APPENDICES

APPENDIX A

Reagent Preparation

I Reagents for bilirubin standard

1. Unconjugated bilirubin standard 400 mg/dL

Dissolved 10 mg unconjugated bilirubin standard in 1 mL 0.1 M of Na_2CO_3 and add with 0.75 mL 0.1 M of NaOH mix throughly and then make up to 2.5 mL with 10 mM of Tris-HCl, pH 7.5.

2. 10 mM Tris hydrochloride buffer, pH 7.5

Dissolved 0.788 g in approximately 400 mL distilled water. Adjust to pH 7.5 with NaOH. Made to 500 mL with distilled water and stored at room temperature.

II. Reagent for studying of the interaction of bilirubin with cationic metal ions

1. Cupric chloride

Dissolved 0.017 g Cu(II)$Cl_2$2H_2O in 25 mL 10 mM Tris-HCl, pH 7.5

2. Ferrous chloride

Dissolved 0.02 g $FeCl_2$4$H_2$0 in 25 mL 10 mM Tris-HCl, pH 7.5

3. Zinc chloride

Dissolved 0.017 g Zinc chloride in 25 mL 10 mM Tris-HCl, pH 7.5

5 Calf thymus DNA

Dissolved calf thymus DNA approximately 0.030 g in 0.75 mL 10 mM Tris-HCl, pH 7.5 and then read absorbance at 260 nm. Calculate amount of DNA by the following formula:

DNA concentration (µg/mL) = (OD_{260}) X (dilution factor) X

(50 µg DNA /mL) / (1 OD_{260} unit)

Then dilute to 500 µg/mL by 10 mM Tris-HCl, pH 7.5

6. Quercetin

Dissolved 4 mg quercetin powder in acetonitrile 12 ml and pipette 1.2 mL of this solution to 10 mM Tris-HCl, pH 7.5. Made to 10 mL with 10 mM Tris-HCl, pH 7.5. The reagent were mixed and freshly prepare before used.

7. Bathocuproine

Dissolved 6 mg bathocuproine powder in methanol 3 ml and heated at 40 ºC for 40 minutes. The solution was mixed every 10 minutes. Adjust to 5 mL with methanol. The reagent were mixed and freshly prepare before used.

8. 50 mM Sodium azide

Dissolved 0.812 g sodium azide in 10 mL of 10 mM Tris-HCl, pH 7.5.

9. 50 mM Thiourea

Dissolved 0.951 g thiourea in 10 mL of 10 mM Tris-HCl, pH 7.5.

10. 50 mM Mannitol

Dissolved 2.277 g mannitol in 10 mL of 10 mM Tris-HCl, pH 7.5. heated at 50 ºC for 5 minutes

11. BSA as molar ratio of bilirubin 0.5:1.0 (253 µM)

Dissolved 0.017 g BSA powder in 10 mL of 10 mM Tris-HCl, pH 7.5.

12. BSA as molar ratio of bilirubin 1.0:1.0 (506 µM)

Dissolved 0.334 g BSA powder in 10 mL of 10 mM Tris-HCl, pH 7.5.

13. BSA as molar ratio of bilirubin 1.0:1.0 (506 µM)

Dissolved 0.501 g BSA powder in 10 mL of 10 mM Tris-HCl, pH 7.5

III Reagant for S_1 nuclease reaction

1. 1X enzyme buffer

To make 1 mL of 1X enzyme buffer diluted 100 µL of 10X enzyme buffer with 900 µL autoclaved distilled water

2. S_1 nuclease

To make 1 mL of 100 unit by 1X enzyme buffer diluted 10 µL of stock S_1 nuclease with 990 µL 1X enzyme buffer and stored at -20 ºC.

3. 1 mM Zinc sulphate in 100 mM Sodium acetate buffer, pH 4.5

Dissolved 0.072 g $ZnSO_47H_2O$ and 2.05 g C_2H_3NaO in approximately 200 mL in distilled water. Adjust to pH 4.5 with glacial acetic acid and made to 250 mL with distilled water and stored at 4 ºC.

