CHAPTER III

RESULTS

I. Spectrophotometric Determination of Bilirubin Absorption Spectra

Figure 3 is a typical absorption spectra of 50 µM (2.9 mg%) unconjugated bilirubin dissolved in 10 mM Tris-HCl buffer, pH 7.5. The maximum absorption is at 424 nm and the absorbance of unconjugated bilirubin is 1.05.

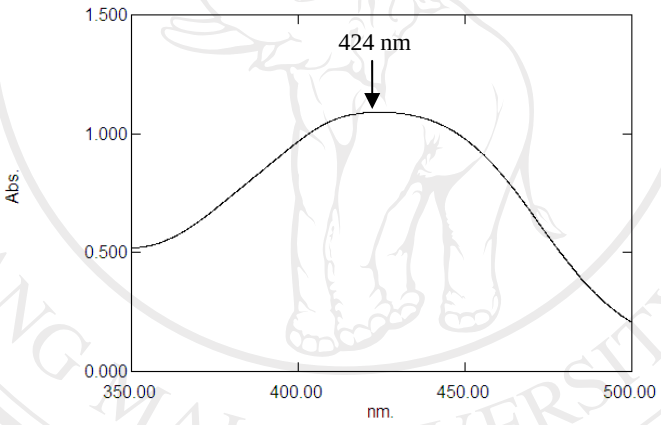

Figure 3. The absorption spectra of 50 µM (2.9 mg%) bilirubin dissolved in 10 mM Tris-HCl buffer, pH 7.5 in a Shimadzu UV-2450 Spectrophotometer.

II. Investigation of the Absorption Spectra of Bilirubin in the Presence of Effectors by Spectrophotometric Method

All absorption spectra were recorded using a Shimadzu UV-2450 spectrophotometer. The absorbance readings between 350 and 550 nm or as indicated were obtained and compared to bilirubin (50 µM) absorption spectrum in Figure 3. After adding of any effectance into the 0.5 mL reaction containing maximum concentration of bilirubin or with various amount of bilirubin, the changes in absorbance spectra were observed. After adding bilirubin in a reaction mixture with interaction metal ion or albumin of various concentrations, the decomposition of bilirubin or the interaction products will cause the shift, decrease or increase of bilirubin absorbance peaks as shown in the following experiments.

1. The effect of transition metal ions on bilirubin absorption spectra

1.1 Types of transition metal ions

Figure 4 is the effect of the types of transition metal ions on the changes in bilirubin absorption spectra after the addition of Cu(II) or Fe(II) or Zn(II) in the reaction mixtures, respectively. Figure 4A shows the results after addition of increasing concentration of $CuCl_2$ (50-500 µM) to the 0.5 mL reaction of 500 µM bilirubin. It was found that the absorption spectrum were all absent and all peaks were shift to the wavelengths which were greater or lower than reference bilirubin absorption spectrum (421 nm), to 594 and 644 nm and 374 nm (see Figure 5). Figure 4B shows the effect of 500 µM $FeCl_2$ on increasing concentration of bilirubin absorption spectra. As shown, the peak of 4B-a, b and c (bilirubin concentrations of 50,100 and 200 µM, respectively) were shift to the left with the maximum absorption of around 400 nm.

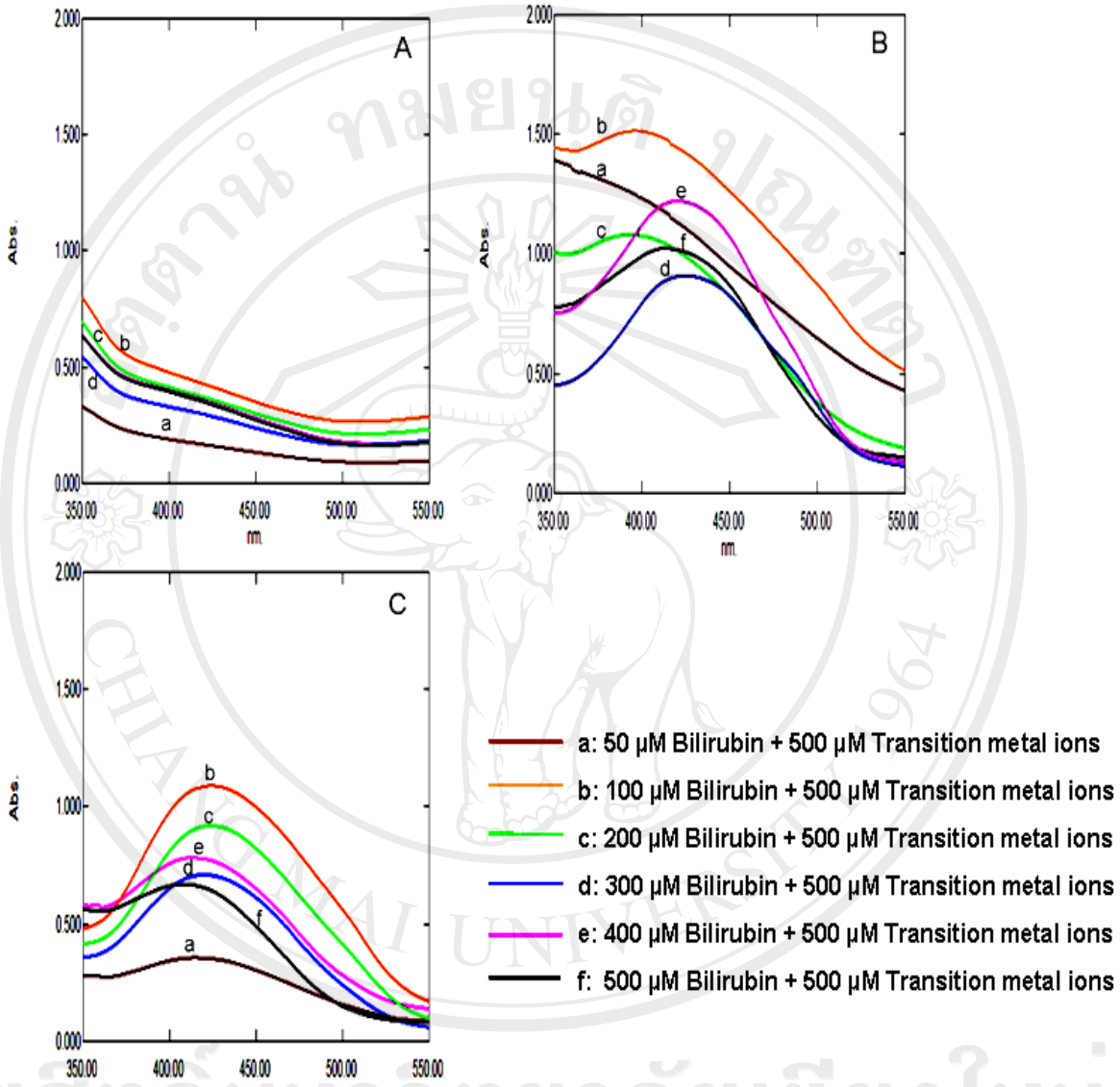

Figure 4. Effect of 3 types of transition metal ions on bilirubin absorption spectra. The 0.5 mL reaction mixtures containing a fix amount of bilirubin (500µM). The concentrations of $CuCl_2$, $FeCl_2$ and $ZnCl_2$ in the reaction mixtures were varied from 50-500 µM. The reaction were incubated at 37 ºC for 1 hour before scanning from 350-550 nm in a UV-2450 Spectrophotometer. [A] Copper, [B] Ferrous and [C] Zinc as interaction metal ion, respectively.

With higher concentrations of bilirubin (Figure 4B-d, e and f, at 300,400 and 500 µM $FeCl_2$,respectively),the peaks of maximum absorption were unchanged when compared with the typical reference bilirubin peak (Figure 3 and 5). In contrast to $FeCl_2$ when the increasing concentration of $ZnCl_2$ were added into the reaction mixtures (shown in Figure 4C), the shifts to the left of bilirubin absorption spectra were observed only in the reactions containing 400 and 500 µM $ZnCl_2$, respectively. Therefore the interaction of metal ions on the fix amount of bilirubin concentration in the reaction mixture were depended on types and concentrations of the interaction metal ions.

The interaction ion which is the most effective on bilirubin absorption spectra is Cu (II). As shown in Figure 5, the peak of bilirubin in the presence of 500 µM $CuCl_2$ was shift from the control (bilirubin alone) (424 nm) to the right at 594 and 644 nm., respectively and to the left at 343 nm.

1.2 The effect of transition metal ion concentrations on the absorption spectra of bilirubin

All the reaction were diluted 1 to 5 folds before scanning. Figure 6 shows the effect of transition metal ion concentrations on the spectral changes of bilirubin on the additions of Cu(II) (Figure 6A), Fe(II) (Figure 6B) and Zn (II) (Figure 6C) , respectively. Figure 6A shows that the changes of bilirubin absorption spectra were dependent on Cu(II) concentrations.

The decrease and disappearance of bilirubin peaks following the interactions with Cu(II) were in the order of $CuCl_2$ concentrations; 50 (a), 200 (b) and 500 µM (c), respectively. As the same increasing concentrations as $CuCl_2$, the effect of $ZnCl_2$

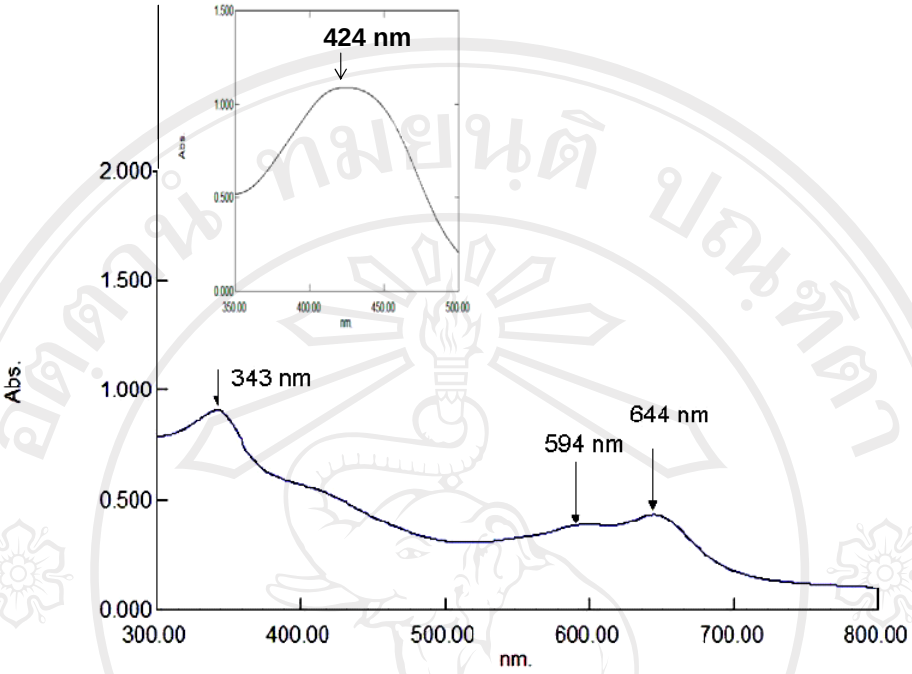

Figure 5. The effect of $CuCl_2$ [Cu(II)] on the absorption spectrum of bilirubin.The concentrations of bilirubin and Cu(II) were at 500 µM. The insert figure is a 50 µM biliubin absorption spectra used as reference for the spectrum without Cu(II). The reaction mixture was scanned in a UV- 2450 Spectrophotometer.

on bilirubin absorption spectra were less than those found with $CuCl_2$ but it demonstrated more or less the same pattern as $CuCl_2$ interaction. There were some slightly changes of absorbance peaks observed after adding 200 µM and 500 µM $ZnCl_2$ into the reaction. For $FeCl_2$, there was no change of absorbance peak and the reduction of bilirubin peak was limited at 50 µM $FeCl_2$. At higher concentrations of Fe Cl_2 in the reaction mixture shows the same characters of bilirubin absorption spectra as at 50 µM.

These results showed that Cu(II), as compared with Fe(II) and Zn(II), was the most powerful interaction ion with bilirubin molecules. The interactions caused the marked change in absorption peak and decrease in the absorbance value of bilirubin concentrations.

