

## CHAPTER 2

### MATERIALS AND METHODS

#### 1. Materials and equipments

##### 1.1 Preparation of *Hibiscus sabdariffa* Linn. water extract

Fresh calyces of *Hibiscus sabdariffa* Linn. (HS) were obtained from Amphur Jana, Songkhla, Thailand. The specimen (NO. SKP 1090819) was deposited in the herbarium of Pharmaceutical Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The calyces of HS were dried at 50°C. Water extraction was carried out by boiling calyces of HS (5 kg) in water (30 l) for 15 min. The water extract was filtered through nylon cloth and then dried using vacuum dry at 40°C for 8-10 hr. The yield (calculated on the dried extract) was 4.6% of the fresh calyces. Dried HS water extract (HSE) was packed in tight containers and kept in a desiccator at room temperature.

The HS calyces and extract were obtained from Associate Professor Dr. Arunporn Itharat, Faculty of Medicine, Thammasat University. HS calyces was accepted by standard of RAISE (Rural and Agricultural Incomes with a Sustainable Environment) (<http://www.raise.com>). It showed the standard values as followed; total ash 7.81% (RAISE standard < 10%), moisture 6.2% (RAISE standard < 12%) and acid insoluble ash 0.12 (RAISE standard < 1.5%). Determination of quantitative active compound of HS water extract by HPLC found that it composed of quercetin 0.433 mg/g and delphinidine-3-sambucoside 3.74 mg/g.

## 1.2 Experimental animals

One hundred eighty male Wistar rats weighing  $229.8 \pm 1.0$  g were obtained from Southern Laboratory Animal Facility (Prince of Songkla University, Songkhla, Thailand). Rats were housed in hanging stainless steel cages under controlled conditions (temperature 23-25°C, humidity 50-55% and 12 hr light/dark cycle). They were given a commercial animal feed (S.W.T., Thailand) and tap water *ad libitum*. Experimental protocols were approved by the Prince of Songkla University Animal Ethics Committee (เลขที่ 0521.11/101).

## 1.3 Chemicals

1. Acetic acid ( $\text{CH}_3\text{COOH}$ ), Merck, Darmstadt, Germany
2. Albumin from bovine serum, Sigma, St. Louis, USA
3. Ammonium sulfamate ( $\text{H}_6\text{N}_2\text{O}_3\text{S}$ ), Fluka, Buchs, Switzerland
4. Anthrone ( $\text{C}_{14}\text{H}_{10}\text{O}$ ), Fluka, Buchs, Switzerland
5. Calcium chloride dihydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ), Merck, Darmstadt, Germany
6. *Cis*-diaminedichloroplatinum [cisplatin,  $(\text{NH}_3)_2\text{Cl}_2\text{Pt}$ ], Dabur Pharma, Brotivala, Distt Solan, India
7. Copper (II) sulfate pentahydrate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ), Sigma, St. Louis, USA
8. D(-)-Fructose ( $\text{C}_6\text{H}_{12}\text{O}_6$ ), Fluka, Buchs, Switzerland
9. D(+)-Glucose anhydrous ( $\text{C}_6\text{H}_{12}\text{O}_6$ ), Fluka, Buchs, Switzerland
10. Di-sodium hydrogen phosphate anhydrous ( $\text{Na}_2\text{HPO}_4$ ), Carlo Erba Reagenti, Milan, Italy

11. Enzymatic colorimetric test for urea (UREA liquicolor), Human,  
Wiesbaden, Germany
12. Glycine ( $\text{NH}_2\text{CH}_2\text{COOH}$ ), Ajax Finechem, Seven Hill, New South Wales,  
Australia
13. Heparin, Leo pharmaceutical, Ballerup, Denmark
14. Hydrochloric acid ( $\text{HCl}$ ), Merck, Darmstadt, Germany
15. 4-(2-Hydroxyethyl)piperazine-1-ethanesulonic acid (HEPES,  
 $\text{C}_8\text{H}_{18}\text{N}_2\text{O}_4\text{S}$ ), Fluka, Buchs, Switzerland
16. Inulin, Sigma, Steinheim, Germany
17. Magnesium sulfate heptahydrate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ), Merck, Darmstadt,  
Germany
18. n-Butanol [ $\text{CH}_3(\text{CH}_2)_3\text{OH}$ ], Lab-Scan Analytical Science, Bangkok, Thailand
19. N-(1-naphthyl)-ethylenediamine dihydrochloride ( $\text{C}_{12}\text{H}_{16}\text{Cl}_2\text{N}_2$ ), Merck,  
Darmstadt, Germany
20. Para-aminohippuric acid ( $\text{C}_9\text{H}_{10}\text{N}_2\text{O}_3$ ), Sigma, London, United Kingdom
21. Pentobarbitone sodium (Nembutal<sup>®</sup>), Ceva Sante Animale, Libourne, France
22. Potassium chloride ( $\text{KCl}$ ), Merck, Darmstadt, Germany
23. Sodium acetate trihydrate ( $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ ), Merck, Darmstadt, Germany
24. Sodium chloride ( $\text{NaCl}$ ), Ajax Finechem, Seven Hill, New South Wales,  
Australia
25. Sodium dihydrogen phosphate dihydrate ( $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ ), Merck,  
Darmstadt, Germany
26. Sodium dodecyl sulfate ( $\text{C}_{12}\text{H}_{25}\text{NaO}_4\text{S}$ ), Fluka, Buchs, Switzerland
27. Sodium hydrogen carbonate ( $\text{NaHCO}_3$ ), Riedel-de Haen, Seelze, Denmark

