CHAPTER II

MATERIALS & METHODS

1. Materials

1.1 Equipments

- Rotary Evaporator (BüCHI Rotavapor RE 120, Becthai Bangkok Equipment & Chemical Co., Ltd., Thailand)
- Lyophylizer (Dyra-Dry II MP, Science Engineer International Co., Ltd., Thailand)
- Accutrend® meters GCT (Roche Diagnostics, Ltd., Germany)
- Balance (Satorius Basic BA610, Scientific Promotion Co., Ltd., Thailand)
- Desiccator (normal with plate 30 cm, China)
- Flexor E Automation (Baker instrument, Thailand)

1.2 Chemicals

- Glucose, anhydrous (Fluka, Switzerland)
- Streptozotocin (STZ) (Sigma Chemicals Co., Ltd., Germany)
- Glukotest® (urine) (Roche Diagnostics, Ltd., Germany)
- Accutrend® Glucose (blood) (Roche Diagnostics, Ltd., Germany)
- Humulin® R (Eli Lilly Asia Inc., Thailand)
- Diethyl ether (E. Merck, Damstadt, Germany)
- Formalin, 10 % (E. Merck, Germany)
- Isopropyl Alcohol, 70%v/v (Siribuncha &Co., Ltd., Thailand)
- Normal saline (0.9% sodium chloride injection) (General hospitial Products Public Co., Ltd., Pathum Thani, Thailand.)
- Commercial pellet food for rats (C.P. Mice Feed; S. W. T. Co. Ltd., Samutprakarn, Thailand)
- Heparin (Leo, Denmark)

- Ethylenediamine tetraacetic acid (EDTA) (E. Merck, Damstadt, Germany)

1.3 Plant material

The dried stem of *Coscinium fenestratum* of family Menispermaceae was purchased from a medicinal herbal drug store (FONG KAEWVONGSA), tambol Kuankao, amphur Siritorn, Ubonratchthani province, region the north-eastern part of Thailand.

2. Methods

2.1 Preparation of crude water extract of *C. fenestratum* (CE)

One hundred grams of dried stem of *C. fenestratum* was cut in to thin pieces and boiled in 500 ml of distilled water for extraction and filtered. The procedure was repeated three times and the water extract was pooled and concentrated using Rotary Evaporator at 70 °C. Extracts were pooled and lyophilized. Sixteen gram of yellowish-brown dried powder (Figure 12) was obtained and stored in a desiccator (25°C) until use. The crude water extract (CE) to be used in the experiment was freshly prepared by dissolving in distilled water.

Figure 12. Dried powder of crude water extract of *C. fenestratum* (CE)

2.2 Preparation of animal model

Male Wistar rats weighed 80-100 g were obtained from National Laboratory Animal Centre, Mahidol University, Bangkok, Thailand. Animals were acclimatized for 1 week before being used in the experiment started under a constant 12 hr (light: dark), temperature of 22-25 ^{o}C and humidity 55 ± 5%. Food and distilled water were given ad libitum. All animals were weighed and placed in individual stainless steel cage (8x10 in) at the day experiment started.

2.2.1 Diabetes mellitus induction in animal model

Normal male Wistar rats were fasted overnight (15-18 hr). They were diabetes mellitus induced with streptozotocin (STZ) which was freshly dissolved in normal saline at dose of 85 mg/kg body weight single intraperitoneal injection. Normal saline was injected in control rat. Animals in all groups consumed water and food as usual. Twenty-four hours after STZ injection, fresh urine sample was kept from each animal and urine glucose level was determined. Diabetes mellitus was confirmed in STZ-treated rats by measuring fasting blood glucose concentration within 48-72 hr after STZ injection. Rats having blood glucose concentration above 200 mg/dl were considered mild diabetes, moderate diabetes, whose basal glycemia ranged between 200 - 300 mg/dl and severe diabetes, whose basal glycemia equal to or higher than 300 mg/dl (Ganda *et al.*, 1976; Chen *et al.*, 1994; Rao *et al.*, 1995; Porte *et al.*, 1997; Pepato *et al.*, 2001; Alarcon-Aguilar *et al.*, 2000b).