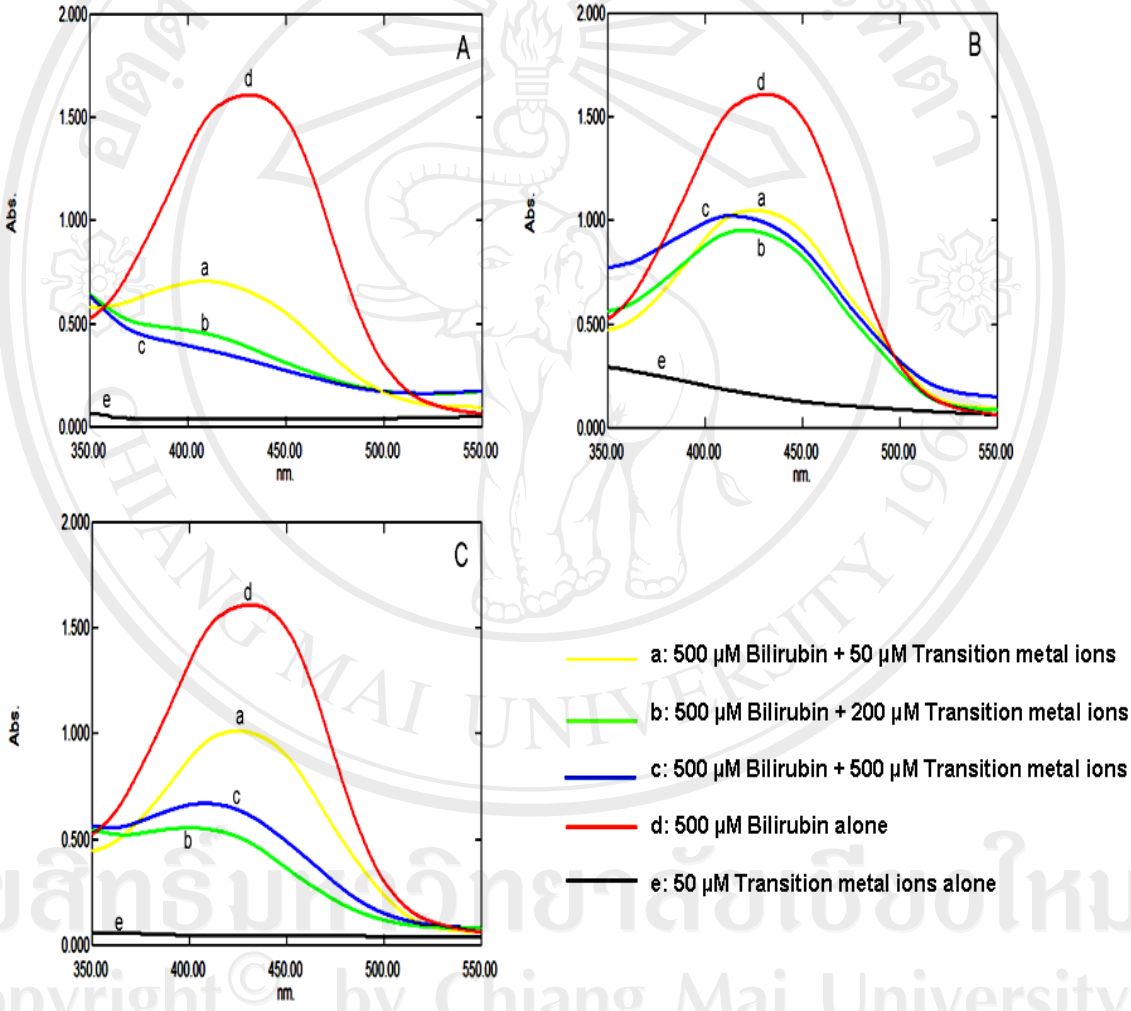

Figure 6. The effect of transition metal ion concentrations on the absorption spectra of bilirubin (500 µM). The concentrations of $CuCl_2$, $FeCl_2$ and $ZnCl_2$ were 50, 250 and 500 µM, respectively. The reaction were incubated at 37 ºC for 1 hour before scanning from 350-550 nm in a UV-2450 Spectrophotometer. [A] Copper, [B] Ferrous and [C] Zinc as interaction metal ion, respectively.

2. Confirmation of bilirubin binding with interaction metal ion

The absorbance peaks of bilirubin interacted with Cu(II) or quercetin were compared with reference bilirubin control. Figure 7 demonstrated the maximum absorption of bilirubin alone which was at 434 nm (peak a) and it was changed to 404 nm when $CuCl_2$ was added in the reaction mixture (peak e). Quercetin alone absorbs at 374 nm (peak b). After the addition of quercetin to the reaction of bilirubin containing $CuCl_2$, the absorption spectrum was changed to 422 nm (peak g). Bilirubin or quercetin interacted with $CuCl_2$ show the peak approximately at 404 nm and 432 nm, respectively. This result proved that bilirubin can interact and form complex with copper and when the complex is formed in the presence of quercetin, the copper is capable to form a co-complex with both bilirubin and quercetin.

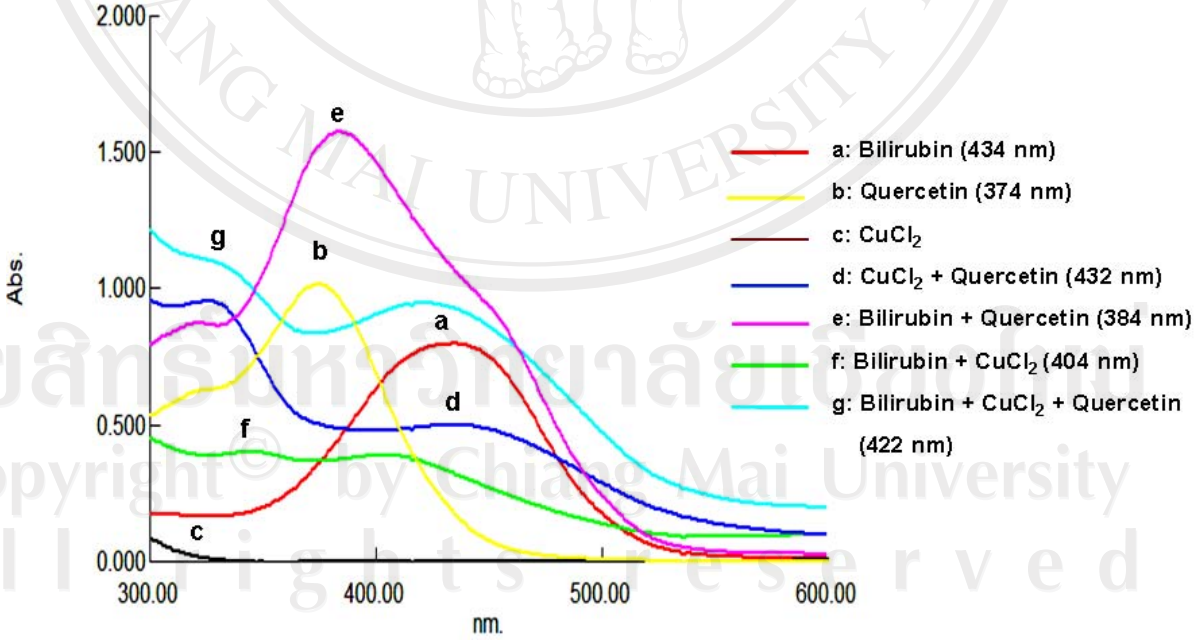

Figure 7. The absorbance peaks of bilirubin or quercetin interacted with Cu(II) were compared with reference bilirubin absorption spectrum

3. The effect of albumin on bilirubin-transition metal ion complex on the absorption spectra of bilirubin

All the reactions were performed as indicated in materials & methods and diluted 1 to 10 folds before scanning from 350 – 550 nm in a UV-2450 Spectrophotometer. Figure 8 shows the effect of albumin as molar ratio of bilirubin; 0.5:1.0 on the spectra of bilirubin-transition metal ion complex in the selected bilirubin optimal concentration, 500 μM. Figure 8 A is the effect of albumin as molar ratio of bilirubin 0.5:1.0 on bilirubin-$CuCl_2$ absorption spectra**.** It was shown that all peaks were shifted to the right to the same absorbance peak as bilirubin-albumin control (without interaction ions) and reach maximum at 451 nm. In the presence of albumin (0.5:1.0 ratio) and equal concentration of bilirubin and $CuCl_2$ (500 μM), there seemed to have some competitive binding to bilirubin molecules by albumin and $CuCl_2$. The effect of albumin (0.5:1.0) on the absorption spectra of bilirubin-$FeCl_2$ complex is shown in Figure 8 B. In the presence of albumin, some bilirubin molecules were bound to albumin and the peaks of bilirubin-albumin interaction were shifted to the same wavelength as bilirubin-albumin control (no interaction ions). The decrease in all absorbance peaks of bilirubin in the presences of both albumin and $FeCl_2$ demonstrated some interactions of Fe (II) with bilirubin and the results were similar to Figure 8 B in which the interactions were independent on the $FeCl_2$ concentrations. Figure 8 C is the effect of albumin (0.5:1.0) on the interaction of zinc and bilirubin molecules. In the presence of albumin, $ZnCl_2$ at any concentrations could not interacted with bilirubin molecules. As shown, all absorbance peaks were shifted to the same wavelength as the bilirubin-albumin control (462 nm). No decrease in peaks

were observed in the reactions containing Zn(II) which means that Zn(II) can hardily interact with bilirubin in the presence of albumin.

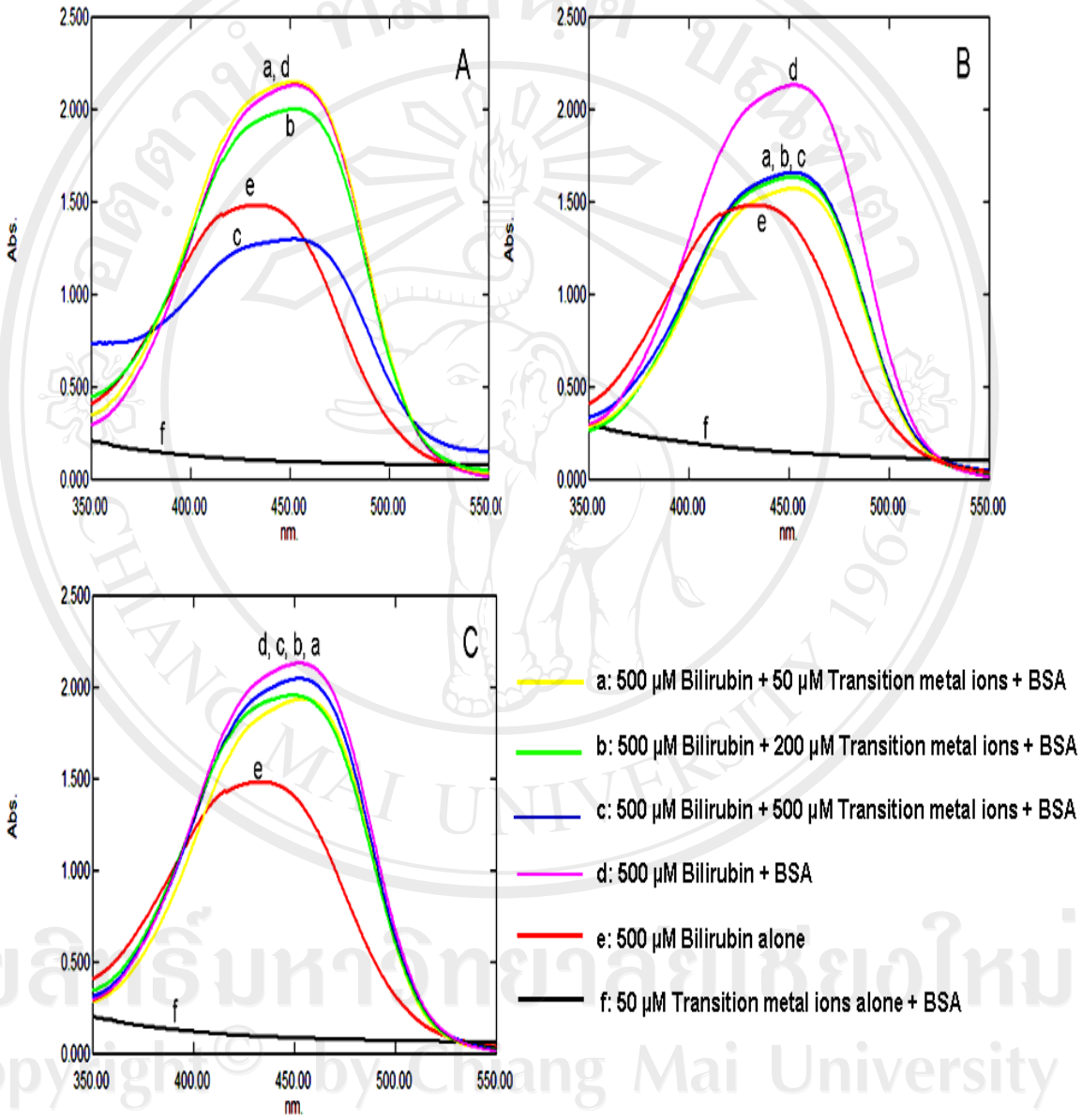

Figure 8. The effect of albumin on the absorption spectra of bilirubin-transition metal ion complex. Albumin as a molar ratio of bilirubin; 0.5:1.0, on the selected optimal bilirubin concentrations (500 µM). The reaction were incubated at 37 °C for 1 hour before scanning from 350-550 nm in a UV-2450 Spectrophotometer. [A] Copper, [B] Ferrous and [C] Zinc as interaction metal ion, respectively.

