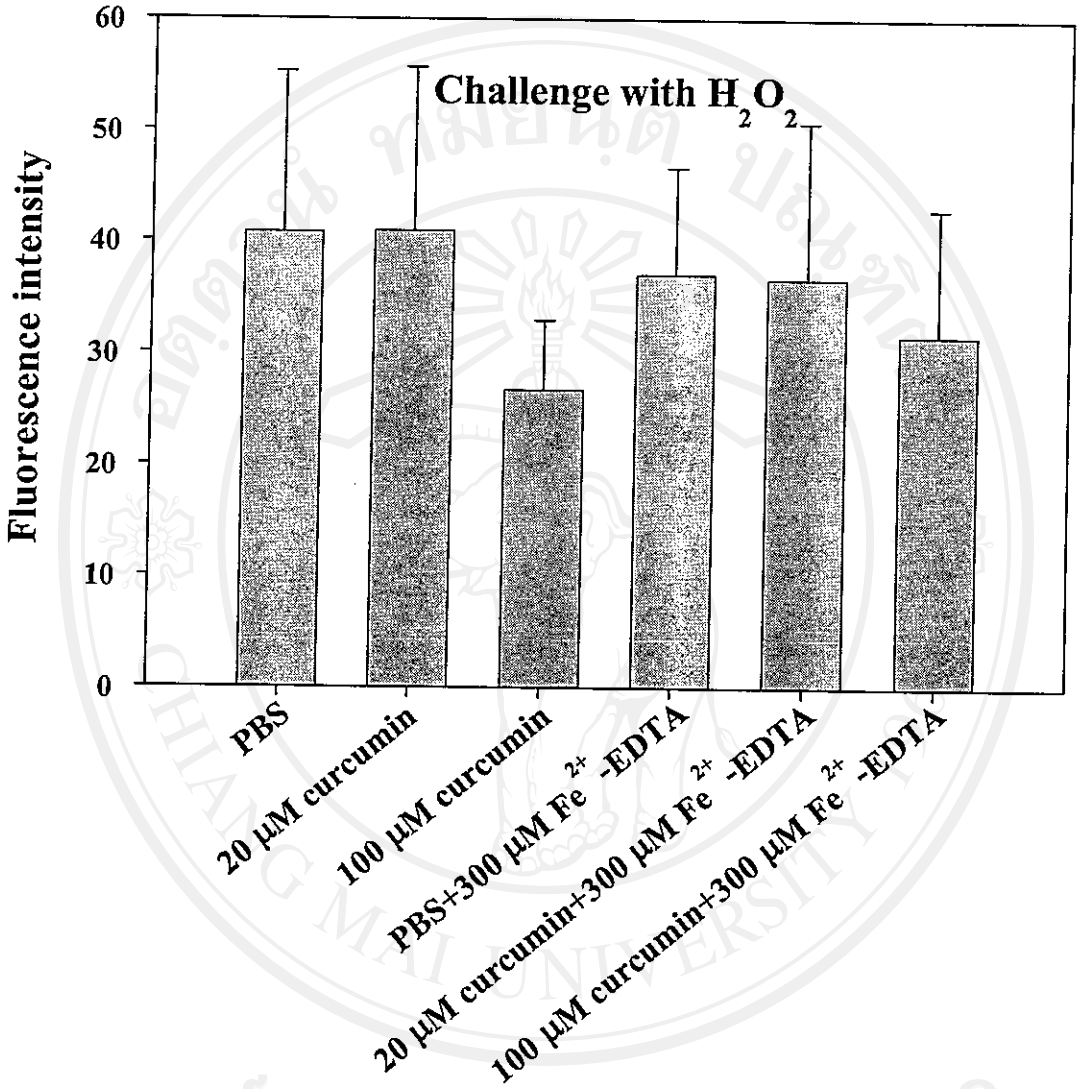


Figure 3.7A Determination of oxidative stress (represented as fluorescence intensity, FI) in whole blood of seven ESRD patients were treated with PBS (as a control), curcumin, Fe^{2+}-EDTA and curcumin coincubated with Fe^{2+}-EDTA. Data are shown as mean±SEM of FI.

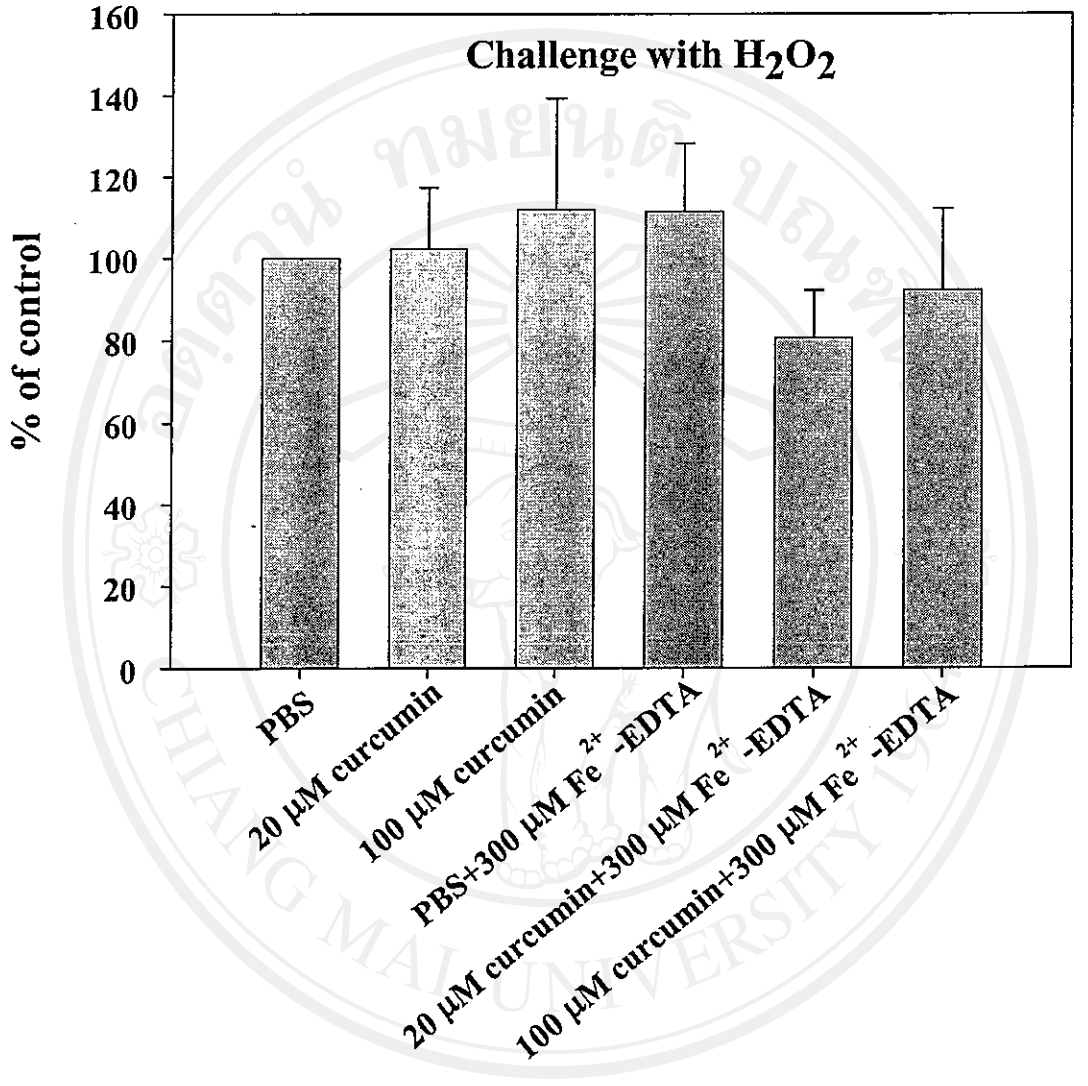


Figure 3.7B Determination of oxidative stress (represented as fluorescence intensity, FI) in whole blood of seven ESRD patients treated in the presence of hydrogen peroxide with PBS (as a control), curcumin, Fe^{2+}-EDTA and curcumin coincubated with Fe^{2+}-EDTA. Data are shown as % of control.

3.7 Effect of curcumin on oxidative stress in RBC suspension

To study the effect of curcumin on ROS scavenging, the oxidative stress in RBC suspension of different seven ESRD patients was determined by flow cytometry. RBC of ESRD patients was incubated with DCFH-DA for 15 minutes and curcumin was added. Later, treated RBC was incubated with Fe-EDTA in the absence and presence of hydrogen peroxide and finally measured using the flow cytometry. The results were shown in Table 3.6, Figure 3.8A, 3.8B, 3.9A and 3.9B. Without hydrogen peroxide challenge (Figure 3.8A and 3.8 B), curcumin in the presence and absence of Fe-EDTA did not affect the red blood cell fluorescence intensity. With hydrogen peroxide challenge (Figure 3.9A and 3.9B), curcumin at 100 μM in the presence and absence of Fe-EDTA was almost significantly decreased red blood cell fluorescence intensity when compared with control (p=0.05). In contrast, curcumin at 20 μM in the presence and absence of Fe-EDTA increased red blood cell fluorescence intensity slightly.

Table 3.6 Determination of oxidative stress in red blood cell suspension of different seven ESRD patients treated with PBS (as a control), curcumin in the presence and absence of Fe-EDTA and hydrogen peroxide. Data are shown as mean±SEM of and % of control of FI.