2.2.2 Blood collection and determination

Blood samples were collected from tail vein. Blood glucose concentration was determined by Glucose-oxidase/mediator reaction test strips (Accutrend® Glucose, Roche Diagnostics, Ltd, Germany) using Accutrend® meters GCT. Blood glucose concentration was expressed in mg/dl.

2.2.3 Glucose determination from urine

Urine was collected for the semi - quantitative determination of glucose for the assessment of diabetes mellitus by Glucose-oxidase/peroxidase reaction (Glukotest®, Roche Diagnostics, Ltd., Germany).

2.2.4 Blood collection for blood clinical biochemistry analysis and hematologic analysis

At the end of experiment, rats were fasted overnight and anaesthetized with diethyl ether. Blood samples (3 ml) were obtained from the heart and divided into two portions. The first portion was collected in a 50 µl heparinized microcentrifuge tube and centrifuged at 3,000 rpm for 5 min. Supernatant (plasma) was separated for blood clinical biochemistry analysis by Flexor E automation. Blood glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), cholesterol, triglyceride, blood urea nitrogen (BUN) and creatinine were determined. The second portion was collected in an EDTA containing bottle. Then, it was mixed for 5 min by Hematology series cell mixer (Baker instrument, Thailand) and Complete Blood Counts (CBC) [total red cell counts, total white cell counts, differential white cell counts, hemoglobin (Hb) and Hematocrit (Hct) measurement] were measured by automate monitor (DANAM HC 5710, USA).

Euthanasia was performed using diethyl ether. Liver and pancreas were immediately isolated, weighed and gross pathology examined. The organs were then trimmed and preserved in 10% neutral formalin solution for at least twenty-four hours at room temperature before performing the Histopathology procedure (Clark *et al.*, 1973; Humason, 1967).

2.3 Experiments

2.3.1 The hypoglycemic effect of crude water extract from *C. fenestratum* (CE) in normal male Wistar rats

2.3.1.1 Single-oral dose of CE in glucose tolerance test (OGTT)

Normal male Wistar rats were randomly divided into 6 groups, each of 15 rats and fed single dose of CE as follows:

- Treatment group 1. 0.1 g/kg body weight.
- Treatment group 2. 0.25 g/kg body weight.

- Treatment group 3. 0.5 g/kg body weight.
- Treatment group 4. 0.75 g/kg body weight.
- Treatment group 5. 1 g/kg body weight.
- Control group. Distilled water 1 ml/kg body weight.

Rats were fasted overnight (15-18 hr). Blood samples were collected from tail vein at 30 min-intervals for determination of fasting blood glucose concentration. Single doses of CE or water were fed 30-min prior to feeding of glucose at 1 g/kg body weight, where blood was collected just before glucose feeding. Blood glucose concentrations was determined at time –30, 0, 30, 60, 90, 120, 150 and 180 min after feeding glucose (Sharma *et al.*, 1997; Peungvicha *et al.*, 1999; Perfumi *et al.*, 1991; Naik *et al.*, 1991).

2.3.1.2 Repeated–oral doses of CE in normal rats

Normal male Wistar rats were randomly divided into 2 groups of 10 rats each, and fed CE every day for 14 days.

- Group 1. 1 g/kg body weight/day.
- Group 2. Distilled water 1 ml/kg body weight as control.

Rats were fasted overnight (15-18 hr). The fasting blood glucose concentration at day 0 was determined. Each group of rats received oral CE or distilled water, once daily for 14 days. Blood samples from tail vein were collected for determination of glucose concentration at 6 hr after CE treated on day 7 and 14. On day 14, 16 hr after an oral CE or distilled water administration, blood as collected for blood clinical biochemistry and hematologic analysis. Finally, the animals were euthanized for necropsy and organs collection (Hawsawi *et al.*, 2001; Vetrichelvan and Jegadeesan, 2002).