Figure 9 and 10 are the effect of albumin as a molar ratio of bilirubin 1.0:1.0 and 1.5:1.0, respectively on the binding of interaction ions with bilirubin. As shown in both figures, all bilirubin absorption spectra were shifted to the same wavelength as bilirubin-albumin control (peak d). There were some interactions of bilirubin with Cu (II) at the 500 μM concentration. Therefore, it can conclude that in the presence of suitable amount of albumin to bilirubin ratio (which was greater than 0.5:1.0), bilirubin preferencially select to bind with albumin rather than metal ions. The binding of transition metal ions to bilirubin were decreased after the concentration of albumin increased. However, these results can be postulated that the concentrations of albumin and bilirubin in serum should be 1 to 1 in ratio. If the concentration of albumin in serum is low in disease, the toxicity of bilirubin will occurred. In neonatal the excess unbound bilirubin in circulation will enter the brain cells and cause some toxicity to the brain which is the cause of kernicterus.

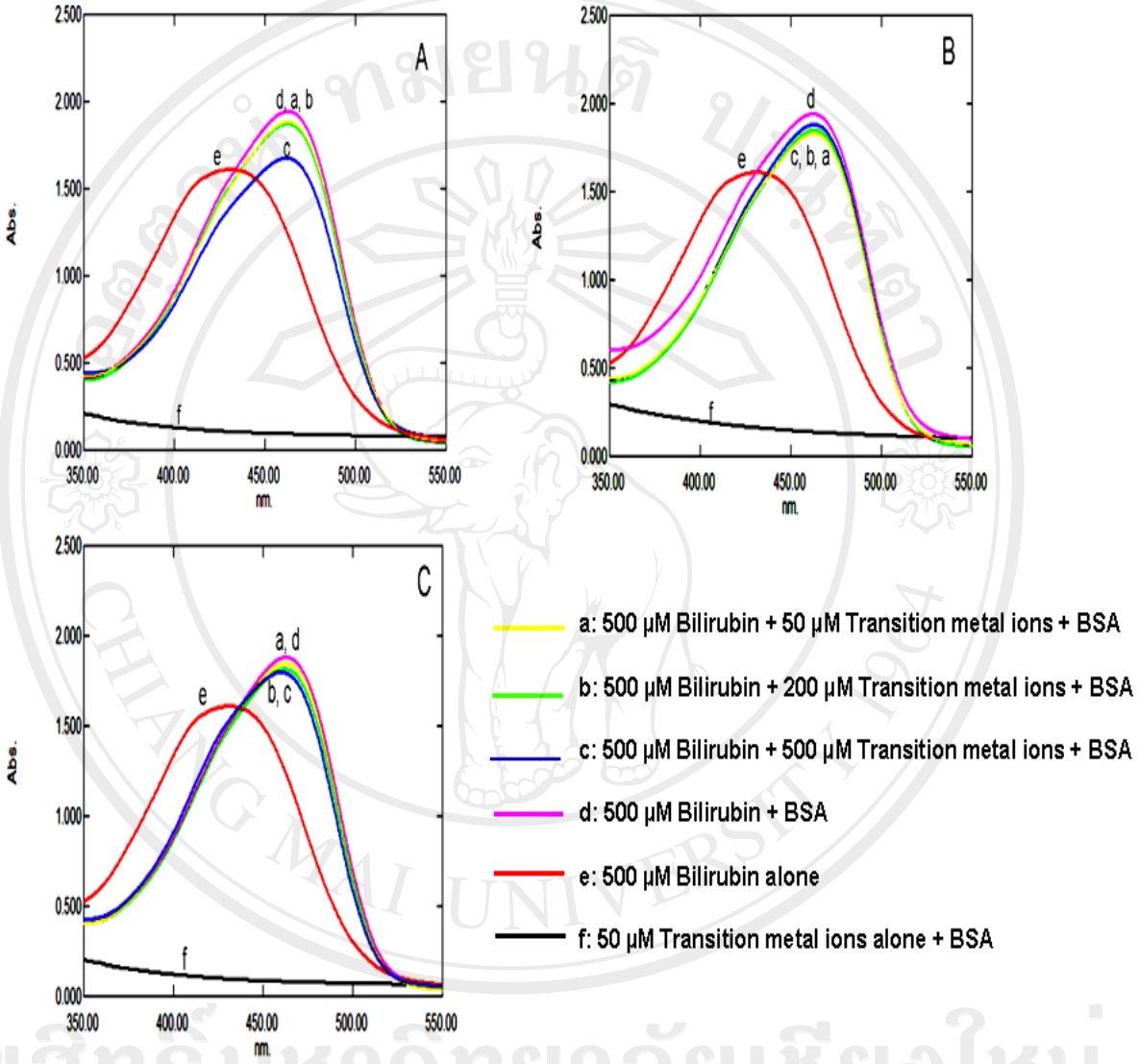

Figure 9. The effect of albumin on the absorption spectra of bilirubin-transition metal ion complex. Albumin as a molar ratio of bilirubin; 1.0:1.0, on the selected optimal bilirubin concentrations (500 µM). The reaction were incubated at 37 °C for 1 hour before scanning from 350-550 nm. in a UV-2450 Spectrophotometer. [A] Copper, [B] Ferrous and [C] Zinc as interaction metal ion, respectively.

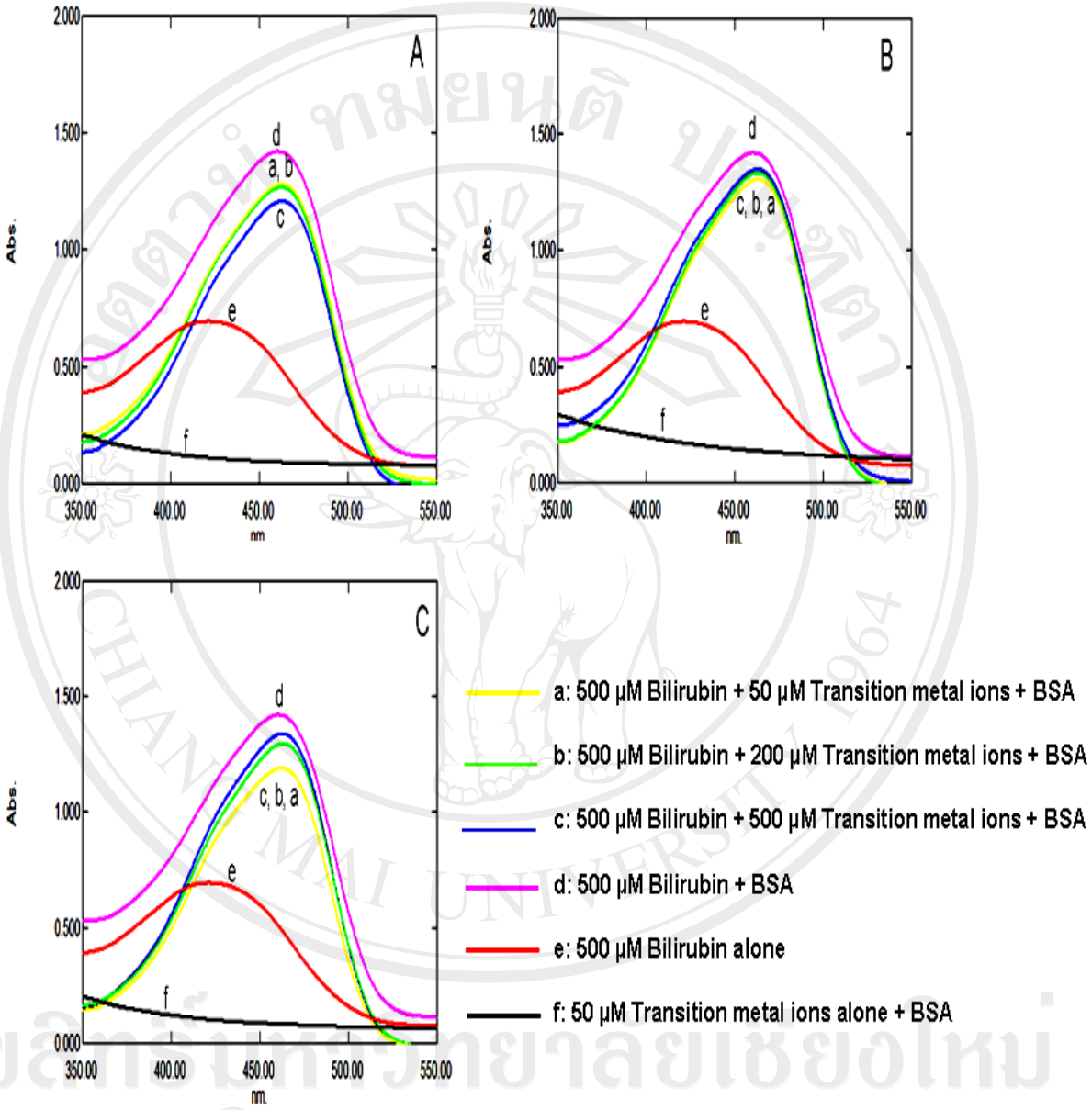

Figure 10. The effect of albumin on the absorption spectra of bilirubin-transition metal ion complex. Albumin as a molar ratio of bilirubin; 1.5:1.0, on the selected optimal bilirubin concentrations (500 µM). The reaction were incubated at 37 ^{O}C for 1 hour before scanning from 350-550 nm. in a UV-2450 Spectrophotometer. [A] Copper, [B] Ferrous and [C] Zinc as interaction metal ions, respectively.

III. Investigation of the Effect of Bilirubin on DNA Degradation *in vitro*.

1. Investigation of the effect of bilirubin and transition metal ions concentrations on DNA degradation *in vitro*.

1.1. Quantitation of DNA degradation

Standard graph of DNA by DPA reaction (Figure 11) is linear up to 1.0 OD at 600 nm, as the concentration of DNA in a tube is 1.0 mg/mL. The concentrations of DNA (mg/mL) were correlated significantly with the absorbance at 600 nm at R = 0.9953.

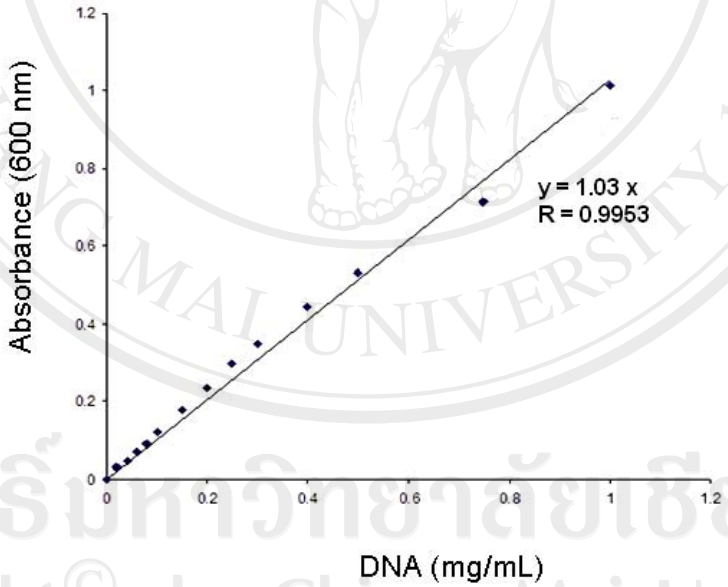

Figure 11 Standard graph of DNA by Diphenylamine reaction

1.2 The effect of bilirubin concentrations on the degradation of DNA *in vitro* with fixed transition metal ion concentration

Figure 12 shows the effect of bilirubin concentrations interacted with fixed transition metal ion concentrations on DNA degradation *in vitro*. The DNA degradation caused by bilirubin and Cu(II) interaction was depended on bilirubin concentrations (lane 2-5). It was shown that the maximum DNA degradation was observed after mixing 500 µM of bilirubin with 500 µM of $CuCl_2$ (lane5). In contrast to $CuCl_2$, the maximum DNA degradation caused by the interaction of $FeCl_2$ or $ZnCl_2$ (both 500 µM) with bilirubin obtained at lower amount of bilirubin (100 µM). When the concentrations of bilirubin increased (200 to 500 µM), the DNA degradation reaction was decreased (lane 6 – 9 for $FeCl_2$ and lane 10-12 for $ZnCl_2$). Control experiment (lane1) was the DNA incubated at 37 ^{O}C for 1 hour in a 0.5 mL of 10 mM Tris buffer, pH 7.5. Lane 12 is the native DNA in 0.5 mL of 10 mM Tris buffer, pH 7.5, without treatment. Control experiments in lane 1 shows some degradation of DNA as compared with lane 12.