Effectors	No challenge with H_2O_2		Challenge with H_2O_2	
	Fluorescence intensity	% of control	Fluorescence intensity	% of control
PBS	16±4	100	159±69	100
20 μM Curcumin	16±4	107±10	183±57	113±16
100 μM Curcumin	15±4	102±8	109±26	70±5
PBS+300 μM Fe-EDTA	18±6	113±15	205±48	128±10
20 μM Curcumin+300 μM Fe-EDTA	18±6	116±13	293±72	152±18
100 μM Curcumin +300 μM Fe-EDTA	15±4	101±9	153±36	101±9

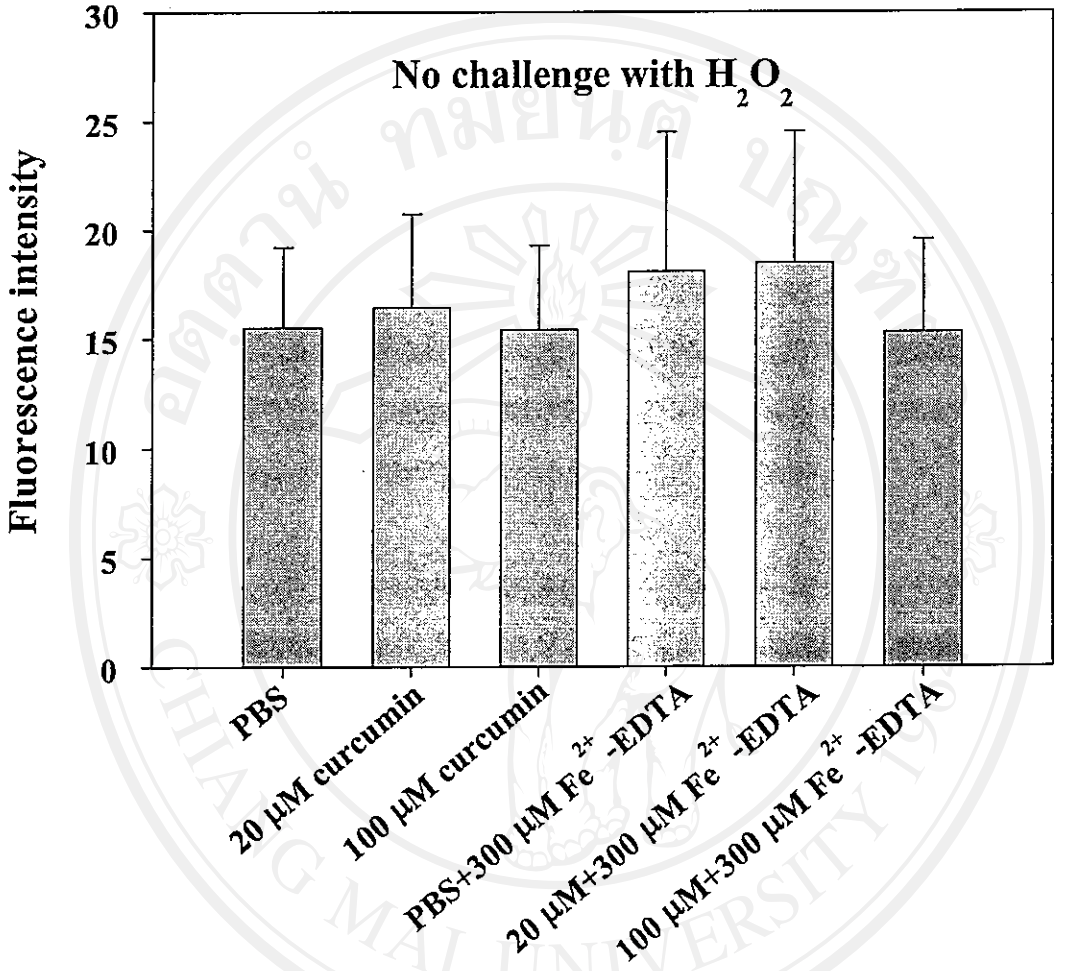


Figure 3.8A Determination of oxidative stress (represented as fluorescent intensity, FI) in red blood cell suspension of different seven ESRD patients treated with PBS (as a control), curcumin in the presence and absence of Fe-EDTA. Data are shown as mean±SEM of FI.

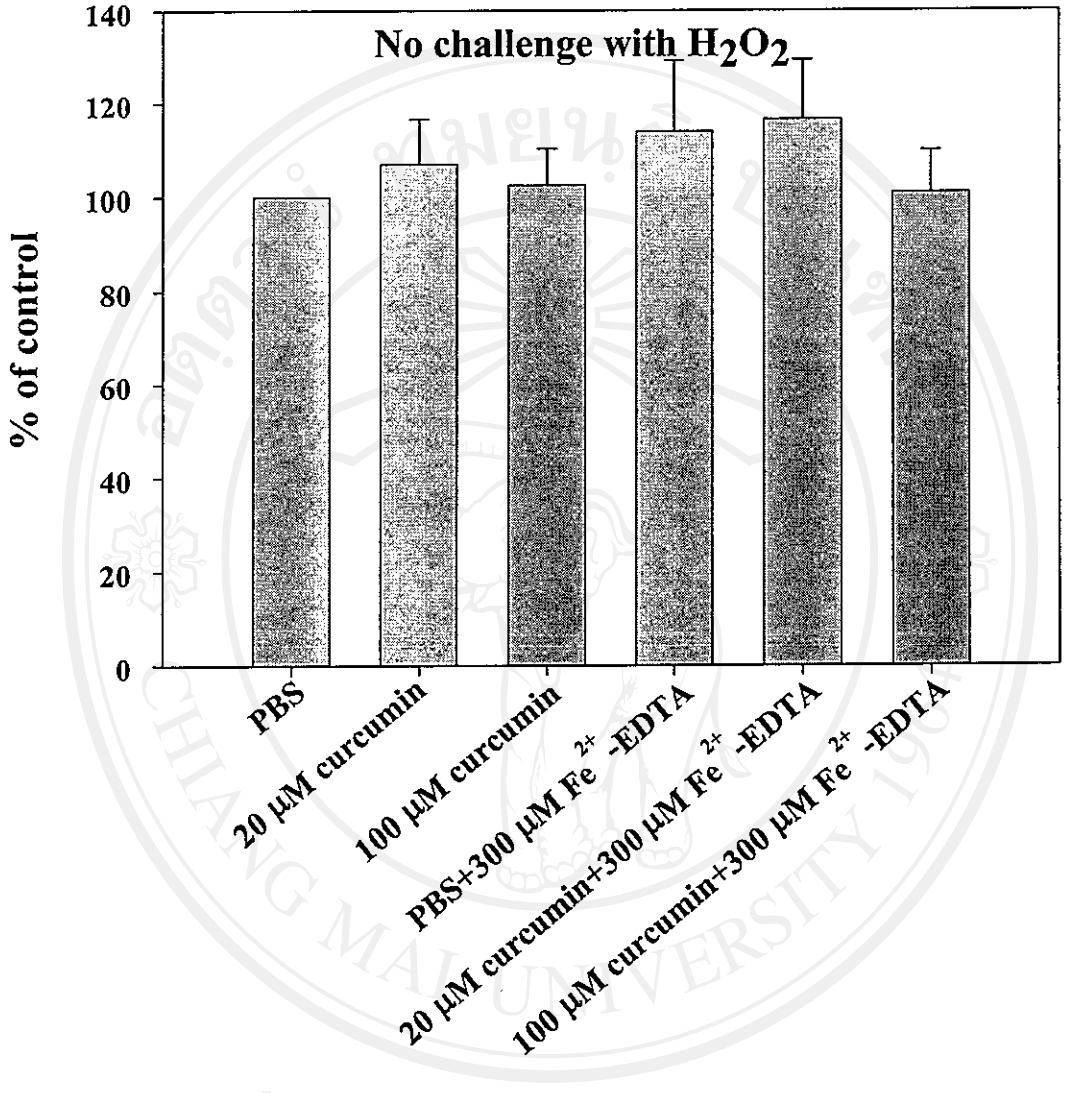


Figure 3.8 B Determination of oxidative stress (represented as fluorescent intensity, FI) in red blood cell suspension of different seven ESRD patients treated with PBS (as a control), curcumin in the presence and absence of Fe-EDTA. Data are shown as % of control.