2.3.2 The hypoglycemic effect of CE in diabetic male Wistar rats

2.3.2.1 Single-oral dose of CE in diabetic rats; non-fasting blood glucose concentration analysis

Diabetic male Wistar rats were randomly divided into 5 groups, 8 rats/group, and one groups of 6 normal rats.

- Treatment group 1; CE, orally at 0.1 g/kg body weight.
- Treatment group 2; CE, orally at 0.5 g/kg body weight.
- Treatment group 3; CE, orally at 1.0 g/kg body weight.
- Standard group; Regular insulin injection (HUMULIN® R) dose 5 IU/kg body weight, subcutaneously.
- Control group; Distilled water orally at 1 ml/kg body weight.
- Normal group; Non-diabetic rats.

Non-fasting blood glucose concentration was determined before administration of CE (at 0 hr). Rats in each group received an oral single-dose of CE, distilled water or subcutaneous injection regular insulin. Blood glucose concentration was measured at 1, 2, 3, 4, 5 and 6 hr after feeding CE (Aybar *et al.*, 2001; Alarcon-Aguilar *et al.*, 2000a).

2.3.2.2 Repeated-oral doses of CE in diabetic rats

Diabetic male Wistar rats were randomly divided into 5 groups, each of 12 rats except for one group of normal rat with only 6 rats.

- Treatment group 1; CE, orally at 0.1 g/kg body weight/day.
- Treatment group 2; CE, orally at 0.5 g/kg body weight/day.
- Treatment group 3; CE, orally at 1 g/kg body weight/day.
- Standard group; Regular insulin injection (HUMULIN® R), at 5 IU/kg body weight, subcutaneously.
- Control group; Distilled water orally at 1ml/kg body weight as control.
- Normal group; Non-diabetic rats.

Diabetic rats and normal rats were fasted overnight (15-18 hr). Fasting blood glucose concentration was measured on day 0 at 8.00 a.m. Each group of rats received orally CE, distilled water or subcutaneous injection regular insulin, once daily for 14 days. Blood samples were collected from tail vein for determination of blood glucose concentration at 22-24 hr after CE administration on day 7 and 14. On day 14, 16 hr after last the 14^{th} dose of CE oral administration, distilled water or injected insulin, blood was collected for blood chemistry and hematology analysis.

Finally, the animals were euthenized for necropsy and organ collection (Alarcon-aguilar *et al.*, 2000a; Zhang, 2000).

2.3.4 The acute toxicity and LD_{50} determination tested in normal male Wistar rats

Normal male Wistar rats were divided into 4 groups, each of 10 rats weighing 80-100 g each. Rats were fed CE in one single dose. The lowest effective dose with hypoglycemic actively in normal rats is 0.1 g/kg body weight.

- Treatment group 1; CE, orally at 5 g / kg body weight.
- Treatment group 2; CE, orally at 10 g / kg body weight.
- Treatment group 3; CE, orally at 20 g / kg body weight.
- Control group; Distilled water orally at 3 ml/kg body weight.

Rat received orally single-dose of CE or distilled water. Food and water consumed during the experimental period were measured. General behavior, clinical signs and any toxicity effect within 1-4 hr after administration were observed i.e., fur, mucous membranes, respiratory system, convulsion, diarrhea, lethargy and coma. In addition, their weights were recorded everyday for a period of five days. Numbers of rats died within 24 hr were recorded. On day 5, blood was collected for blood clinical biochemistry and hematologic analysis. Euthanasia, necropsy and organs collection was performed (OCEC, 1981; Al-Khazraji *et al.*, 1993; Majnarich, 1996; Abdel-Barry *et al.*, 1997b; Peungvicha *et al.*, 1999).

2.4 Statistical analysis

All data were expressed as mean ± standard error of mean (S.E.M.). Statistical comparisons were made using analysis of variance (ANOVA), with post-hoc range tests followed by the Least Significant Difference test (LSD).