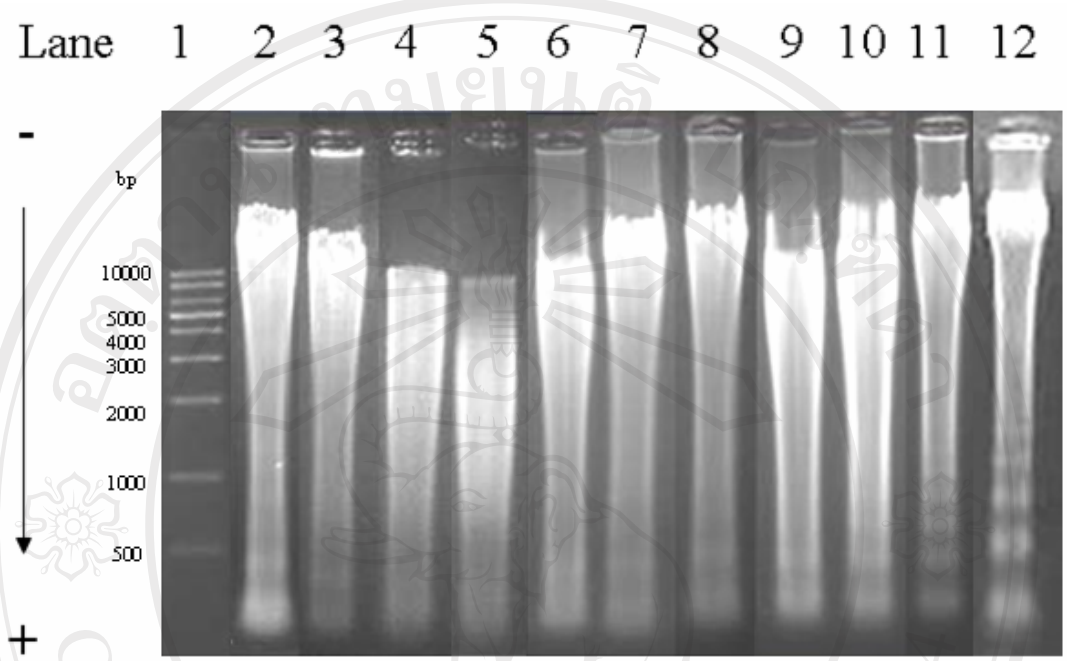

Figure 12. The effect of increasing bilirubin concentrations interacted with a fixed concentration of transition metal ion on DNA degradation *in vitro*. The 0.5 mL reaction mixtures contained 500 µM fixed concentration of transition metal ions and increasing concentrations of bilirubin from 100-500 µM in the presence of 500 µg of calf thymus DNA. The reactions were mixed and incubated at 37 ^{O}C for 4 hours and the DNA degradation were detected by agarose gel electrophoresis.

Lane 1, DNA marker; lane 2, DNA alone; lane 3-5, 100, 250 and 500 µM bilirubin in the presence of 500 µM $CuCl_2$; lane 6-7, 100, 250 and 500 µM bilirubin in the presence of 500 µM $FeCl_2$, lane 9-11, 100, 250 and 500 µM bilirubin in the presence of 500 µM $ZnCl_2$, respectively and lane 12, native calf thymus DNA in the same reaction buffer without treatment.

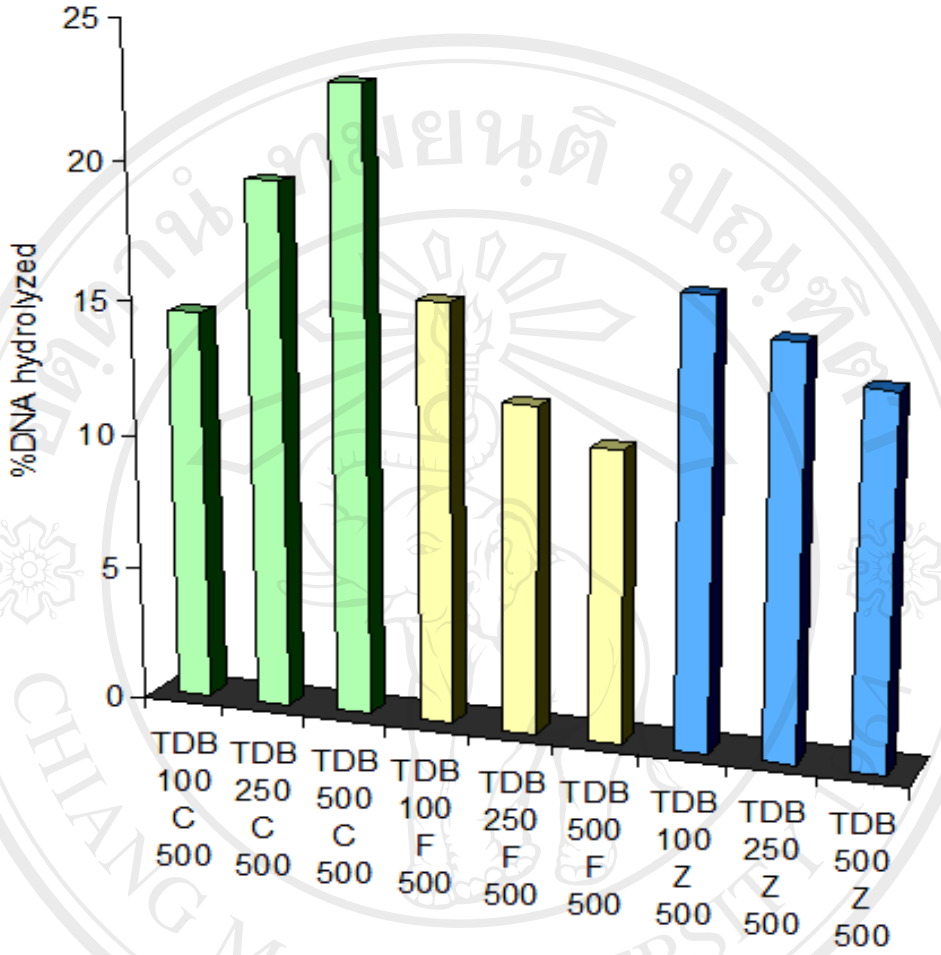

Figure 13. The acid soluble DNA hydrolyzed (%) estimated by the DPA reaction. The DNA degradation (the products of the reactions in Figure 12.), resulted by the interactions of 500 μM bilirubin with increasing concentrations of transition metal ions, were incubated with S_1 Nuclease for 4 hours for the DNA strand scission before reacting with diphenylamine reagent. DNA hydrolyzed (%) is defined as the quantity of DNA cleavage in the reaction mixture compared with that obtained from the total DNA hydrolyzed (100% degradation) reaction.

For abbreviations; TDB = Tris buffer containing DNA and bilirubin, C = Copper, F = Ferrous, Z = Zinc, and the numbers indicate the concentrations of bilirubin or interaction metal ions for example; TDB 500 C 500 is the reaction in 10 mM Tris buffer pH 7.5 containing DNA, 500 μM bilirubin and 500 μM Cu(II), respectively.

The degradation of DNA in the incubated reaction in the presence of interaction metal ions and increasing bilirubin concentrations reaction shown in Figure 12 were also assessed by the diphenylamine reaction. The acid soluble nucleotides obtained from the conversion of double stranded DNA by the S_1 nuclease of all reactions were compared with the 100% DNA hydrolysis. The increase in DNA hydrolysis was observed when DNA was incubated in the reactions containing 500 μM $CuCl_2$ with increasing concentrations of bilirubin. The decrease in DNA hydrolysis was observed when DNA was incubated with 500 μM $FeCl_2$ or 500 μM $ZnCl_2$ with increasing concentrations of bilirubin ranged from 100-500 μM (Figure13). These results were agreed with the DNA patterns detected by agrose gel electrophoresis in Figure 12 The percentages of DNA hydrolyzed obtained from maximum DNA degradation of 3 types of 500 μM transition metal ions interacted with the optimal bilirubin concentrations were 23.0% , 15.6% and 16.4% respectively.

1.3 The effect of transition metal ions concentrations on DNA degradation *in Vitro* at fixed 500 μM bilirubin concentrations

Figure 14 shows the effect of increasing concentration of transition metal ions interacted with fixed bilirubin concentrations on DNA degradation by agarose gel electrophoresis. It was found that the maximum DNA degradation was observed in the reaction of DNA containing 500 μM bilirubin and 500 μM $CuCl_2$. DNA degradation caused by the lower concentrations of $CuCl_2$ or by various concentrations of $FeCl_2$ or $ZnCl_2$ were not dependent on the transition metal ion concentrations. The DPA reaction in Figure 15 were agreed with the DNA degradation results. The percentages of DNA hydrolyzed obtained from maximum DNA degradation caused by interaction of 500 μM $CuCl_2$ and 500 μM bilirubin was 23.0%.

Figure 14. The effect of increasing interaction ion concentrations interacted with fixed concentration of bilirubin caused DNA degradation *in vitro*. The 0.5 mL reaction mixtures contained 500 µM concentration of bilirubin and increasing concentration of intraction metal ions from 100-500 µM in the presence of 500 µg of calf thymus DNA. The reactions were mixed and incubated at 37 ^{O}C for 4 hours before detection of DNA degradation by agarose gel electrophoresis.

Lane 1, DNA marker; lane 2, DNA alone; lane 3-5, 100, 250 and 500 µM $CuCl_2$ in the presence of 500 µM bilirubin; lane 6-7, 100, 250 and 500 µM $FeCl_2$ in the presence of 500 µM bilirubin, lane 9-11, 100, 250 and 500 µM $ZnCl_2$ in the presence of 500 µM bilirubin, respectively and lane 12, native calf thymus DNA in the same reaction buffer without treatment.

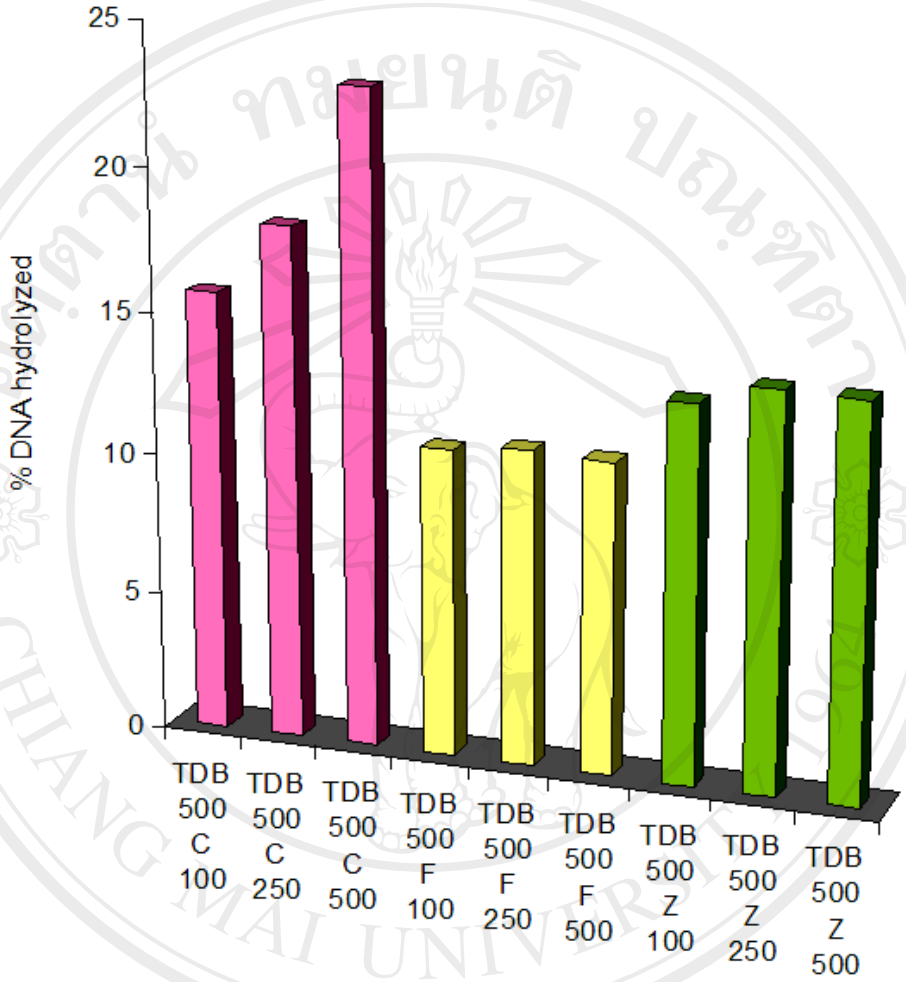

Figure 15. The acid soluble DNA hydrolyzed(%) estimated by the DPA reaction. The DNA degradation (the products of the reactions in Figure 12), resulted by the interactions of increasing transition metal ions with 500 µM bilirubin, were incubated with S_1 Nuclease for 4 hours for the DNA strand scission before reacting with diphenylamine reagent. DNA hydrolyzed(%) is defined as the quantity of DNA cleavage in the reaction mixture compared with that obtained from the total DNA hydrolyzed (100% degradation) reaction.