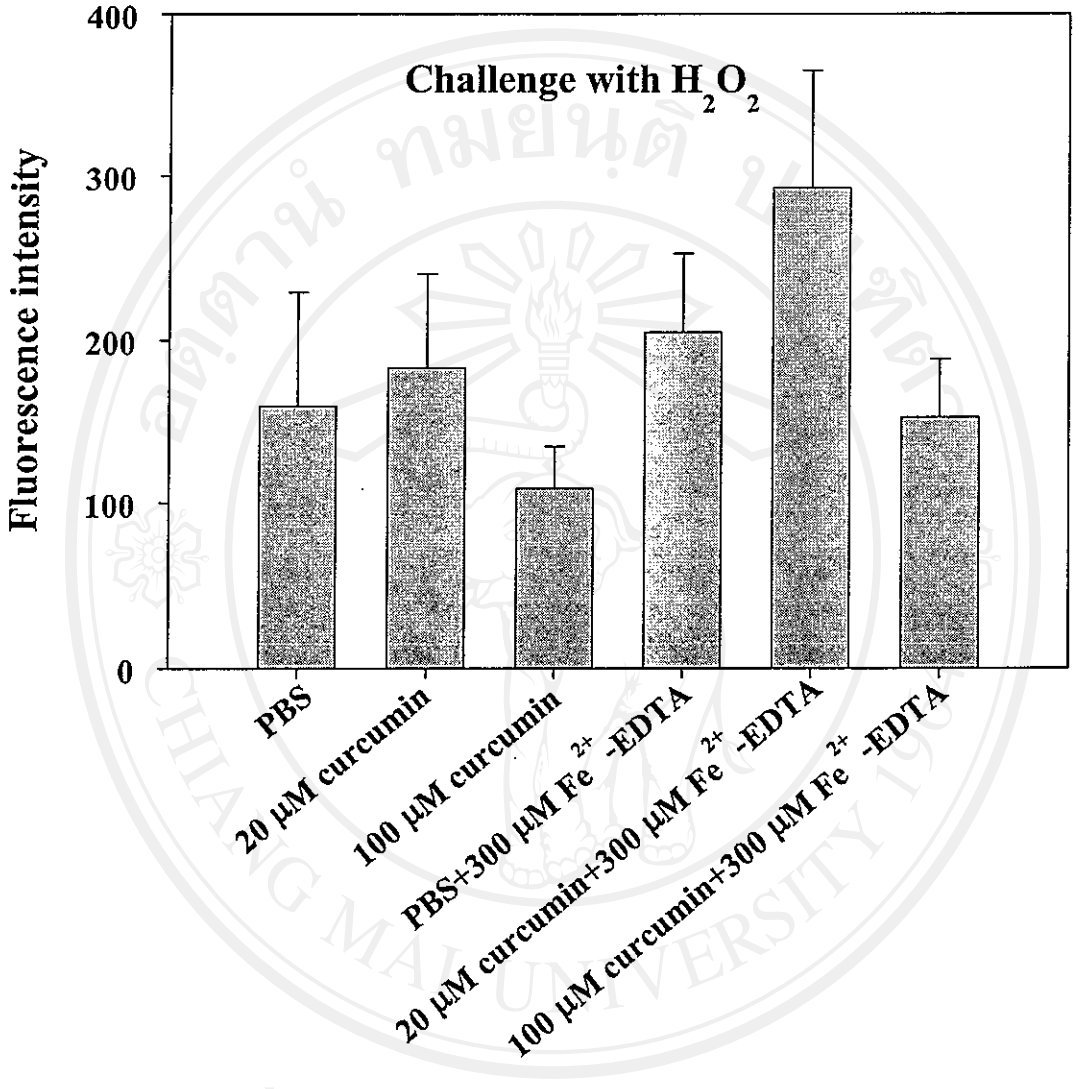


Figure 3.9A Determination of oxidative stress (represented as fluorescent intensity, FI) in red blood cell suspension of different seven ESRD patients treated with PBS (as a control), curcumin in the presence and absence of Fe-EDTA with hydrogen peroxide stimulation. Data are shown as mean±SEM of FI.

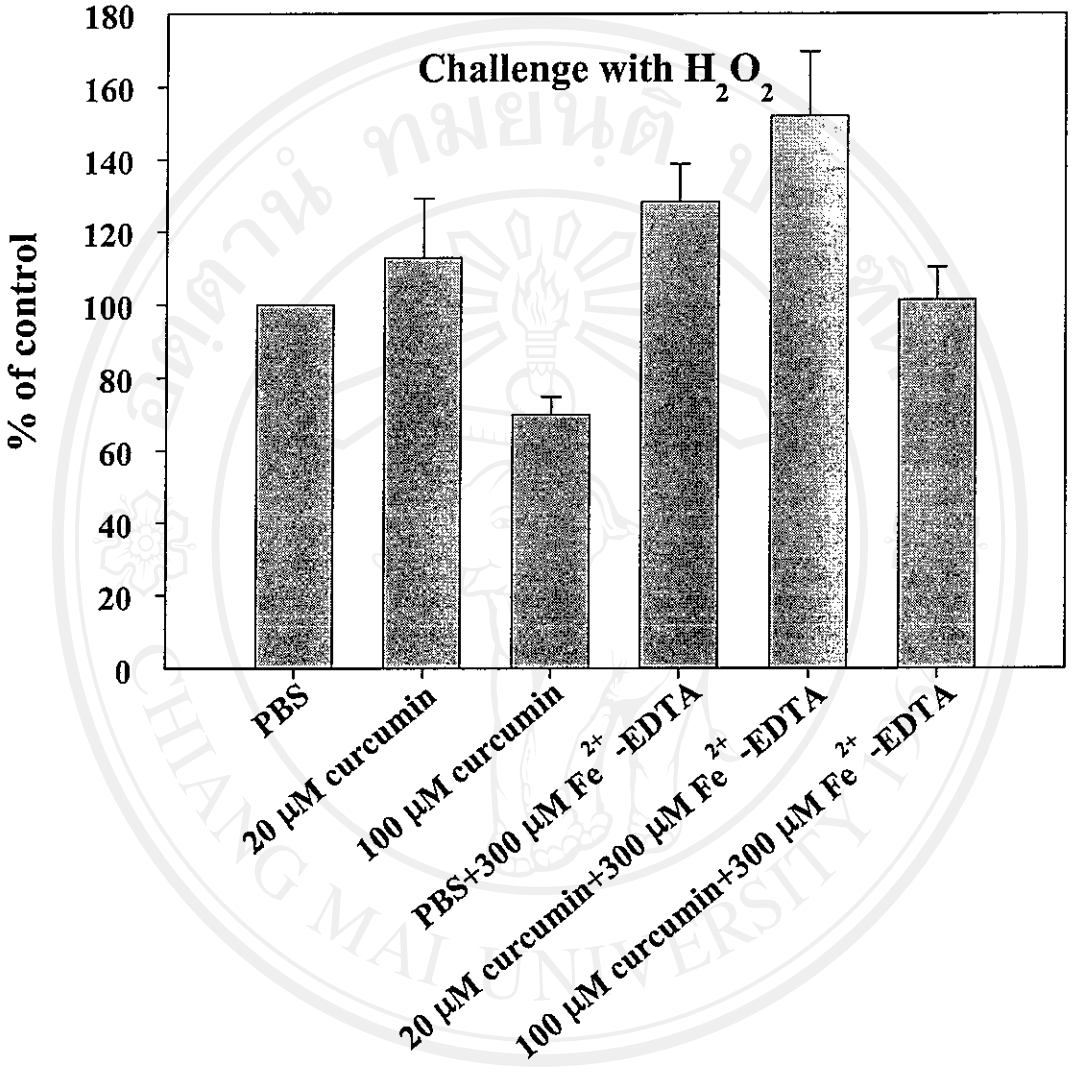


Figure 3.9B Determination of oxidative stress (represented as fluorescent intensity, FI) in red blood cell suspension of different seven ESRD patients treated with PBS (as a control), curcumin in the presence and absence of Fe-EDTA with hydrogen peroxide stimulation. Data are shown as % of control.

3.8 Effect of *in vivo* hemodialysis on plasma NTBI levels

When four ESRD patients were regularly hemodialysed for 4 hours using the hemodialysis machine with the flow rate of dialysate fluid at 500 ml/minute and blood flow at 250 ml/minute, plasma NTBI levels in all four patients were decreased from 5.77±1.24 μM to 3.59±0.62 μM insignificantly ($p = 0.63$) (Figure 3.10) (Table 3.7). Regarding to the NTBI difference between pre-hemodialysis and post-hemodialysis, the decrease of NTBI value was approximately 2.18±0.75 μM or 35.7±5.0%. This suggests that hemodialysis is potential to reduce plasma NTBI concentrations in these patients. Possibly, the low MW NTBI persisting in the plasma passed through dialysis membrane and diffused into the dialysate passively and gradually. Nevertheless, some of NTBI remained in the plasma after 4-hour hemodialysis, which was presumed as the high MW iron that loosely bound to other ligands such as plasma proteins (especially albumin), citrate, phosphate and some amino acids.

Table 3.7 Effect of *in vivo* hemodialysis for 4 hours on plasma NTBI levels in four ESRD patients. Data are shown as individual NTBI concentration and %decrease of NTBI.