4. 1 mg/dL Bovine Serum Albumin (BSA) in distilled water

Dissolved BSA powder 0.5 g in 50 mL autoclaved distilled water and stored at 4 ºC.

5. 14% Perchloric acid

Add approximately 7 mL of 12.63 N $HClO_4$ to distilled water. Made to 50 mL with distilled water and stored at 4 ºC.

IV Reagant for Diphenylamine reaction

1. 1N Perchloric acid

Add approximately 8.6 mL of 12.63 N $HClO_4$ to distilled water. Made to 100 mL with distilled water and stored at 4 ºC.

2. 1.6% acetaldehyde

Add approximately 2 mL of ice cold Conc. Acetaldehyde and made to 100 mL

with ice-cold distilled water then stored at 4 ºC.

3. Diphenylamine reagent

1.6% acetaldehyde	0.25	mL
Diphenylamine powder	0.75	mL
Conc. H_2SO_4	0.75	mL
Glacial acetic aid	50.0	mL

All reagent were mixed and freshly prepare before used.

V Reagent for electrophoresis on agarose gel

1. 0.5 M EDTA , pH 8.0

Dissolved EDTA 3.722 g in approximately 10 mL distilled water. Adjust to pH 8.0 with NaOH. Made to 20 mL with distilled water

2. 10X Tris-Acetate-EDTA (TAE) buffer

Tris (hydroxymethyl aminomethane)	48.4	g
Glacial actetic acid	11.42	mL
0.5 M EDTA , pH 8.0	20	mL

Made to 1000 mL with distilled water and stored at 4 ºC.

3. 1X Tris-Acetate-EDTA (TAE) buffer

To make 1 L of 1X TAE buffer diluted 100 mL of 10X TAE buffer with 900 mL distilled water

4. 1% Agarose gel

Dissolved agarose powder 0.5 g in 50 mL TAE buffer

5. 6X loading dye

Bromphenol blue	0.025	g

Sucrose 4 g

Dissolved in 10 mL distilled water and then filter through the filter membrane. Stored at 4 ºC.

6. 0.5 µg/mL Ethidium bromide

Add 10 µL of 10 µg/ml Ethidium bromide to TAE buffer 200 mL

VI Reagent for Malondialdehyde assay

1. 1% (W/V) Thiobarbituric acid in 0.05 M NaOH

Dissolved NaOH 0.1 g in in approximately 45 mL distilled water and then dissolved Thiobarbituric acid 0.5 g in this solution. Made to 50 mL by distilled water

2. 2.8% (W/V) Trichloroacetic acid

Dissolved trichloroacetic acid 1.4 g in 50 mL distilled water

APPENDIX B

Confirmation of Cu(I) Production by Bathocuproine

Method:

The 2 ml reaction mixtures contained 10 mM Tris-HCl, pH 7.5 and 15 µM bilirubin, 50 µM $CuCl_2$ and 300 µM bathocuproine. The spectra was recorded immediately on the addition of components indicated.

Result :