For abbreviations; TDB = Tris buffer containing DNA and bilirubin, C = Copper, F = Ferrous, Z = Zinc, and the numbers indicate the concentrations of bilirubin or interaction metal ions for example; TDB 500 C 100 is the reaction of 10 mM Tris buffer pH 7.5 containing DNA, 500 µM bilirubin and 100 µM $CuCl_2$, respectively.

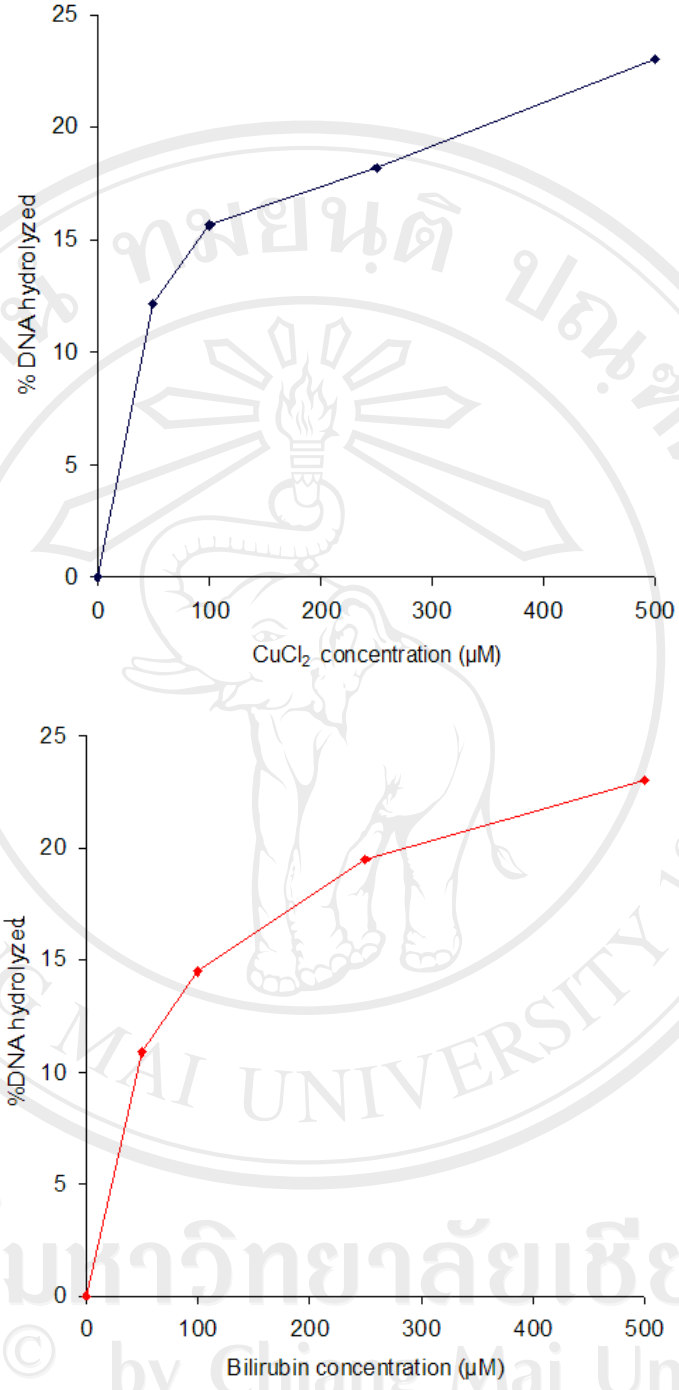

Figure 16. Degradation of calf thymus DNA by the bilirubin-Cu (II) complex detected by DPA reaction.

Upper: The effect of increasing concentrations of $CuCl_2$ interacted with 500 µM bilirubin on DNA degradation.

Lower: The effect of increasing concentrations of bilirubin interacted with 500 µM $CuCl_2$ on DNA degradation.

The concentrations dependent degradation of calf thymus DNA by bilirubin-Cu(II) complex was shown in Figure 16. $CuCl_2$ showed a dose dependent increase in calf thymus DNA degradation (Figure 16; upper). A similar increase in DNA hydrolysis was observed when calf thymus DNA was incubated with 0.5 mM Cu(II) and with increasing concentrations of bilirubin (Figure 16; lower).

2. Time dependent kinetic of DNA degradation

Figure 17 shows the effect of time on DNA degradation. The highest degradation was observed in the incubated reaction at 8 hours in the reaction of DNA treated with the equal concentration of bilirubin and $CuCl_2$ at 250 µM and slightly increased up to 48 hours after incubation at 37 ^{O}C. DNA degradation was increased as a function of incubation time. This experiment also shows the concentration dependent of bilirubin and $CuCl_2$ concentrations. Molar ratio of bilirubin and $CuCl_2$ at 1: 1 was required for the maximum DNA degradation.

The result of DNA degradations pattern on agarose gel shown in Figure 17 shows good agreement with the percentage of DNA hydrolysis found in Figure 18. The highest percentage of DNA hydrolysis of DNA degradation was demonstrated in the reaction of 250 µM bilirubin interacted with 250 µM $CuCl_2$ at 37 ºC and at 48 hour incubation. The DNA hydrolyzed was 12.5% which is significant higher than those of other interaction concentrations.

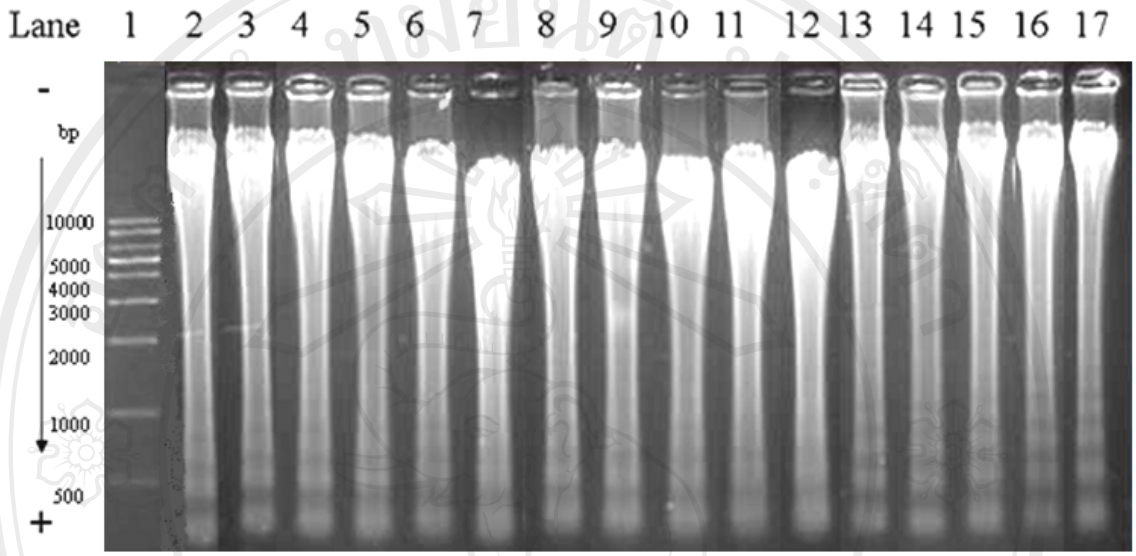

Figure 17. Time dependent kinetic of DNA degradations caused by the interactions of bilirubin with transition metal ions. The rates of DNA degradations were observed by incubating three sets of reaction mixtures (five in each set containing identical reaction mixtures) at 37 ºC for 4, 8, 12, 24 and 48 hours, respectively. At the end of the incubation time, the effect of bilirubin interacted with metal ion on the DNA degradation were determined by agarose gel electrophoresis

Lane 1, DNA marker; lane 2, DNA alone; lane 3-7 were 500 µM $CuCl_2$ in the presence of 50 µM bilirubin and 500 µg calf thymus DNA, lane 8-12 were 250 µM $CuCl_2$ in the presence of 250 µM bilirubin and 500 µg calf thymus DNA and lane 13-17 were 50 µM $CuCl_2$ in the presence of 500 µM bilirubin and 500 µg calf thymus DNA, respectively. The 5 identical reaction mixtures of each set were incubated at 37 ºC for 4, 8, 12, 24 and 48 hours, respectively.

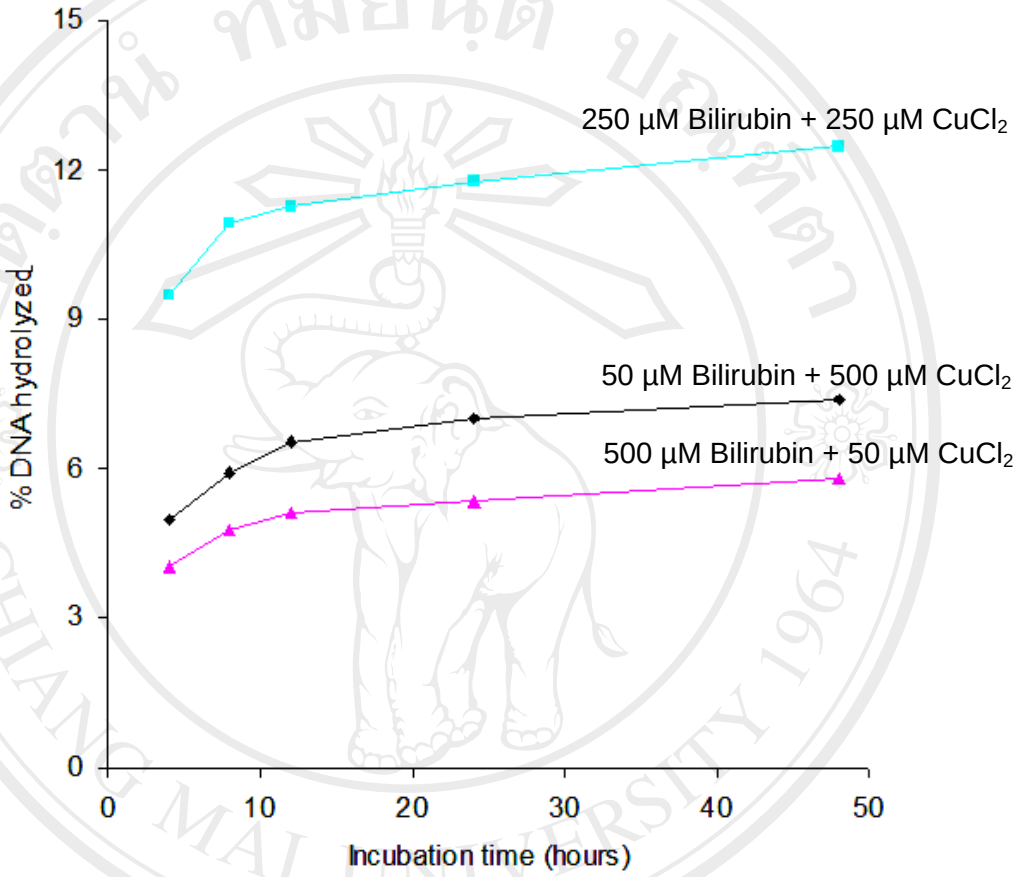

Figure 18. The time dependent kinetic of DNA degradations by the bilirubin-Cu(II) complex. Five identical reaction mixtures were incubated at 37 ºC for 4, 8, 12, 24 and 48 hours, respectively (the reaction mixtures from Figure 16). At the end of the incubation time, S_1 nuclease digestion was performed as described previously. Acid soluble deoxyribonucleotides were determined by the DPA reaction.

IV. Investigation of the Mechanism of Bilirubin Interacted with Metal Ion Influenced DNA Degradation *in vitro*

The causes of DNA degradation mostly concerned with the reactive oxygen species (ROS) especially the hydroxyl radical ($OH^{\cdot}$) produced in the reaction mixture. Therefore, in order to understand the mechanism of DNA degradation caused by the interaction of bilirubin with the interaction metal ions, the further experiments were conducted to detect malondialdehyde (MDA) which is the oxidized product of the degraded DNA.