	NTBI concentration (μM)		ΔNTBI (μM)	Decreased NTBI (%)
	Pre-hemodialysis	Post-hemodialysis		
ESRD1	3.80	1.80	2.00	52.6
ESRD2	5.54	3.69	1.85	33.4
ESRD3	8.70	4.35	4.35	50.0
ESRD4	6.15	4.50	1.65	26.8
Mean±SEM	6.05±1.01	3.59±0.62	2.46±0.6	40.71±6.3

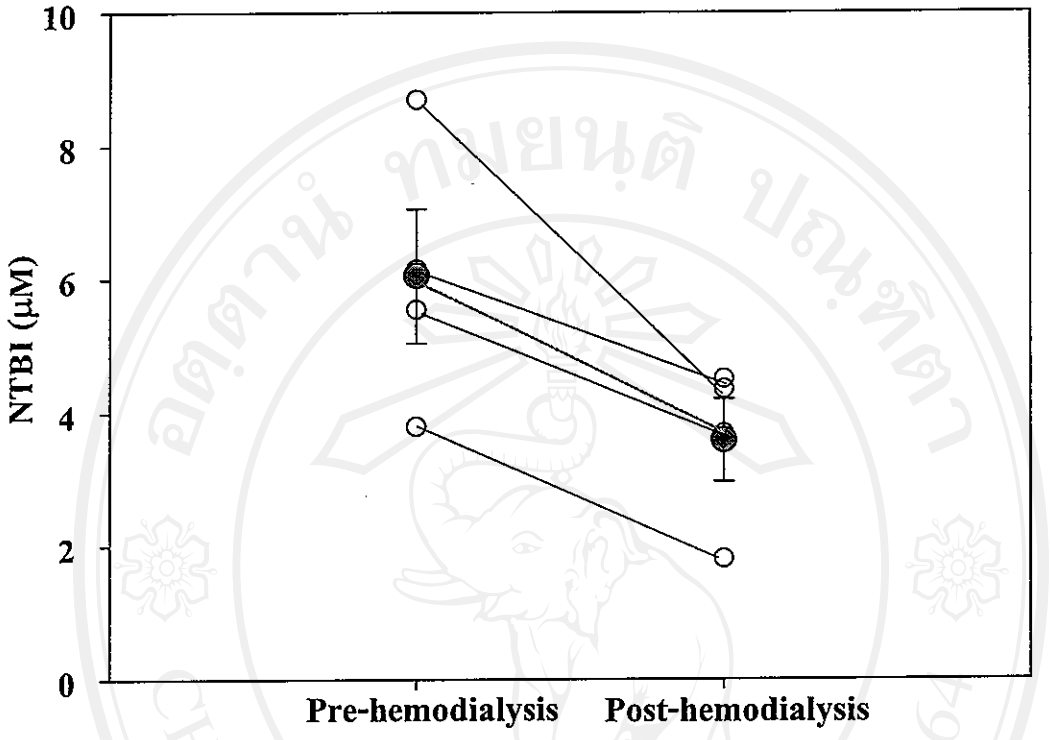


Figure 3.10 Effect of *in vivo* hemodialysis for 4 hours on NTBI levels in four ESRD patients. Data are shown as individual NTBI concentration (opened circle) and mean±SEM (closed circle).

3.9 Effect of *in vitro* hemodialysis on plasma NTBI levels

To study the effect of *in vitro* hemodialysis on plasma NTBI levels, the whole blood of four ESRD patients was collected into dialysis membrane and dialyzed in dialysate buffer for 4 hours. The plasma NTBI in the presence of PBS was decreased from 6.0±1.0 μM to 4.2±0.6 μM insignificantly (p=0.18) (Table 3.8 and Figure 3.11). In the presence of 100 μM L1, plasma NTBI was almost significantly decreased from 6.0±1.0 μM to 3.3±0.3 μM (p= 0.05). The result suggests that L1 could chelate the low MW NTBI fraction in plasma to form $Fe(L1)_3$ complex that was subsequently passed through dialysis membrane while the high MW NTBI fraction remained unchelated.

Table 3.8 Effect of *in vitro* hemodialysis for 4 hours on NTBI levels in whole blood of four ESRD patients (control) chelated with 100 μM L1 compared with PBS. Data are shown as individual NTBI concentration and % decrease of NTBI.

Patients	Control	PBS			100 μM L1		
	NTBI (μM)	NTBI (μM)	ΔNTBI (μM)	Decreased NTBI (%)	NTBI (μM)	ΔNTBI (μM)	Decreasd NTBI (%)
ESRD1	3.8	2.7	1.1	28.9	2.5	1.3	34.2
ESRD2	5.5	4.8	0.7	12.7	4.1	1.4	25.4
ESRD3	8.7	3.7	5.0	57.5	3.6	5.1	58.6
ESRD4	6.1	5.6	0.5	8.2	3.0	3.1	50.8
Mean±SEM	6.0±1.0	4.2±0.6	1.8±1.1	26.8±11.1	3.3±0.3	2.7±0.9	42.3±7.6

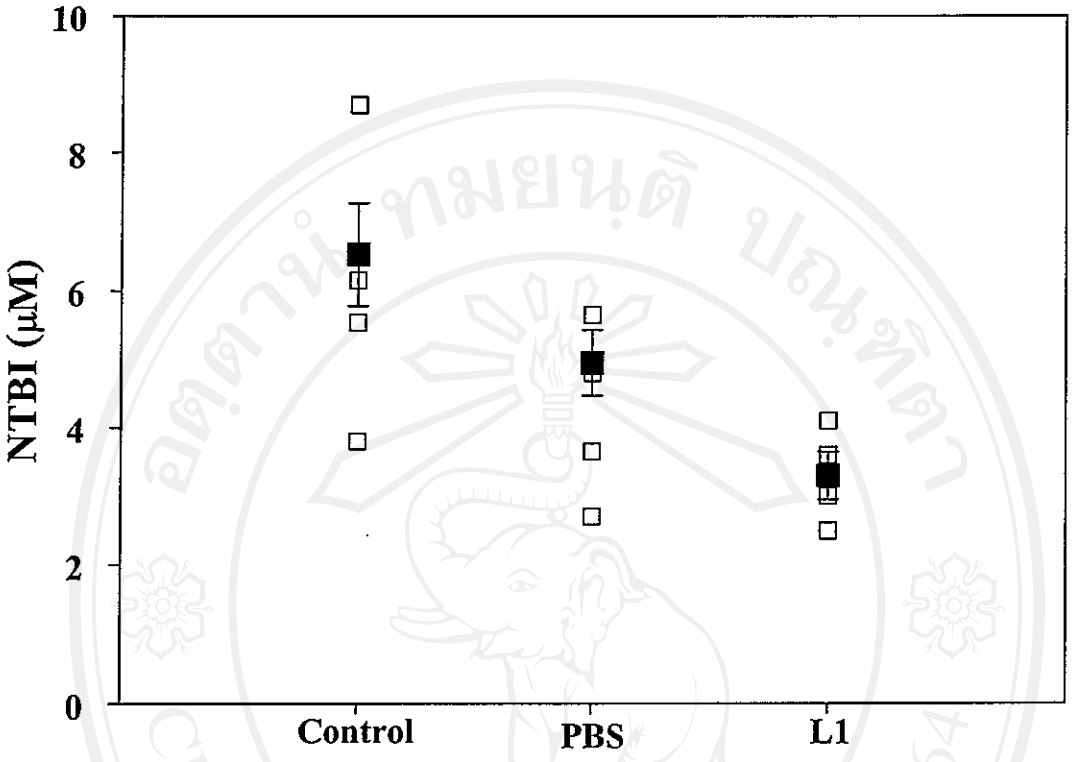


Figure 3.11 Effect of *in vitro* hemodialysis for 4 hours on NTBI levels in whole blood of four ESRD patients (control) that were chelated with 100 μM L1 compared with PBS. Data are shown as individual NTBI concentration (opened squared) and mean±SEM (closed squared).

3.10 Effect of curcumin and deferiprone to remove plasma NTBI during *in vitro* hemodialysis

To study the effect of curcumin and deferiprone to remove plasma NTBI during *in vitro* hemodialysis, whole blood of four ESRD patients was collected into dialysis membrane pre-incubated with 100 μM L1 and curcumin at different concentration and dialyzed in dialysate buffer solution for 4 hours. Chelation with the curcumin at 20 μM and 100 μM, in the presence of 100 μM L1, did not significantly decrease the plasma NTBI level from 11.5±1.9 μM to 9.0±0.4 μM (p=0.33) and to 9.5±0.6 μM (p=0.3), respectively (Table 3.9) (Figure 3.12). Curcumin at 800 μM and L1 at 100 μM decreased the plasma NTBI from 11.5±1.9 μM to 8.6±1.4 μM insignificantly (p=0.15). The result suggested that 800 μM curcumin together with 100 μM L1 was more effective than 20 μM and 100 μM curcumin to chelate NTBI from plasma of ESRD patients under hemodialysis process.

Table 3.9 Effect of curcumin and deferiprone on *in vitro* hemodialysis for 4 hours to remove NTBI levels in whole blood of four ESRD patients chelated with different curcumin concentrations in the presence of 100 μM L1. Data are shown as individual NTBI concentration and %decreased NTBI.

Patients	PBS	20μM curcumin+L1		100μM curcumin+L1		800 μMcurcumin+L1	
	NTBI (μM)	NTBI (μM)	Decreased NTBI (%)	NTBI (μM)	Decreased NTBI (%)	NTBI (μM)	Decreased NTBI (%)
ESRD 1	6.3	9.4	-49.92	8.8	-39.7	5	20.6
ESRD 2	12.7	9.1	28.19	10.4	18.11	9.7	23.6
ESRD 3	11.6	9.4	19.27	8.3	28.8	11.4	1.3
ESRD 4	15.2	7.9	47.83	10.7	29.5	8.4	44.8
Mean±SEM	11.5±1.9	9±0.4	11.3±21.2	9.5±0.6	9.17±16.5	8.6±1.4	22.6±8.9