28. Sodium hydroxide (NaOH), Carlo Erba Reagenti, Milan, Italy
29. Sodium nitrite (NaNO<sub>2</sub>), Carlo Erba Reagenti, Milan, Italy
30. Sulfuric acid (H<sub>2</sub>SO<sub>4</sub>), Merck, Darmstadt, Germany
31. 1,1,3,3-Tetramethoxypropane (C<sub>7</sub>H<sub>16</sub>O<sub>4</sub>), Fluka, Buchs, Switzerland
32. 2-Thiobarbituric acid (C<sub>4</sub>H<sub>4</sub>N<sub>2</sub>O<sub>2</sub>S), Sigma, Deisenhofen, Germany
33. Trichloroacetic acid (CCl<sub>3</sub>COOH), Carlo Erba Reagenti, Milan, Italy

#### **1.4 Equipments**

1. Automatic pipettes, Eppendorf, Geritebau, Hamburg, Germany
2. Balance, Model CC023D10ADBAAA, Avery Barkel, United Kingdom.
3. Centrifuge, Model 4232, A.L.C., Italy
4. Digital infusion syringe pump, Model SP101i, World Precision Instruments, Sarasota, Florida, USA
5. Electrolyte analyzer, Model AVL 988-3AVL, AVL List GmbH Medizintechnik, Hersteller, Graz, Austria
6. Hand homogenizer grinder, Model 41640-323, China
7. Homeothermic blanket control unit, Harvard, Southnatick, Massachusetts, USA
8. IEC Micro-Hematocrit centrifuge, Model MB, Needham Heights, Massachusetts, USA
9. Infusion pump, Model 075 A, Harvard, Southnatick, Massachusetts, USA
10. pH meter, Model UB-5, Denver Instruments, Denver, USA
11. Polyethylene tube, Clay Adams, Parsippany, New Jersey, USA
12. Polygraph, Model RPS7C 8B, Grass, Massachusetts, USA
13. Pressure transducer, Model Statham P23XL, Grass, Massachusetts, USA

14. Spectrophotometer, Model Spectro SC, Labomed, Culver City, California, USA
15. Spectrophotometer, Model Ultrospec III 80-2097-62, Pharmacia LKB  
Biochrom, Cambridge, United Kingdom
16. Surgery set
17. Tachograph preamplifier, Model 7P44A, Grass, Massachusetts, USA
18. Thermostat-heater-circulator, Model D1, HAAKE, Denmark
19. Ultrasonic cleaning bath, Model 460/H, Elma, Germany

## **2. Method**

### **2.1 Experimental design**

The experiments were divided into 2 parts. Part I was designed to investigate the dose-response of cisplatin on renal functions and a minimal dose that induced ARF will be chosen for Part II study. Criteria of ARF are acute reduction in glomerular filtration rate (< 50%) and increase in blood urea nitrogen. In Part I, rats were injected intraperitoneally (i.p.) with cisplatin at the doses of 4.5, 6, 7.5 and 9 mg/kg and 0.9% NaCl (18 ml/kg) was used as vehicle control. The numbers of rats treated with each dose of cisplatin were between 12-17. Three days after the injection, renal functions (using clearance study) and renal lipid peroxidation (using MDA measurement) were assessed.

Part II study was designed to investigate the protective effect of HSE on renal functions and renal lipid peroxidation in cisplatin-induced ARF rats. HSE treatments were planned as short and long term treatments. In short term treatment group, oral administration of HSE at the dose of 250 mg/kg (5 ml/kg) was performed

twice, 24 hr and 10 min prior either cisplatin or vehicle injection and distilled water was used as vehicle solvent of HSE. In long term treatment group, the animals were received HSE (similar dose to short term treatment) by gavage daily for 7 days before injection of either cisplatin or vehicle and a dose on the day of injection and following another 2 days after the injection. Three days after cisplatin was given, all rats were experimented with similar protocol for measuring renal functions and renal MDA. The numbers of rats in each group were between 11-26.