1. Standard graph of malondialdehyde (MDA)

Figure 19 is the standard graph of MDA by TBA reaction. The graph was linear when the concentrations of MDA in the reactions were from 0-40 µM, giving the absorbance values from 0 to 0.4. The absorbance of the determination was correlated with the absorbance at 532 nm (Figure 20) at $y = 0.0091x$ and R is equal to 0.9998.

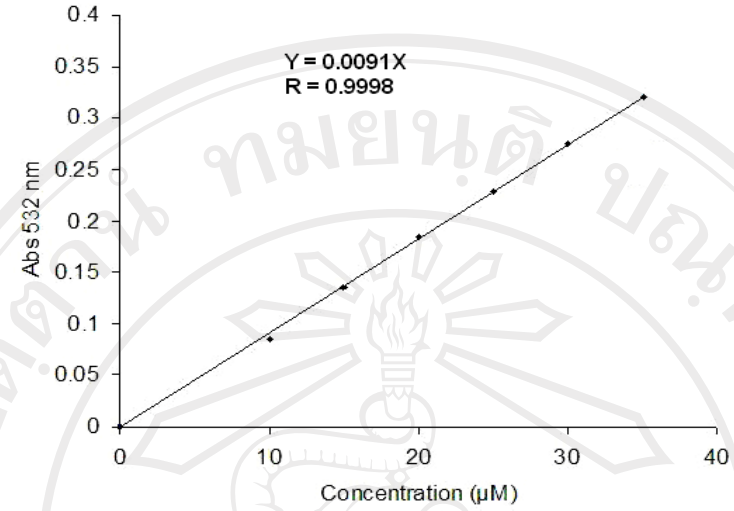

Figure 19. Standard graph of MDA assay

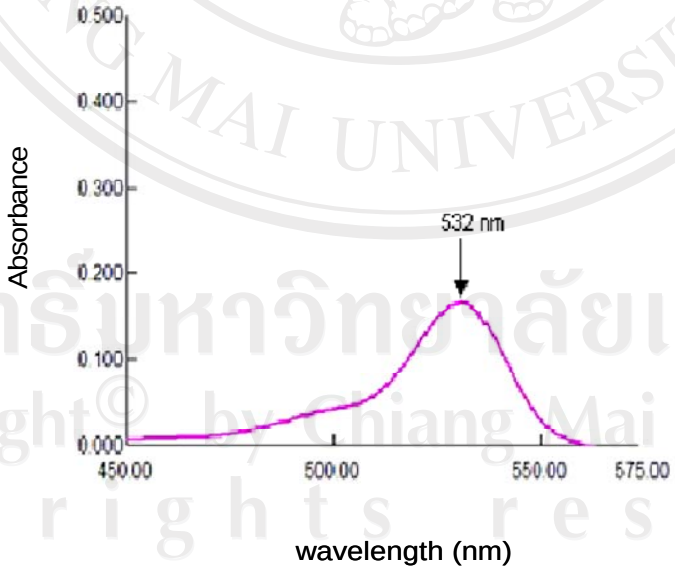

Figure 20. The typical absorbance peak of MDA by TBA reaction

2. Free radical generation resulted by the interaction of bilirubin with metal ions

This experiment tried to prove that there was the generation of free radical as a result of the interaction of bilirubin with the transition metal ions. Three types of hydroxyl scarvengers *i.e*. thiourea, sodium azide and manitol were added in the reaction mixtures to scarvenge of the free radical formed. Results of scarvenging activities were reported as % inhibition of DNA hydrolysis shown in the following figures.

2.1. The inhibition effect of free radical scavengers on bilirubin – $CuCl_2$ complex

Figure 21 is the DNA patterns by agarose gel electrophoresis. Lane 3-6 are the effect of bilirubin alone on the degradation of DNA used as a control of the experiment without interaction metal ions. Lane 7-10 are the effect of $CuCl_2$ alone on the degradation of DNA used as a control of experiment without bilirubin. Lane 11-14 are the interested reactions contained both bilirubin and $CuCl_2$ (1:1 molar ratio). Lane 3, 7 and 10 were the experiments without scarvengers and lane 4, 8 and 12 contained 50 mM thiourea, lane 5, 9 and 13 contained 50 mM sodium azide, and lane 6, 10 and 14 contained contained 50 mM mannitol, respectively. DNA in each lane was degraded (also in the DNA alone in lane 2) as a result of the incubation at 37 ^{O}C for 4 hours (degraded about 18%). The reactions in the presence of scarvenger in each lane inhibited more DNA degradation than that found in the controls. Figure 22 is the inhibition of DNA degradation reported as % DNA hydrolysis. From the results, it can be concluded that thiourea was the most potent free radical scavenger to inhibit DNA degradation caused by the interaction of bilirubin and $CuCl_2$ *in vitro.* The DNA hydrolyzed (%) was decreased from 30.5% to 28.2, 16.0 and 1.2% when the reaction

were added with mannitol, sodium azide and thiourea respectively (comparing only in the reaction mixtures contained DNA, bilirubin and $CuCl_2$). There were good agreement of the DNA patterns in Figure 21 and % DNA hydrolysis in Figure 22.

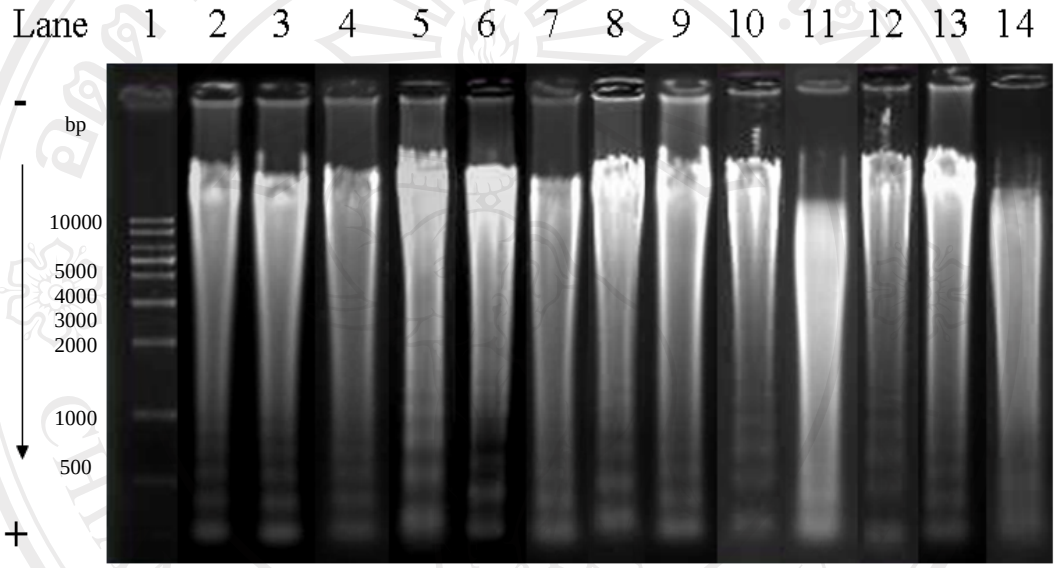

Figure 21. The inhibition effect of free radical scavengers on DNA degradation caused by bilirubin-$CuCl_2$ interaction. All reaction mixtures were incubated at 37 ^{O}C for 4 hours before agarose gel electrophoresis.

Lane 1, DNA marker; lane 2, DNA alone; Lane 3, DNA treated with 200 µM bilirubin; Lane 4-6, DNA treated with 200 µM bilirubin in the presence of 50 µM sodium azide, thiourea and mannitol, respectively; Lane 7, DNA treated with 200 µM $CuCl_2$; Lane 8-10, DNA treated with 200 µM $CuCl_2$ in the presence of 50 µM sodium azide, thiourea and mannitol, respectively; Lane 11, DNA treated with 200 µM bilirubin and 200 µM $CuCl_2$; Lane 12-14, DNA treated with 200 µM bilirubin and 200 µM $CuCl_2$ in the presence of 50 µM sodium azide, thiourea and mannitol, respectively.

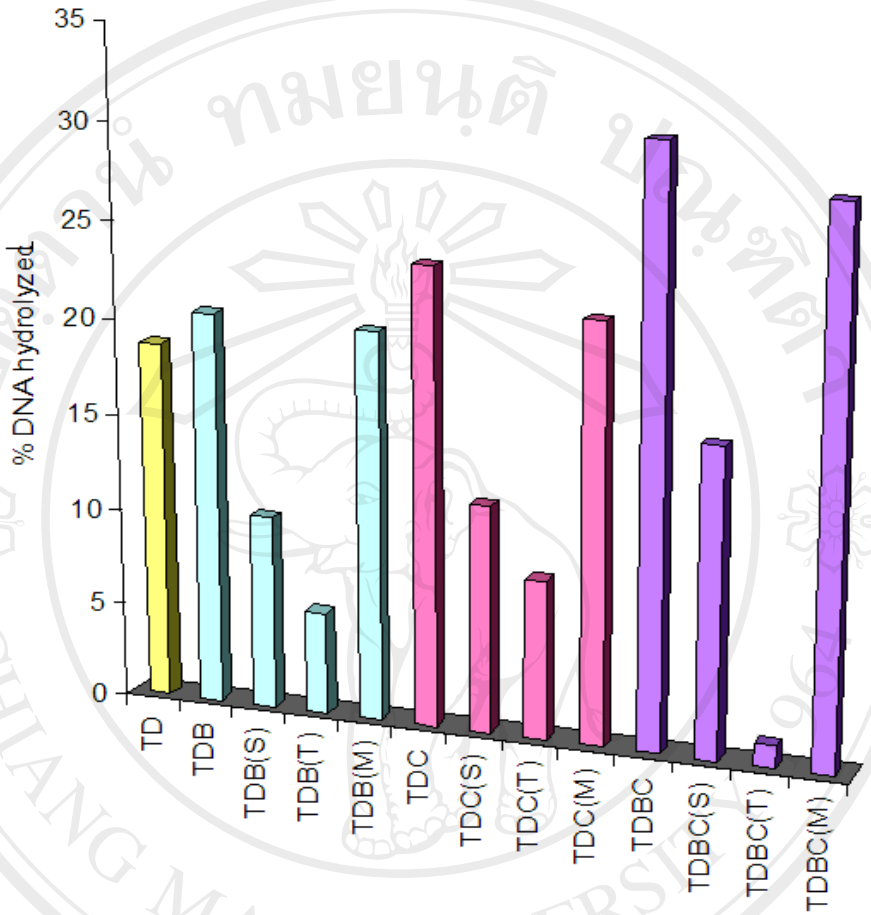

Figure 22. The decrease in percentage of DNA hydrolysis by the inhibition effect of free radical scavengers. The reaction mixtures contained 200 μg of DNA, 200 μM bilirubin and 200 μM $CuCl_2$ in the presence and absence of scavengers; 50 μM sodium azide, 50 μM thiourea or 50 μM manitol. The DNA degradation caused by bilirubin-$CuCl_2$ complex produced free hydroxyl radical was inhibited by different scavengers with various degrees.

The examples of abbreviations; TD = Tris buffer containing DNA; TDB = Tris buffer containing DNA and Bilirubin, *etc*. C = Copper, (S) = Sodium azide, (T) = Thiourea and (M) = Manitol.

2.2 The inhibition effect of free radical scavengers on bilirubin – $FeCl_2$ complex

Alike experiment VI.2 (2.1), The inhibition effect of free radical scavengers on bilirubin–$FeCl_2$ complex were shown in Figure 23 for DNA degradation patterns and Figure 24 for % of DNA hydrolyzed. Lane 3-6 are the effect of bilirubin alone on the degradation of DNA used as a control of the experiment without interaction metal ions. Lane 7-10 are the effect of $FeCl_2$ alone on the degradation of DNA used as a control of experiment without bilirubin. Lane 11-14 are the interested reactions contained both bilirubin and $FeCl_2$ (1:1 molar ratio). Also, Lane 3, 7 and 10 were the experiments without scarvengers and lane 4, 8 and 12 contained 50 mM thiourea, lane 5, 9 and 13 contained 50 mM sodium azide, and lane 6, 10 and 14 contained contained 50 mM mannitol, respectively. DNA in each lane was degraded (also in the DNA alone in lane 2) as a result of the incubation at 37 ^{O}C for 4 hours (degraded about 18%). The reactions in the presence of scarvenger in each lane inhibited more DNA degradation than that found in the controls. Figure 24 is the inhibition of DNA degradation reported as % DNA hydrolyzed. The percentages of DNA hydrolysis patterns was more likely to be in the same order as the effect of bilirubin-CuCl2 interaction (Figure 22). The DNA hydrolyzed (%) by the effect of bilirubin-$FeCl_2$ complex was decreased from 25.8% to 22.4, 9.1 and 4.4% when the free radical in reactions were scarvenged by mannitol, sodium azide and thiourea respectively.