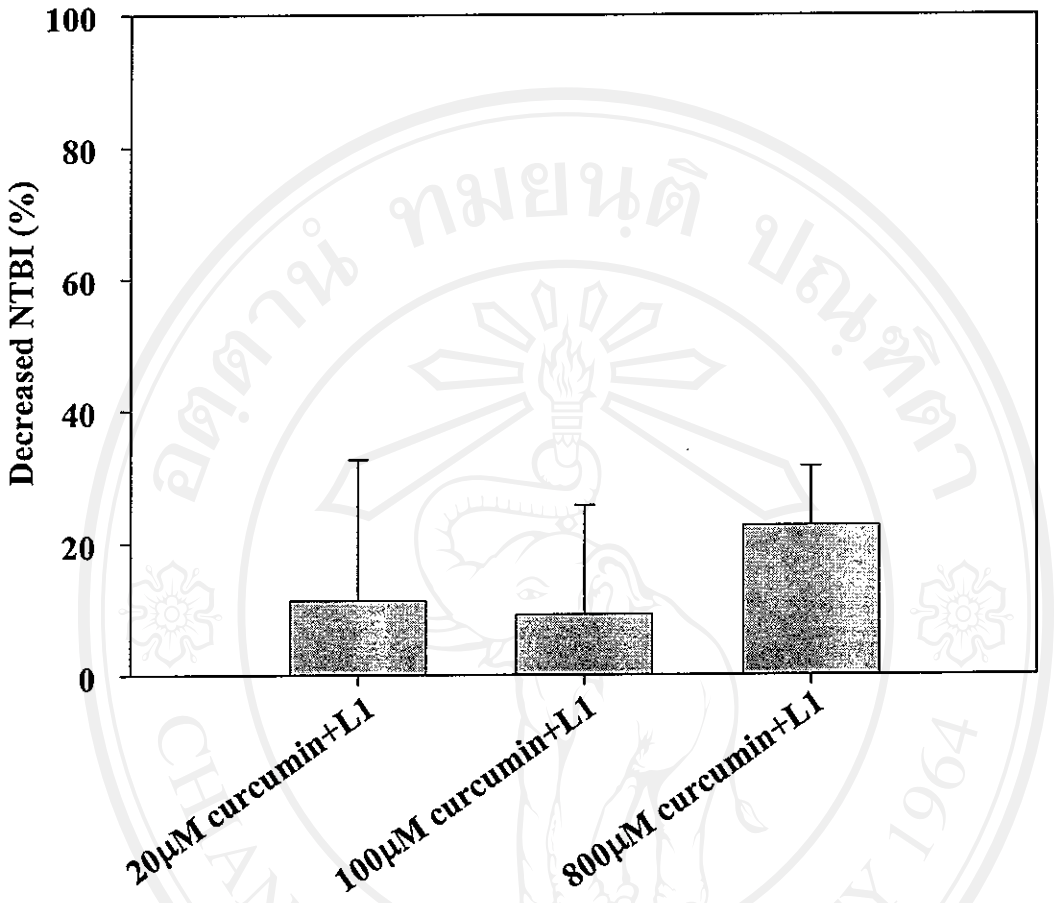


Figure 3.12 Effect of curcumin and deferiprone on *in vitro* hemodialysis for 4 hours to remove NTBI levels in whole blood of four ESRD patients chelated with different curcumin concentrations in the presence of 100 μM L1. Data are shown as mean values of %decreased NTBI ±SEM.

3.11 Iron-binding activity of curcumin *in vitro*

3.11.1 Spectrum analysis of binding of curcumin with ferric nitrate

Curcumin solution at a final concentration of 800 μM was incubated with ferric nitrate (0, 20, 40, 80 and 100 μM) at room temperature for 30 minutes. The absorbance of colored product was subsequently measured between 300-700 nm, which curcumin solution itself was used as a reagent blank. In Figure 3.13, it was found that curcumin was able to complex with ferric nitrate to form a colored curcumin-iron complex that gave a predominant peak at 500 nm.

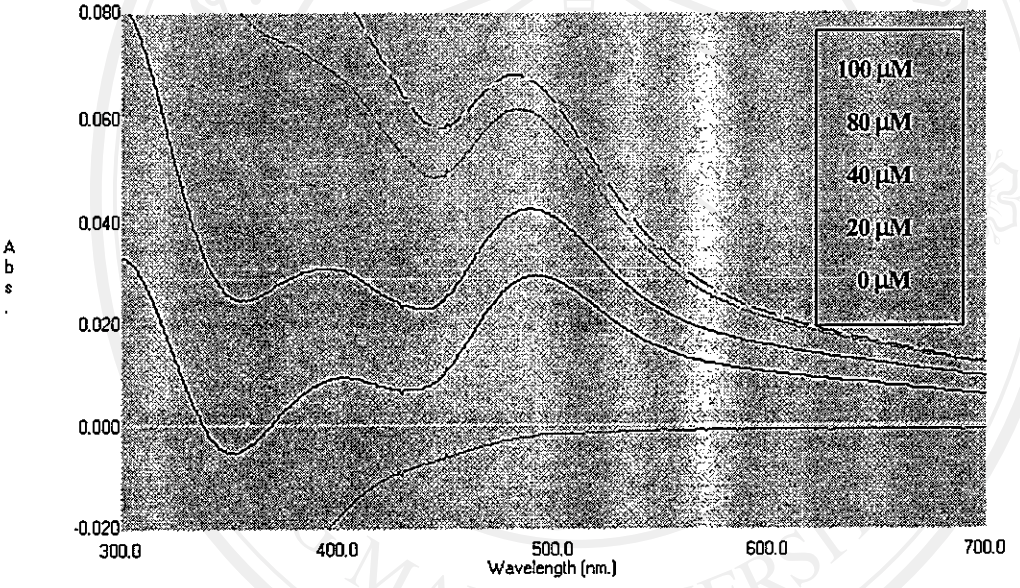


Figure 3.13 Spectrum of curcumin-ferric complex occurring when ferric nitrate at different concentrations (0, 20, 40, 80 and 100 μM at final concentrations) was bound to curcumin 800 μM *in vitro*.

3.11.2 Spectrum analysis of binding of curcumin with ferric citrate

Working curcumin solution at a final concentration 800 μM was incubated with ferric citrate solution (0, 20, 40, 80 and 100 μM) buffered in 100 mM MOPS, pH 5.9 at room temperature for 30 minutes. The absorbance of colored product was subsequently measured between 300-700 nm. Curcumin solution itself was used as a reagent blank. As shown in Figure 3.14, it was found that curcumin bound ferric-citrate to form the colored curcumin-iron complex and gave a predominant peak at 500 nm.

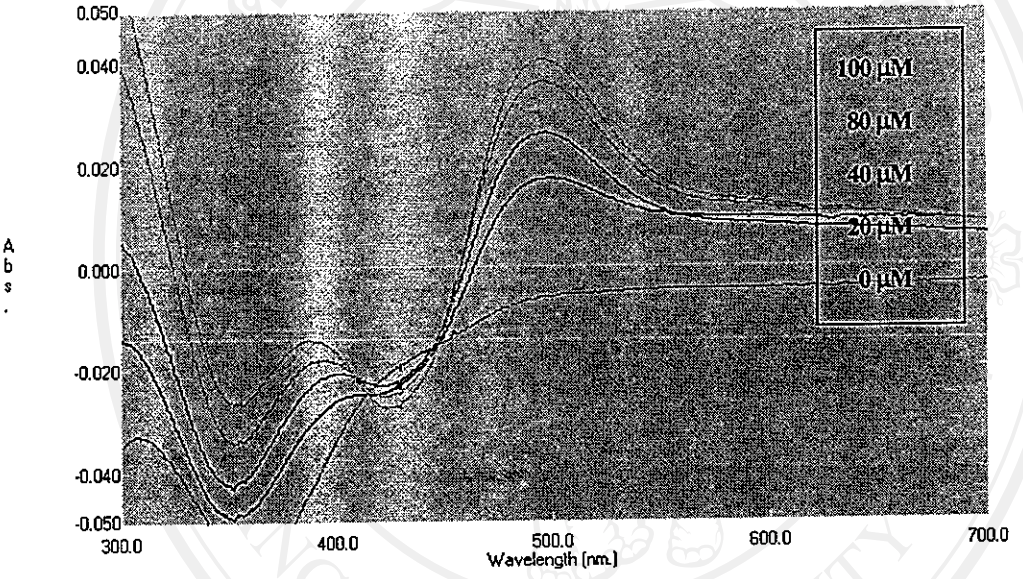


Figure 3.14 Spectrum of curcumin-ferric complex formation occurring when ferric citrate at different concentrations (0, 20, 40, 80 and 100 μM at final concentrations) was bound to curcumin at 800 μM.

Curcumin dose-dependently bound with both ferric citrate and ferric nitrate at pH 5.9 to form a colored product giving the same predominant peak at approximately 500 nm.

3.12 Effect of iron concentrations on curcumin-iron complex formation

3.12.1 Effect of ferric nitrate concentration

To examine the effect of ferric nitrate concentration on curcumin-ferric complex formation, the different concentrations of ferric nitrate (20, 40, 80, 100 μM) was incubated with 1 mM curcumin (a final concentration) for 30 minutes at room temperature. Absorbance was measured at 500 nm. Curcumin solution itself was used as a reagent blank. The result showed that the concentration of curcumin at 1 mM strongly bound ferric-nitrate with a concentration-dependent manner (Figure 3.15).