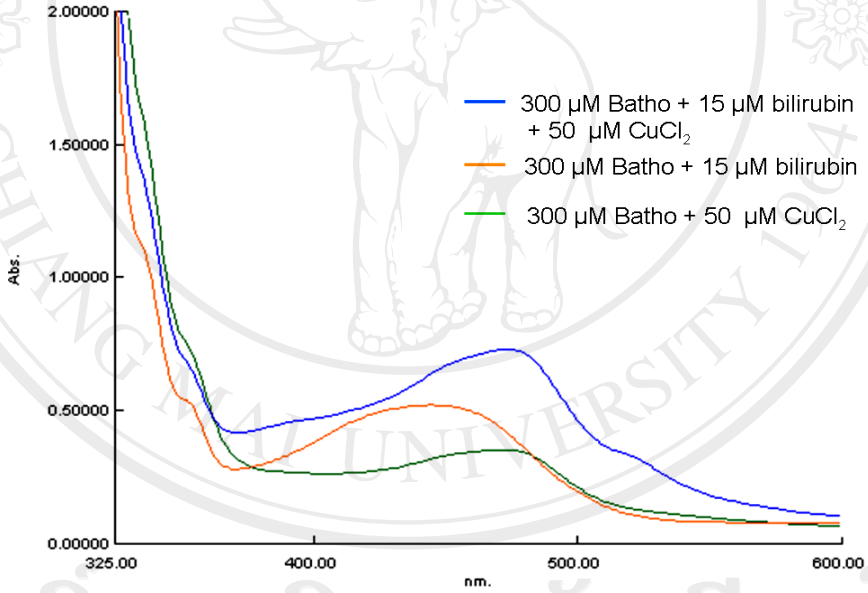

Figure 30. Detection of bilirubin induced Cu(I) production by bathocuproine.

Bathocuproine as a selective Cu(I) sequestering agent. The Cu(I) chelates have absorption maximum at 490 nm. Under our experimental conditions, neither Cu(I) nor bilirubin interferes with this maximum, whereas bilirubin and Cu(II) react to generate Cu(I) (figure 30). The implication of this finding is that Cu(II) is reduced by bilirubin in the complex to generate Cu(I).

APPENDIX C

Absorption spectra of bilirubin-Cu(II) complex with and without scavenger (thiourea)

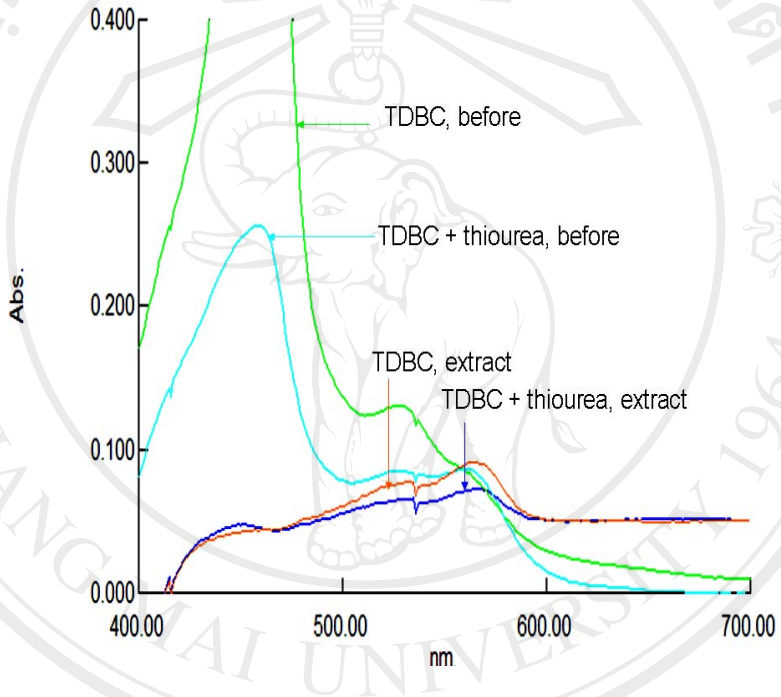

Figure 31. The scanning of the reaction of bilirubin-$CuCl_2$ in the presence and absence of scavenger (thiourea). Bilirubin-$CuCl_2$ + DNA before and bilirubin-$CuCl_2$ + DNA + thiourea before are the absorption spectra of the product(s) in the TBA reaction. Bilirubin-$CuCl_2$ + DNA extract and bilirubin-$CuCl_2$ + DNA + thiourea extract are the absorption spectra of the aqueous buffer (s) after separating bilirubin molecule into the chloroform .

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