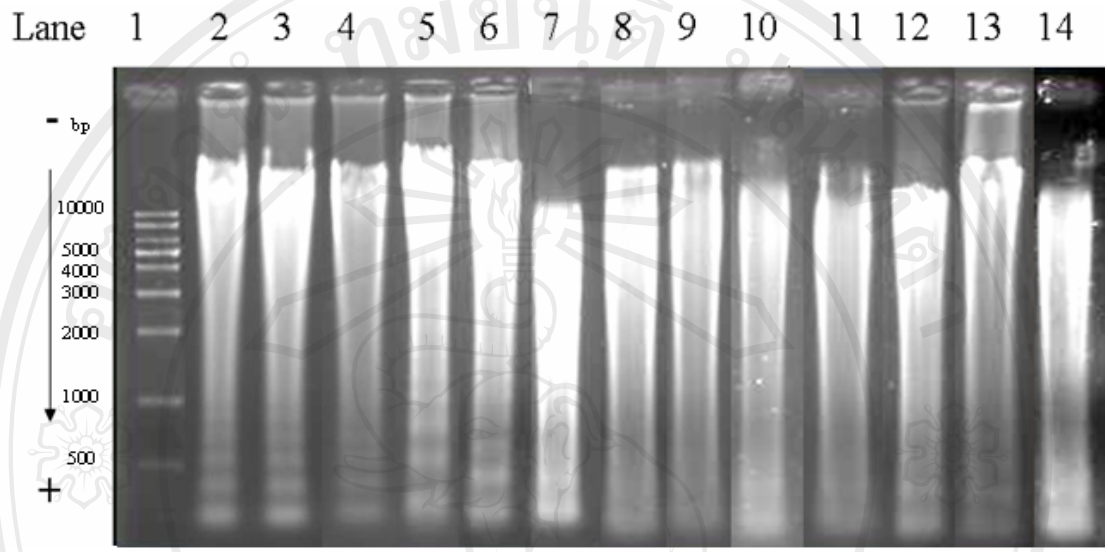

Figure 23. The inhibition effect of free radical scavengers on DNA degradation caused by bilirubin-$FeCl_2$ interaction. All reaction mixtures were incubated at 37 ^{O}C for 4 hours before agarose gel electrophoresis.

Lane 1, DNA marker; lane 2, DNA alone; Lane 3, DNA treated with 200 µM bilirubin; Lane 4-6, DNA treated with 200 µM bilirubin in the presence of 50 µM sodium azide, thiourea and mannitol, respectively; Lane 7, DNA treated with 200 µM $FeCl_2$; Lane 8-10, DNA treated with 200 µM $FeCl_2$ in the presence of 200 µM sodium azide, thiourea and mannitol respectively; Lane 11, DNA treated with 200 µM bilirubin and 200 µM $FeCl_2$; Lane 12-14, DNA treated with 200 µM bilirubin and 200 µM $FeCl_2$ in the presence of 50 µM sodium azide, thiourea and mannitol, respectively.

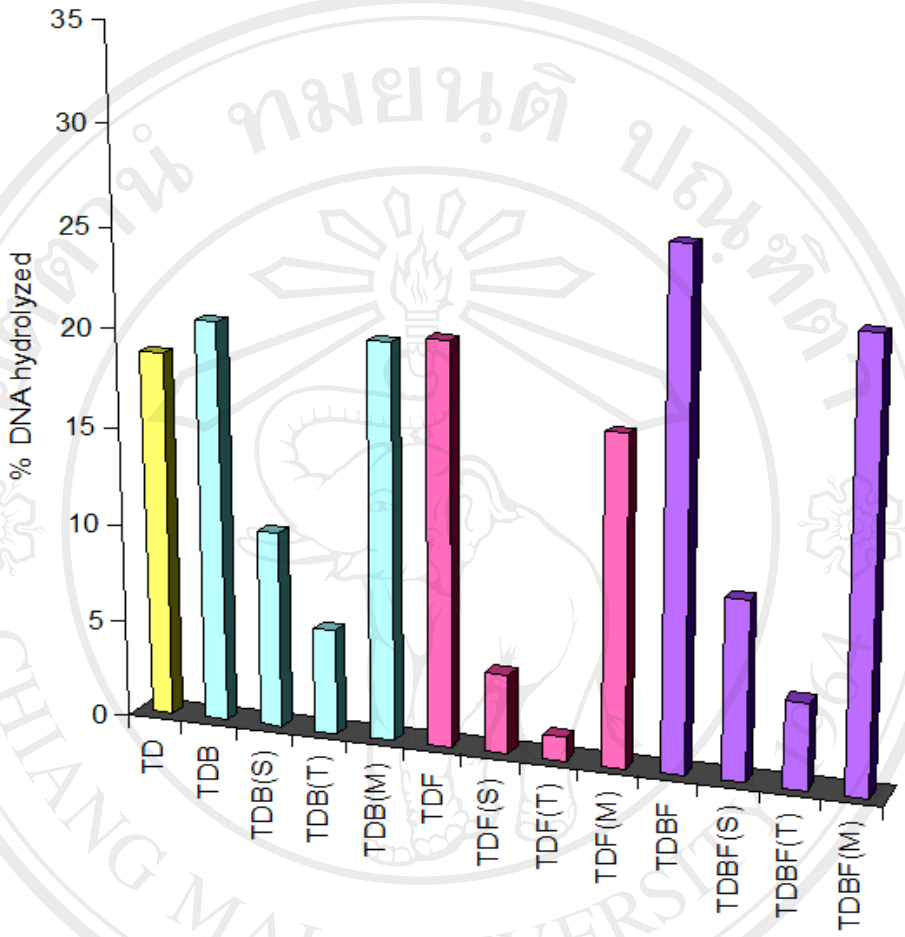

Figure 24. The decrease in percentage of DNA hydrolysis by the inhibition effect of free radical scavengers. The reaction mixtures contained 200 μg of DNA, 200 μM bilirubin and 200 μM $FeCl_2$ in the presence and absence of scavengers; 50 μM sodium azide, 50 μM thiourea or 50 μM mannitol. The DNA degradation caused by bilirubin-$FeCl_2$ complex produced free hydroxyl radical was inhibited by different scavengers with various degrees.

The examples of abbreviations; TD = Tris buffer containing DNA; TDB = Tris buffer containing DNA and bilirubin, *etc.* C = Copper, (S) = Sodium azide, (T) = Thiourea and (M) = Manitol.

2.3 The inhibition effect of free radical scavengers on bilirubin–$ZnCl_2$ complex

The scarvenger effects of mannitol, sodium azide and thiourea on the free radical produced as a result of bilirubin formed complex with $ZnCl_2$ were shown in Figure 25 and 26. Figure 25 is the DNA degradation patterns and Figure 26 is the % of DNA hydrolysis, respectively. The control of experiments were as in Figure 23 and 24. Lane 11-14 are the interested reactions contained both bilirubin and $ZnCl_2$ (1:1 molar ratio). Also, Lane 3, 7 and 10 were the experiments without scarvengers, and lane 4,8 and 12 contained 50 mM thiourea, lane 5, 9 and 13 contained 50 mM sodium azide, and lane 6, 10 and 14 contained 50 mM mannitol, respectively. DNA in each lane was degraded (also in the DNA alone in lane 2) as a result of the incubation at 37 ^{O}C for 4 hours (degraded about 18%). The reactions in the presence of scarvenger in each lane inhibited more DNA degradation than that found in the controls. The DNA hydrolyzed (%) by the effect of bilirubin-$ZnCl_2$ complex was decreased from 28.7% to 25.8%, 11.9% and 5.3% when the free radical in the reaction mixtures were scarvenged by mannitol, sodium azide and thiourea respectively.

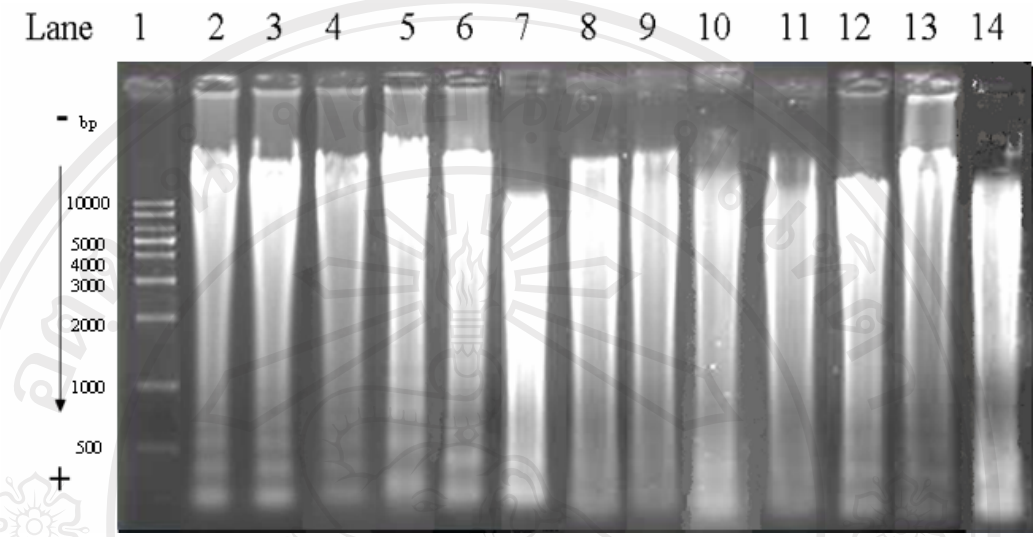

Figure 25. The inhibition effect of free radical scavengers on DNA degradation caused by bilirubin-$ZnCl_2$ interaction. All reaction mixtures were incubated at 37 ^{O}C for 4 hours before agarose gel electrophoresis. Lane 1, DNA marker; lane 2, DNA alone; Lane 3, DNA treated with 200 µM bilirubin; Lane 4-6, DNA treated with 200 µM bilirubin in the presence of 200 µM Sodium azide, Thiourea and Mannitol, respectively; Lane 7, DNA treated with 200 µM $ZnCl_2$; Lane 8-10, DNA treated with 200 µM $ZnCl_2$ in the presence of 200 µM Sodium azide, Thiourea and Mannitol respectively; Lane 11, DNA treated with 200 µM bilirubin and 200 µM $ZnCl_2$; Lane 12-14, DNA treated with 200 µM bilirubin and 200 µM $ZnCl_2$ in the presence of 200 µM Sodium azide, Thiourea and Mannitol, respectively.

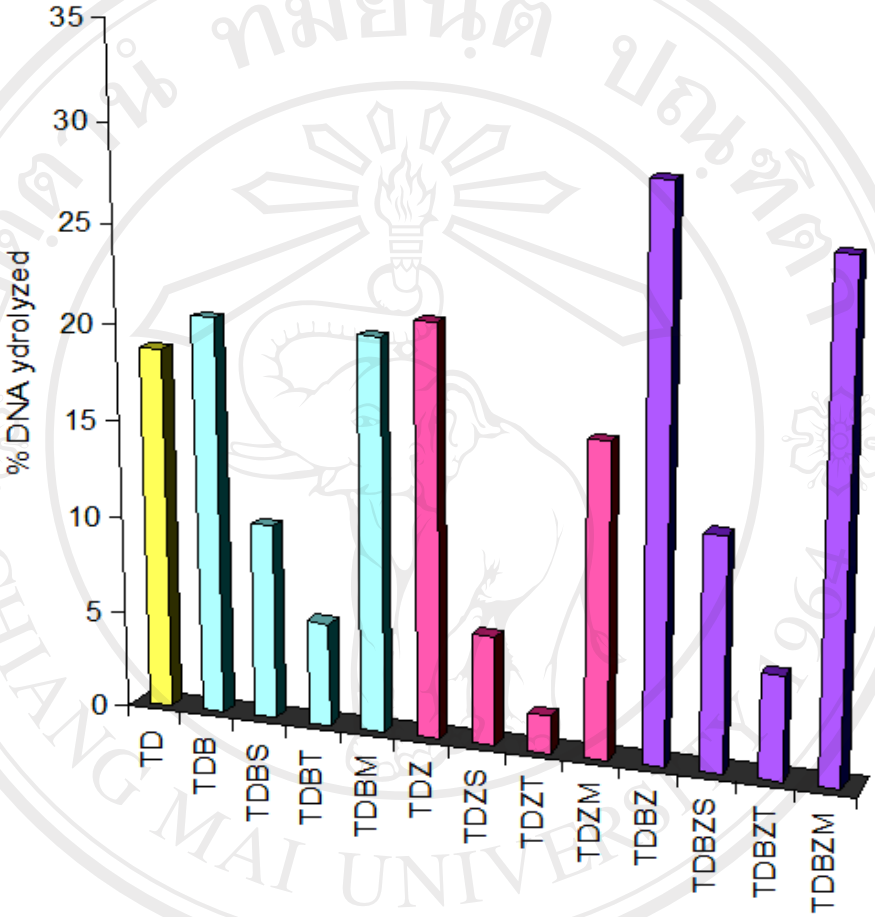

Figure 26. The decrease in percentage of DNA hydrolysis by the inhibition effect of free radical scavengers. The reaction mixtures contained 200 μg of DNA, 200 μM bilirubin and 200 μM $ZnCl_2$ in the presence and absence of scavengers; 50 μM sodium azide, 50 μM thiourea or 50 μM mannitol. The DNA degradation caused by bilirubin-$ZnCl_2$ complex produced free hydroxyl radical was inhibited by different scavengers with various degrees.