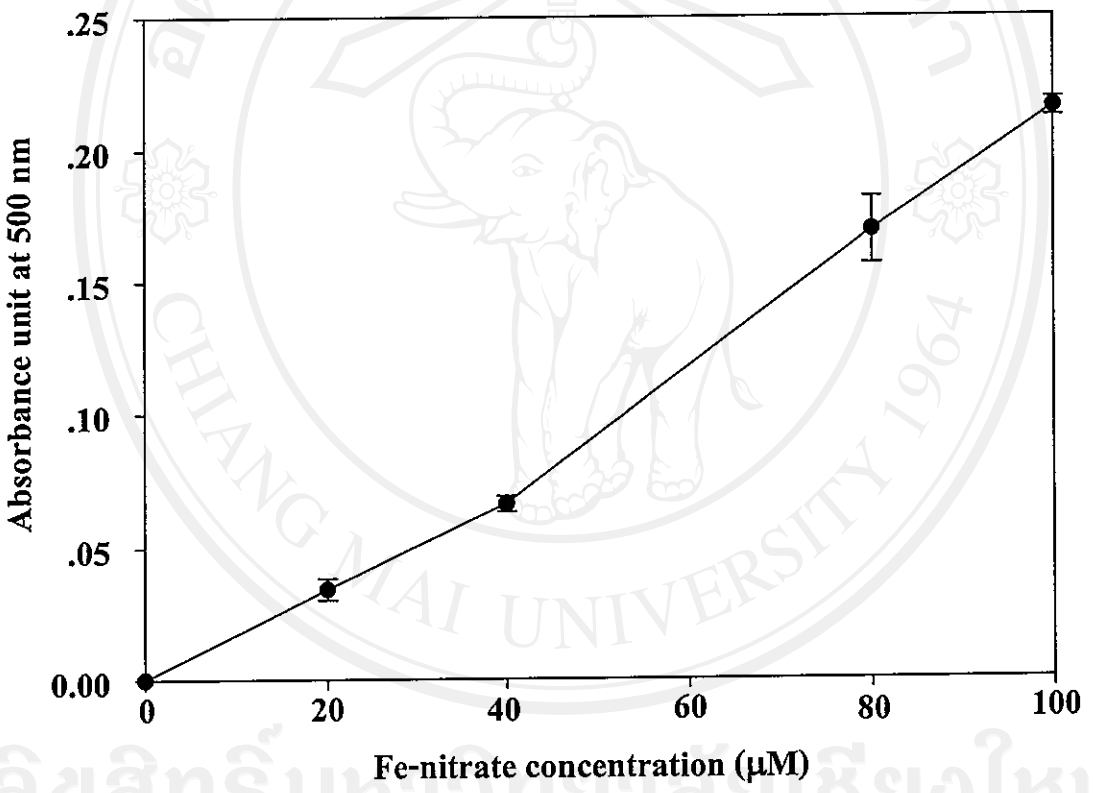


Figure 3.15 Interaction of curcumin solution (1 mM) with different concentrations of ferric nitrate (20, 40, 80 and 100 μM at a final concentration). Data obtained from a triplicate experiment were expressed as the mean±SEM.

3.12.2 Effect of ferric citrate concentration

To examine the effect ferric citrate concentration on curcumin-ferric citrate complex formation, the different concentration of ferric citrate (20, 40, 80 and 100 μM) was incubated with 1 mM curcumin for 30 minutes at room temperature. Absorbance was measured at 500 nm. Curcumin solution itself (1 mM at a final concentration) was used as a reagent blank. The result demonstrated that the concentration of curcumin at 1 mM strongly bound ferric-citrate with a concentration-dependent manner and it reached the saturation at 80 μM ferric citrate (Figure 3.16).

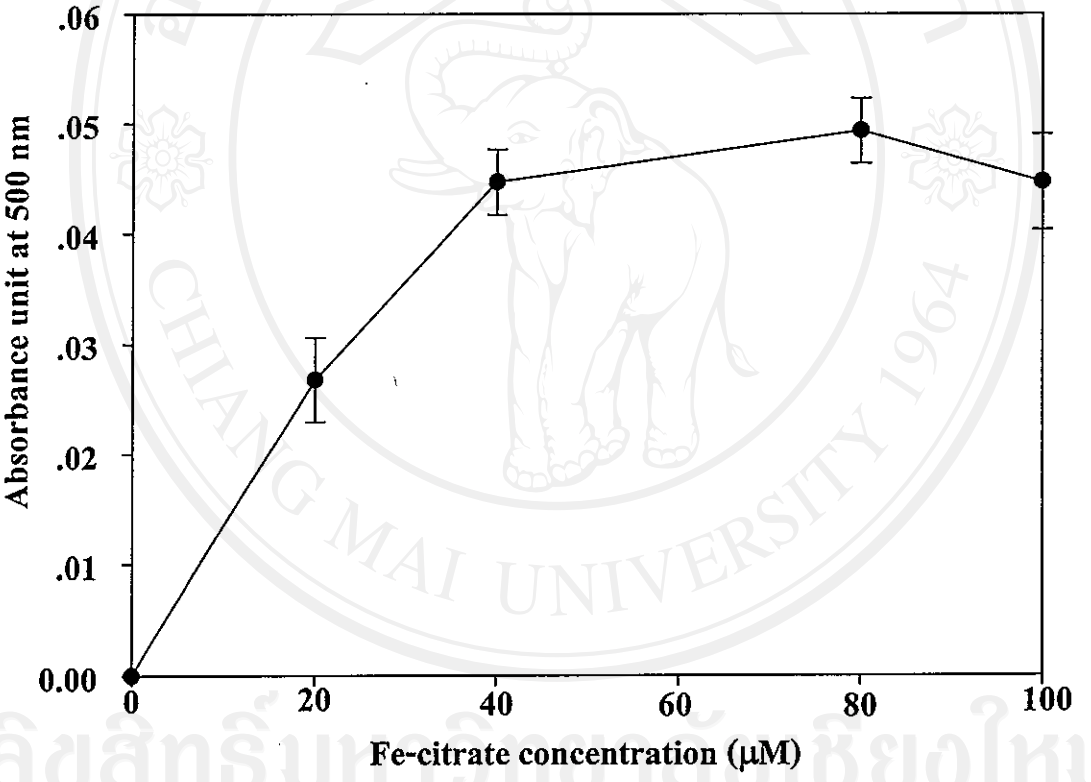


Figure 3.16 Interaction of curcumin (1 mM at a final concentration) with ferric citrate solution (20, 40, 80, 100μM at a final concentration). Data obtained from a triplicate experiment were expressed as mean±SEM.

3.13 Effect of curcumin concentration on curcumin-iron complex formation

3.13.1 Effect of curcumin concentration on curcumin-ferric nitrate complex formation

To study the effect of curcumin concentration on curcumin-ferric complex formation, different concentrations of curcumin (100, 200, 400 and 800 μM) was incubated with 100 μM ferric nitrate solution for 30 minutes at room temperature. Absorbance was measured at 500 nm. Curcumin solution alone at equivalent concentrations was used as a reagent blank. The result indicated that ferric nitrate (100 μM) strongly bound curcumin with a concentration-dependent manner (Figure 3.17).

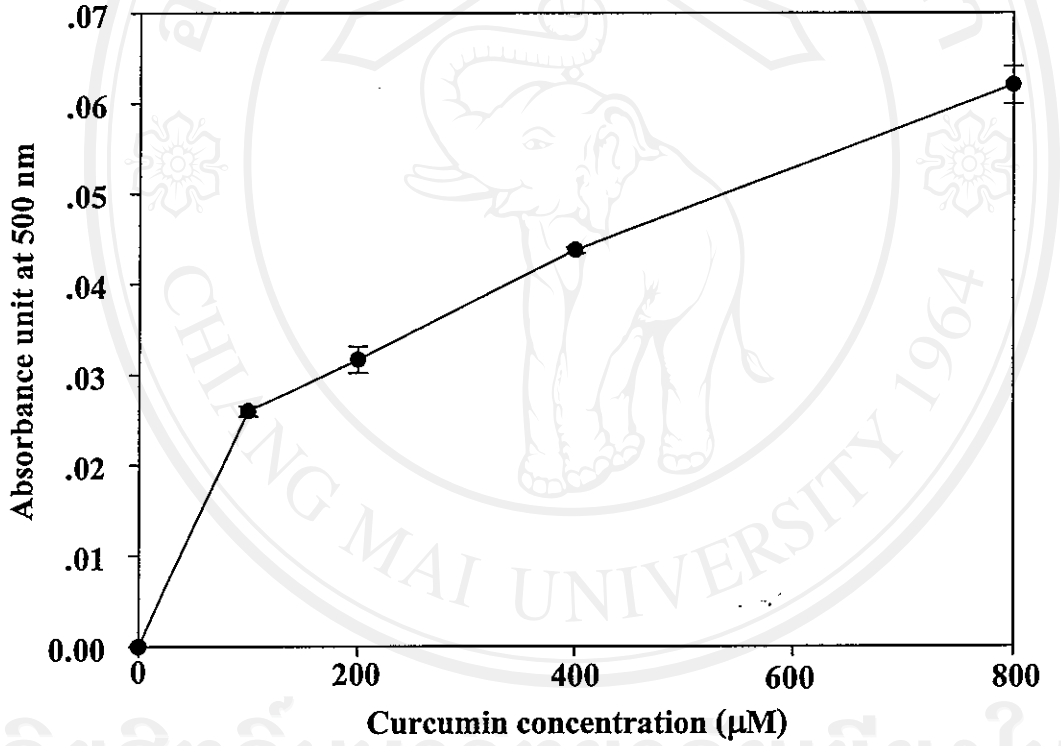


Figure 3.17 Interaction of ferric-nitrate (100 μM at a final concentration) with curcumin (100, 200, 400 and 800 μM at final concentrations). Data obtained from a triplicate experiment were expressed as mean±SEM.

3.13.2 Effect of curcumin concentration on curcumin-ferric citrate complex formation

To study the effect of curcumin concentration on curcumin-ferric complex formation, different concentrations of curcumin solution (100, 200, 400 and 800 μM at final concentrations) was incubated with 100 μM ferric citrate solution for 30 minutes at room temperature. Absorbance was measured at 500 nm. Curcumin solution alone at equivalent concentrations was used as a reagent blank. The result indicated that ferric citrate (100 μM) strongly bound curcumin with a concentration-dependent manner (Figure 3.18).