## **2.2 Experimental protocol for clearance study**

### **2.2.1 Animal preparation**

On the day of experiment, rat was anaesthetized by i.p. injection with pentobarbitone sodium (Nembutal<sup>®</sup>) at the dose of 60 mg/kg. Additional dose(s) was given when necessary. The animal was then placed on a homeothermic blanket control unit to maintain body temperature at 37°C. A tracheostomy, using a short piece of polyethylene tube-240 (PE-240), was performed in order to facilitate the respiration. The right carotid artery was cannulated using PE-50. This was done in order to collect blood samples and to directly and continuously measure the arterial blood pressure using pressure transducer connected to polygraph. The left jugular vein was catheterized using PE-50 for perfusion of clearance markers solution. Urine samples were collected in pre-weighed vial through a cannula (PE-200) placed in the urinary bladder via suprapubic midline incision.

### **2.2.2 Protocol of clearance study**

After surgical preparation, isotonic saline solution containing 0.5% para-aminohippuric acid (PAH) and 0.5% inulin was perfused intravenously at a rate of 1.6 ml/hr/100 g throughout the experiment.

Clearance study was performed for 2 hr after 60 min of equilibration period. The duration of urine collection in each sample was 30 min. Blood samples (0.7 ml) were taken three times. Two samples were taken at the midpoint of the first and the last urine period and another one at the end of the second urine sample collection. A small amount of this blood was used to determine the hematocrit. The remained blood was centrifuged (4000 rpm) for 5 min. Then, the plasma was collected and stored frozen for determination of the concentrations of clearance markers, BUN and electrolytes. The packed blood cells were resuspended to the original volume in isotonic saline and returned to the animal via the jugular vein catheter.

At the end of the experiments, the animals were sacrificed by intravenous injection of saturated magnesium sulfate solution. Both kidneys were decapsulated and weighed immediately after blotting with a tissue paper.

### **2.2.3 Analytical methods**

#### **2.2.3.1 Estimation of blood urea nitrogen**

Blood urea nitrogen (BUN) was estimated by enzymatic method using urease enzyme kit. Absorbance was read at 578 nm by spectrophotometer.

### **2.2.3.2 Determination of Na<sup>+</sup> and K<sup>+</sup> in plasma and urine**

Na<sup>+</sup> and K<sup>+</sup> concentrations in plasma and urine samples were measured using an electrolyte analyzer. Urine samples were diluted with urine diluent (120 mM NaCl) and deionized distilled water.

### **2.2.3.3 Determination of inulin in plasma and urine**

Inulin concentration in plasma and urine samples was estimated by a spectrophotometric method (APPENDIX A). Absorbance was read at 620 nm using fructose as standard (Davidson *et al.*, 1963).

### **2.2.3.4 Determination of para-aminohippuric acid in plasma and urine**

PAH concentration in either plasma or urine samples was estimated by a spectrophotometric method (APPENDIX B). Absorbance was read at 540 nm (Smith *et al.*, 1945).

## **2.2.4 Calculations of clearance study**

2.2.4.1 Mean arterial blood pressure (MABP) was calculated from diastolic pressure + 1/3 pulse pressure. Pulse pressure is the difference pressure between systolic and diastolic pressure.

2.2.4.2 Urine flow rate was determined gravimetrically by collecting urine into pre-weighed vials and assuming a density of 1 g/ml.



1 MgSO<sub>4</sub>, 10 CH<sub>3</sub>COONa, 10 HEPES, 3 glucose and 2 glycine. When the kidneys were blood cleared, they were removed, decapsulated and chopped into a small piece with a scalpel and homogenized in an ice-cold 1.15% KCl (4 ml/g tissue) using hand homogenizers. After that, the homogenated samples were sonicated for one hour.

## **2.3.2 Analytical methods**

### **2.3.2.1 Determination of malondialdehyde content in renal tissue homogenate**

The malondialdehyde (MDA) content was assayed in the form of thiobarbituric acid reacting substances (modified from Ohkawa *et al.*, 1979). The amount of thiobarbituric acid reactive substances was determined from the standard curve generation by MDA from the acid hydrolysis of 1,1,3,3-tetramethoxypropane. The values of MDA were expressed as nmol/mg protein (APPENDIX C).

### **2.3.2.2 Determination of protein content in renal tissue homogenate**

Protein content of the renal tissue homogenate was determined by the micro-biuret method (Itzhaki and Gill, 1964), using albumin from bovine serum as standard (APPENDIX D).

## **2.4 Statistical analysis**

All data were expressed as mean  $\pm$  S.E.M. Analysis of variance (ANOVA) was employed to analyze the data. Multiple comparison was performed using Student-Newman-Keuls post hoc test to test the difference between the mean values.  $P < 0.05$  was considered statistically significant.