The examples of abbreviations; TD = Tris buffer containing DNA; TDB = Tris buffer containing DNA and bilirubin, *etc.* C = Copper, (S) = Sodium azide, (T) = Thiourea and (M) = Manitol.

From all results it can be concluded that thiourea was the most powerful free radical scavengers to inhibit DNA degradation by the reaction of bilirubin–$CuCl_2$, bilirubin–$FeCl_2$ and bilirubin–$ZnCl_2$ complex in *vitro.* The DNA hydrolyzed (%) in the control groups show the same patterns of inhibitions by those scarvengers as in the reactions in the presence of bilirubin-metal ion complex. The inhibition effects of all scarvengers were demonstrated in Figure 27.

The bilirubin-transition metal ions DNA breakage reaction was inhibited by various radical scavengers; mannitol, sodium azide and thiourea. Thiourea was the most powerful inhibitor of the DNA breakage *in vitro.* Results of % inhibition of DNA breakage by scarvengers were reverted of the % DNA hydrolyzed. The greater of the % inhibition of DNA degradation, the lesser of the % DNA hydrolyzed.

Table 1 shows the concentrations of MDA (μM) produced in the reaction mixtures containing various conditions of bilirubin interacted with metal ions in the absences (controls) and presences of free radical scavengers. Results of % inhibition of DNA degradation were agreed with the concentrations of MDA produced in each condition. These results can be concluded that a cause of DNA degradation *in vitro* in the reaction mixtures was the free radical produced by the bilirubin - interaction ion complex.

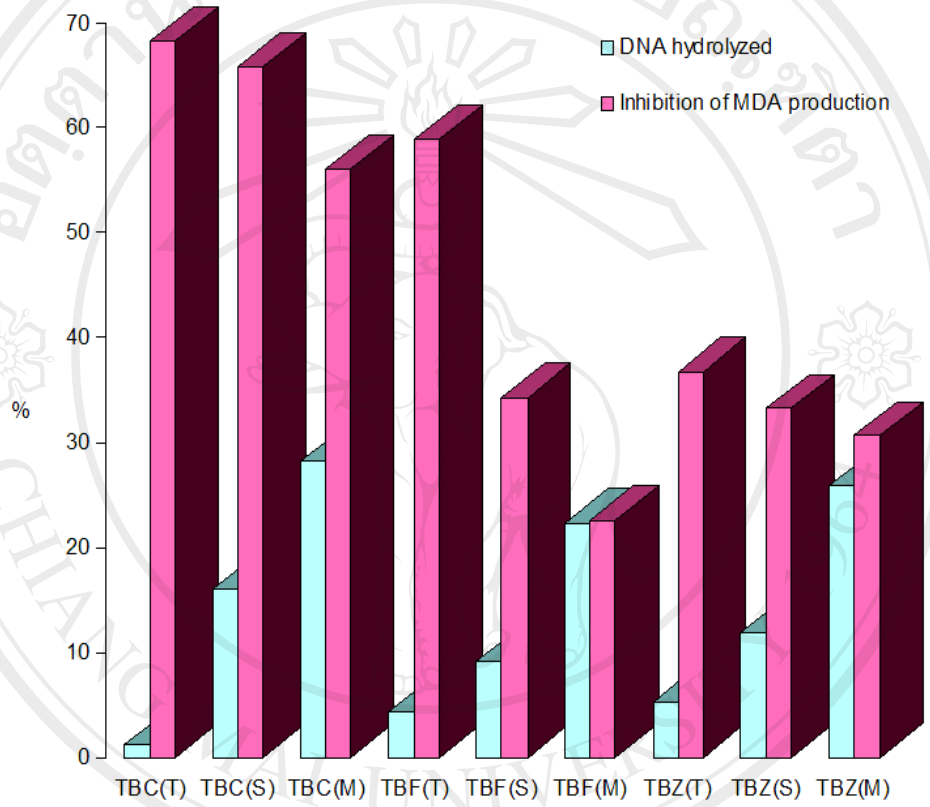

Figure 27. Inhibition of calf thymus DNA degradation after treatment with bilirubin and transition metal ions in the presence of free radical scavengers (thiourea, sodium azide and mannitol).

All reaction mixtures contained 200 μg calf thymus DNA. The examples of abbreviations; TBC(T) = Tris buffer containing DNA and bilirubin, in the presence of thourea, *etc.* (T) = Thiourea, (S) = Sodium azide, (M) = Manitol, C = Copper, F = Ferrous and Z = Zinc, respectively.

Table 1. Inhibition of calf thymus DNA degradation after treatment with bilirubin and transition metal ions (200 µM each) in the presence of free radical scavengers (50 mM in all cases)

Conditions	DNA hydrolyzed (%)	Inhibition (%)	MDA (µM)
DNA + Bilirubin + $CuCl_2$	30.6	-	29.0
DNA + Bilirubin + $CuCl_2$+ thiourea	1.2	68.2	9.2
DNA + Bilirubin + $CuCl_2$ + sodium azide	16	65.8	9.9
DNA + Bilirubin + $CuCl_2$ + mannitol	28.2	56.0	12.6
DNA + Bilirubin + $FeCl_2$	25.9	-	16.0
DNA + Bilirubin + $FeCl_2$ + thiourea	4.4	58.9	6.6
DNA + Bilirubin + $FeCl_2$ + sodium azide	9.2	34.2	10.5
DNA + Bilirubin + $FeCl_2$ + mannitol	22.4	22.6	12.4
DNA + Bilirubin + $ZnCl_2$	28.7	-	12.5
DNA+ Bilirubin + $ZnCl_2$ + thiourea	5.4	36.8	7.9
DNA + Bilirubin + $ZnCl_2$ + sodium azide	12.0	33.3	8.4
DNA + Bilirubin + $ZnCl_2$ + mannitol	25.9	30.7	8.7

3. Investigation of free radical generation by the MDA assay by spectrophotometric scanning.

MDA of bilirubin (alone) in the TBA reaction yielded the yellow-brown colour product giving the maximum absorption at 464 nm. After the component in the reaction was extracted with chloroform the solution was separated into 2 parts. Bilirubin was dissolved in chloroform (yellow colour) and the result of MDA product (pink colour) dissolved in aqueous buffer. The maximum absorption peak obtained when scanning aqueous part is at 565 nm indicated that the pink colour formed in the reaction was not the product of bilirubin in the reaction reacted with TBA color reaction.

TBA reaction of bilirubin in the presence of $CuCl_2$ gave a final colour product with the maximum absorption observed at 464 nm (yellow-brown colour) After the reaction was extracted with chloroform the solution in the aqueous part was colourless which means that there was no MDA product in this reaction mixture. This results can be concluded that no hydroxyl radicals produced in the absence of DNA(Figure 28).

Figure 29 shows the scanning of the reactions containing bilirubin and DNA in the presence and absence of $CuCl_2$. The peak of bilirubin in the presence of DNA and $CuCl_2$ before extraction was also at 464 nm. The peak of MDA at 532 nm appeared at the right shoulder of the absorption spectrum scanning. No peak of MDA appeared in the aqueous extract. However there was the other product of bilirubin reacted with TBA and absorbed at 565 nm.

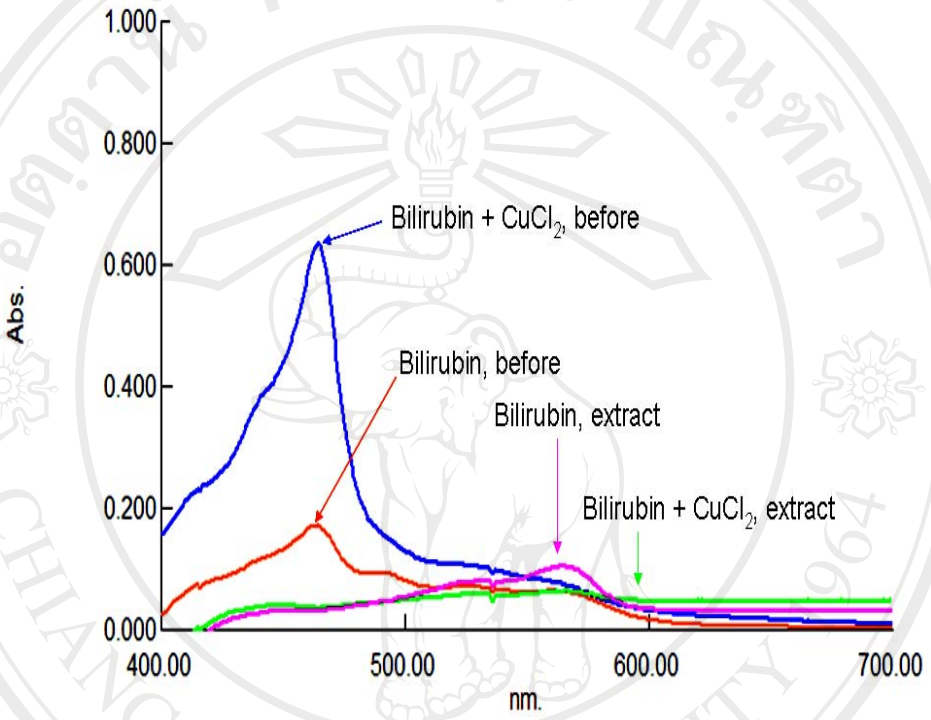

Figure 28. The scanning of the reaction of bilirubin in the presence and absence of $CuCl_2$. Bilirubin alone before and bilirubin+$CuCl_2$ before are the absorption spectra of the product(s) in the TBA reaction. Bilirubin alone, extract and bilirubin+$CuCl_2$ extract are the absorption spectra of the aqueous buffer (s) after separating bilirubin molecule into the chloroform.

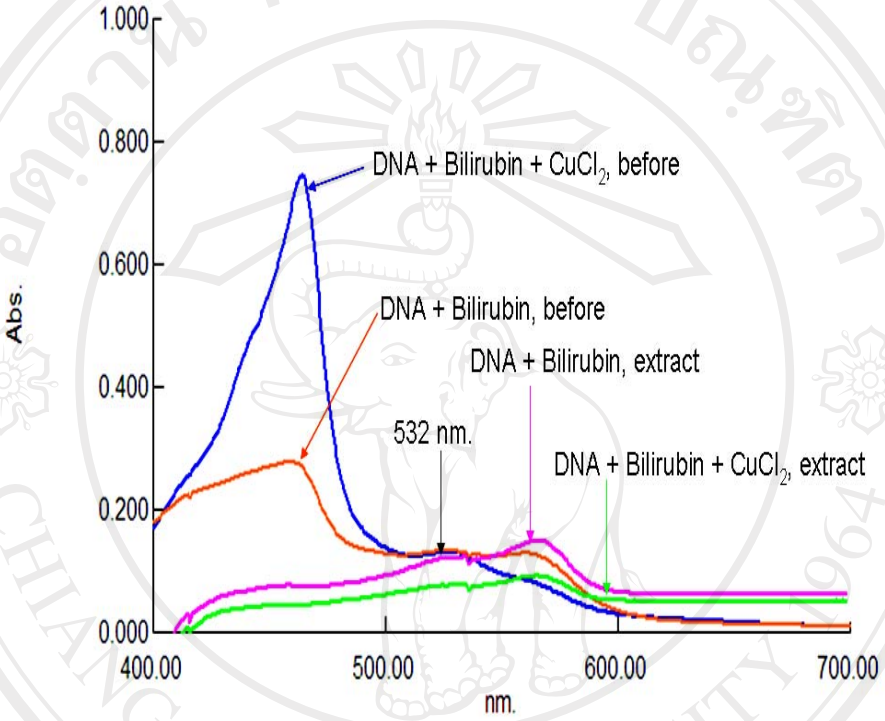

Figure 29. The scanning of the reaction of bilirubin in the presence and absence of $CuCl_2$. Bilirubin+DNA before and bilirubin+$CuCl_2$+DNA before are the absorption spectra of the product(s) in the TBA reaction. Bilirubin+ DNA, extract and bilirubin+$CuCl_2$+DNA extract are the absorption spectra of the aqueous buffer (s) after separating bilirubin molecule into the chloroform .