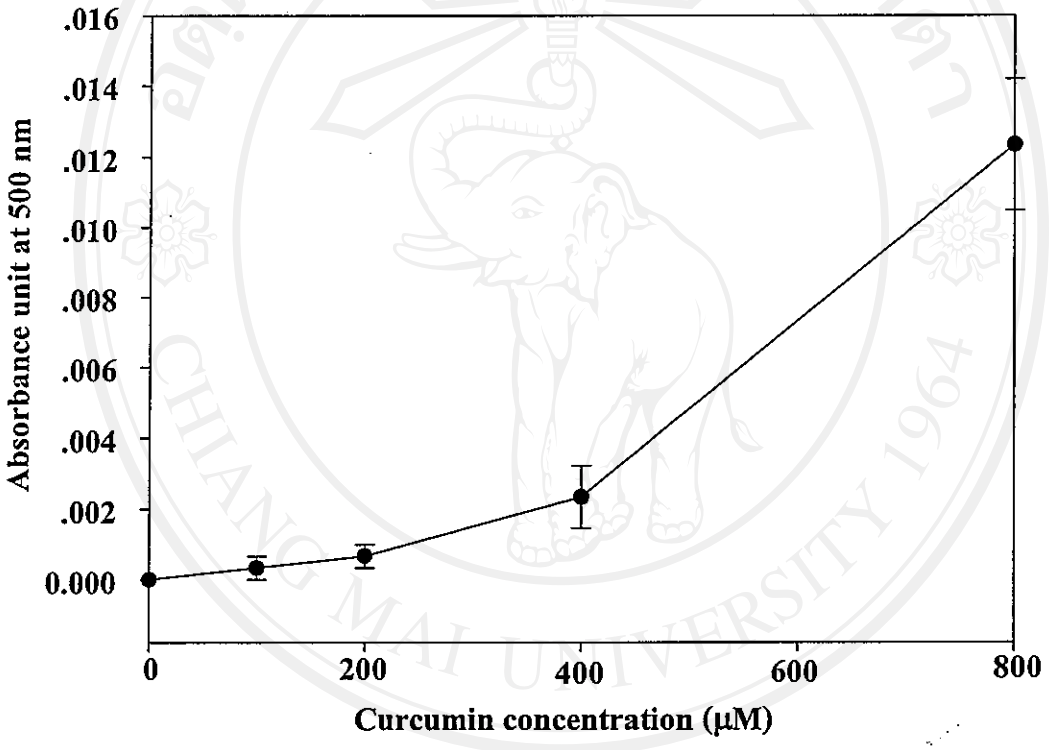


Figure 3.18 Interaction of ferric-citrate (100 μM at a final concentration) with different concentrations of curcumin (100, 200, 400 and 800μM at final concentrations). Data obtained from a triplicate experiment were expressed as mean±SEM.

3.14 Kinetic study of curcumin and iron binding

3.14.1 Time course of curcumin-ferric complex formation *in vitro*

To study the time-course of curcumin-ferric complex formation *in vitro*, curcumin solution (1 mM at a final concentration) was incubated with ferric nitrate solution (20 and 80 μM) for different time (0, 1, 3, 10, 20 and 30 minutes) at room temperature. Absorbance was measured at 500 nm. Curcumin solution itself (1 mM) was used as a reagent blank. The result showed that rate of iron binding onto curcumin molecule was initially fast and subsequently slow. The binding was dependent upon ferric nitrate concentrations in the assay mixture and reached the saturation within 30 minutes at room temperature (Figure 3.19).

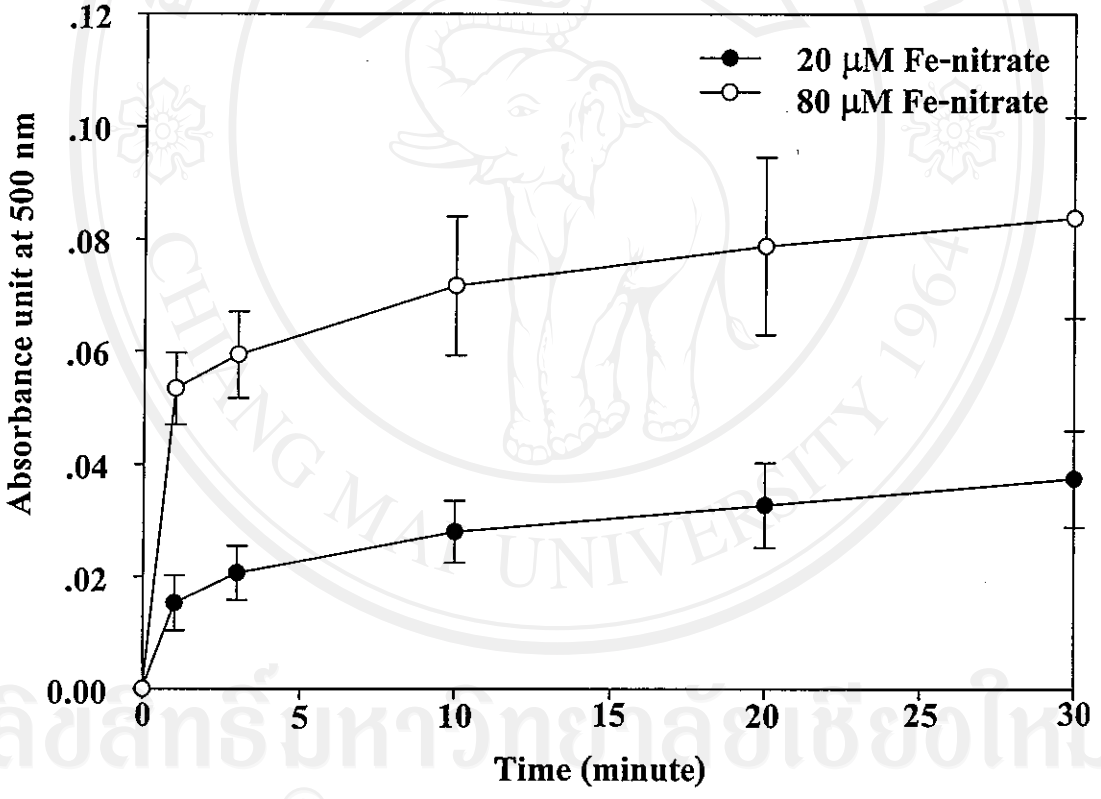


Figure 3.19 Time-course binding of ferric nitrate (20, 80 μM at a final concentration) to curcumin (1 mM). Data obtained from a triplicate experiment are shown as the mean± SEM.

3.14.2 Time course of curcumin-ferric citrate complex formation *in vitro*

To study time-course of curcumin-ferric complex formation *in vitro*, curcumin solution (1 mM at a final concentration) was incubated with ferric citrate solution (20 and 80 μM) for different time (0, 1, 3, 10, 20 and 30 minutes) at room temperature. Absorbance was measured at 500 nm. Curcumin solution itself was used as a reagent blank. The result showed that iron was bound to curcumin slowly and gradually. Anyway, the binding seemed to be concentration- and time-dependent pattern, the saturation did not occur within 30 minutes of incubation (Figure 3.20).

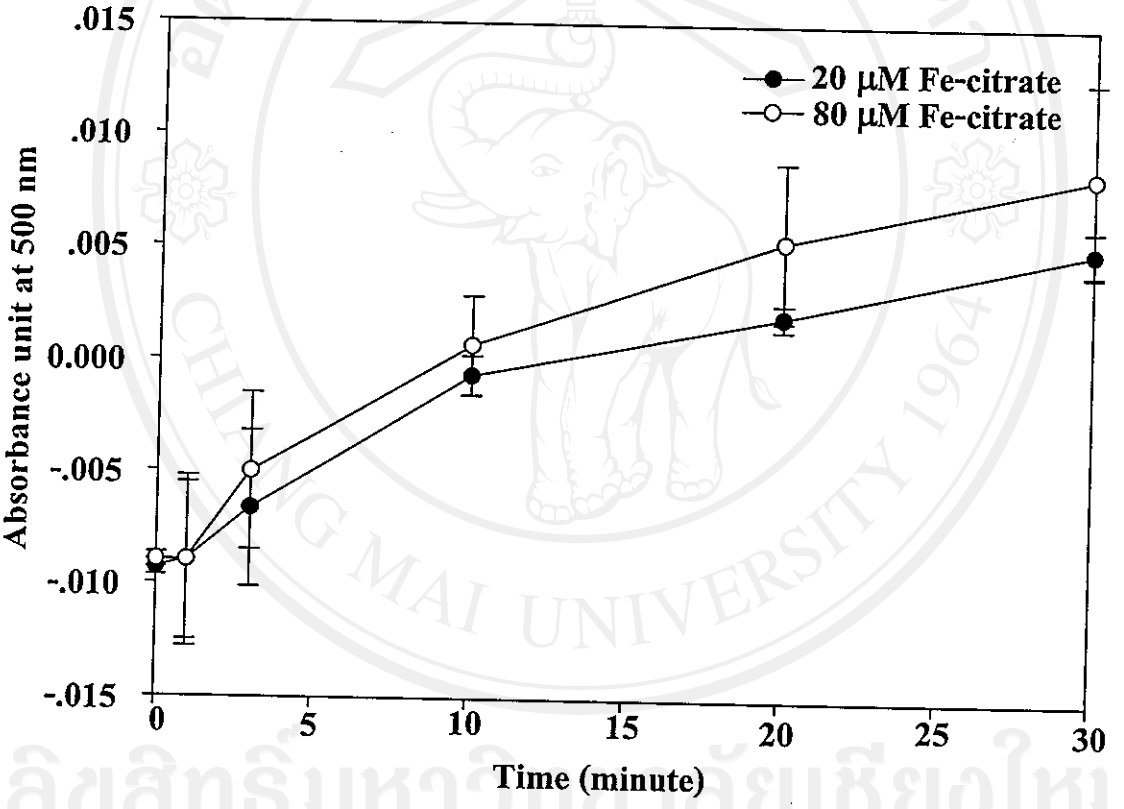


Figure 3.20 Time-course of ferric citrate (20 and 80 μM at a final concentration) binding to curcumin (1 mM at a final concentration). Data obtained from a triplicate experiment were shown as mean±SEM

3.15 Effect of curcumin and deferiprone on the removal of plasma NTBI

To study the effect of curcumin and deferiprone to decrease plasma NTBI, plasma of four ESRD patients was co-incubated with different concentrations of curcumin (100, 200 and 400 μM) and in the presence of 100 μM deferiprone at 37°C for 1 hour. NTBI concentration was measured using the NTA chelation/HPLC-based assay previously described in Section 2.7. The result showed that the concentration of curcumin at 100 and 200 μM in the presence of 100 μM derferiprone reduced plasma NTBI level from 6.6±0.4 to 4.6±0.3 μM (p=0.9) and to 3.9±0.5 μM (p=0.5), respectively compared with control PBS. Moreover, 400 μM curcumin together with 100 μM deferiprone reduced plasma NTBI concentration from 6.6±0.4 to 3.1±0.3 μM compared with control PBS (p=0.06) (Table 3.10) (Figure 3.20). The maximal chelated NTBI was approximately 50% by 400 μM curcumin in the presence of 100 μM deferiprone (Figure 3.222). This result suggests that curcumin (100-400 μM) in the presence of 100 μM deferiprone removed plasma NTBI in a dose-dependent pattern.

Table 3.10 *In vitro* removal of NTBI by curcumin and deferiprone in plasma of four ESRD patients. Data were shown as individual NTBI concentrations and %decreased NTBI.

Patients	PBS	100μM curcumin+L1		200μM curcumin+L1		400 μMcurcumin+L1	
	NTBI (μM)	NTBI (μM)	Decreased NTBI (%)	NTBI (μM)	Decreased NTBI (%)	NTBI (μM)	Decreased NTBI (%)
ESRD 1	5.8	4.5	22.1	3.3	43.8	4.2	28.3
ESRD 2	7.8	5.1	39.3	4.7	39.1	3.0	61.6
ESRD 3	6.7	4.8	28.4	3.0	56.0	2.6	60.8
ESRD 4	6.2	3.9	37.9	4.7	24.5	2.8	55.3
Mean±SEM	6.6±0.4	4.6±0.3	31.9±4.1	3.9±0.5	40.8±6.5	3.1±0.3	51.5±7.9

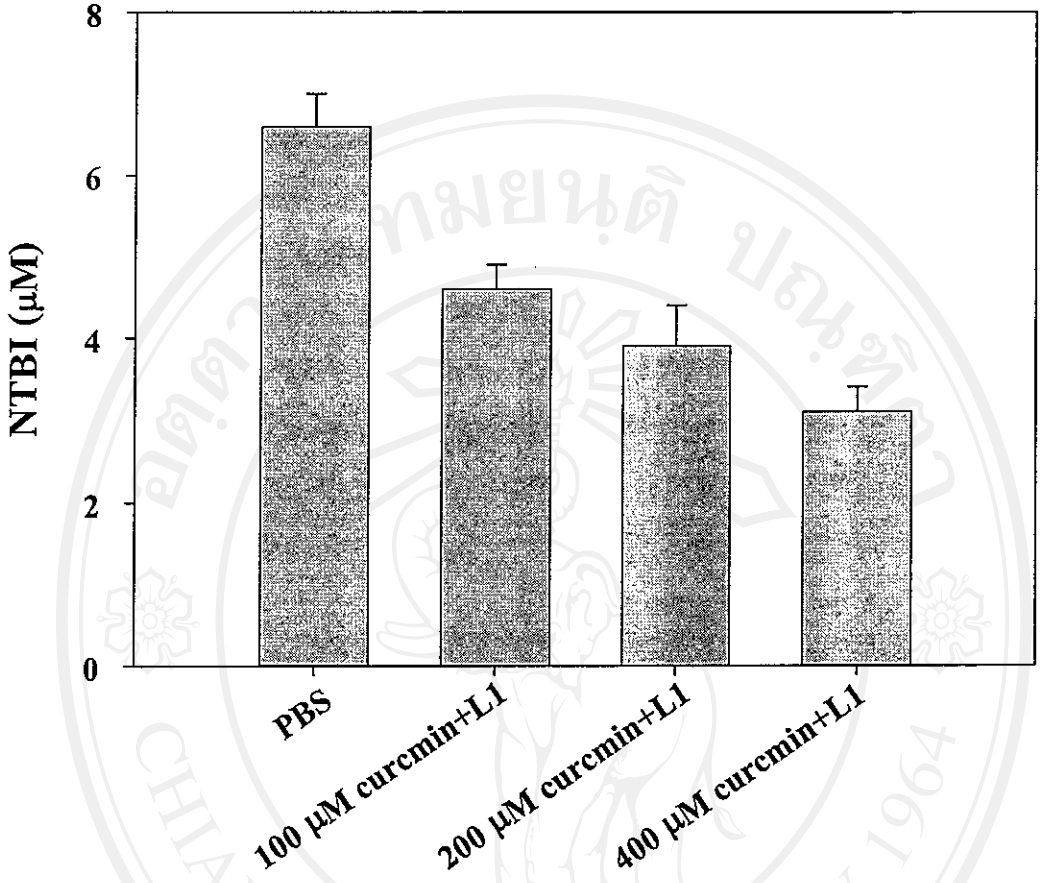


Figure 3.21 *In vitro* removal NTBI by deferiprone and curcumin in plasma of four ESRD patients that were chelated with different concentration of curcumin and 100 μM L1 compared with PBS (control). Data were shown as average values of NTBI±SEM.

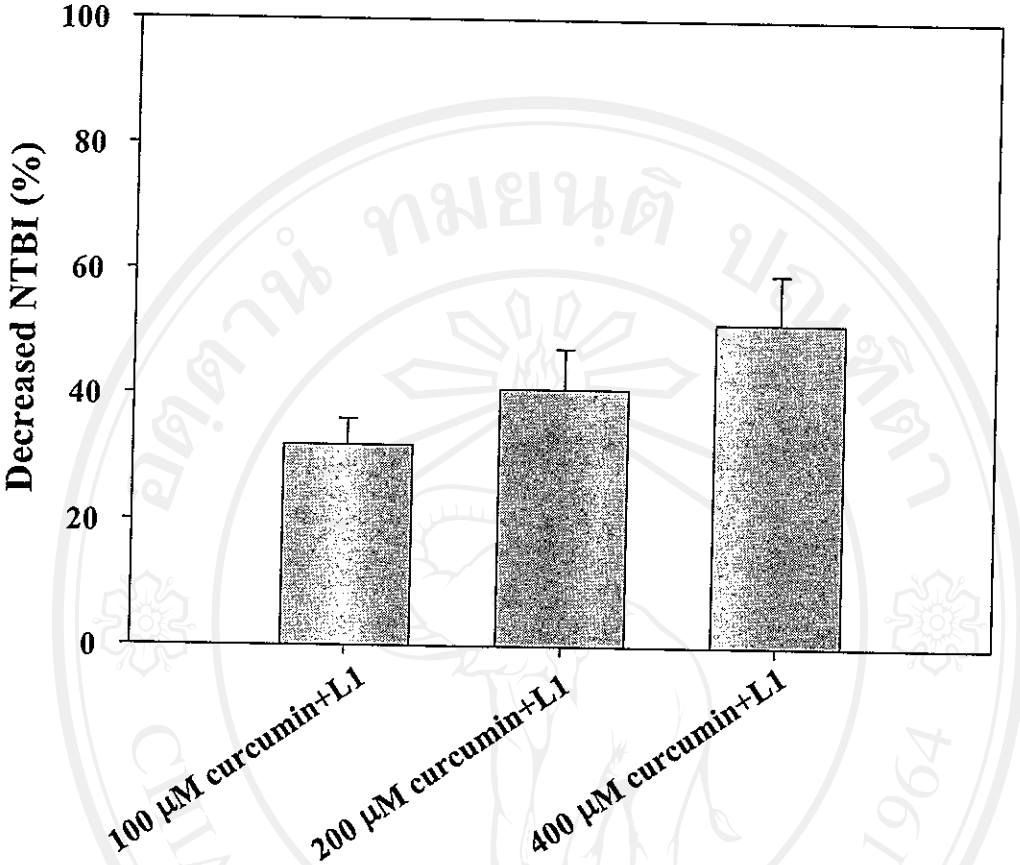


Figure 3.22 *In vitro* removal NTBI by deferiprone and curcumin in plasma of four ESRD patients that were chelated with different concentration of curcumin and 100 μM L1 compared with PBS (control). Data were shown as %decreased NTBI±SEM.