CHAPTER II

MATERIALS & METHODS

1.Materials

1.1 Equipments

- Rotary Evaporator (BUCHI Rotavapor RE 120, Becthai Bangkok Equipment & Chemical Co., Ltd., Thailand)
- IR Spectrometer (Perkin Elmer, FTIR spectrometer spectrum 2000)
- Nuclear Magnetic Resonance Spectrometer JNM-A500
- Spectronic® GENESYS™5 Spectrophotometer
- pH meter (METTLER TOLEDO MP 230, Thailand)
- Balance (METTLER TOLEDO MT/UMT Balance, Thailand)
- Hot plate
- Desiccator (normal with plate 30 cm., China)
- Accutrend® meter GCT (Roche Diagnostics, Ltd., Germany)

1.2 Chemicals

- Absolute ethanol (Merck., Germany)
- Methanol (HPLC grade: Merck., Germany)
- Ammonia solution 25% (analytical grade : Merck., Germany)
- Silica gel 60 (0.063-0.200 mm.) (Merck., Germany)
- Silica gel 60 F_{254} TLC plate (Merck., Germany)
- Berberine hemisulfate (SIGMA, Germany)

- Potassium bromide (for IR spectroscopy : Merck., Germany)
- Tetradeuteromethanol
- Glucose, anhydrous (Fluka, Switzerland)
- Accutrend® Glucose (blood) (Roche Diagnostics, Ltd., Germany)
- Mice Feed (C.P. Mice Feed; S.W.T.Co.Ltd., Samutprakarn, Thailand)

2.Methods

2.1 Preparation of plants material

The dried stems of HAMM were collected from a medicinal herbal drug store (Fong Kaewvongsa), Sirintorn district, Ubon Ratchatani province, the North-eastern part of Thailand.

2.1.1 Macroscopic characterization of HAMM

Macroscopic character of Hamm had been studied by examining leave and stem in comparative with the authentic of other Khamin Khruea in the standard Herbarium (Museum of Natural Medicine, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.1.2 Preparation of crude water extract of HAMM

The dried pieces of HAMM (100 g) was boiled in 500 ml of water and kept at 60-70^{0}C for 20 minutes, then filtered. The same procedure was repeated twice. The water extract was pooled and concentrated using rotary evaporator at

70^0C and dried using spray dryer. The dried powder of HAMM are stored in a desiccator (25^0C) until use.

2.1.3 Purification of HAMM

Sixteen grams of crude water extract from *C. fenestratum* was dissolved in 1 litre distilled water and filtered through a Whatman ® filter paper No. 93. The pH of the solution was adjusted to 2 with 4 M hydrochloric acid. A yellow precipitate was formed, the precipitate was filtered and dried (Cf). The precipitate was dissolved 1 gram in 20 ml distilled water and further purified by column chromatography using (7x 30 cm^2) of silica gel 60 with methanol- water-25% ammonia (8:1:1) as mobile phase. The column was eluated and 20 ml-fractions were collected. The fractions were identified by TLC. Those fractions of similar pattern were combined and evaporated to dryness under reduced pressure at 50^0C, three major fractions (Cf_1, Cf_2, Cf_3) were separated. Cf_2 was crystallized in methanol, Cf_2-crystal was obtained.

2.1.4 Thin layer chromatography

The yellow precipitate of *C. fenestratum* (Cf) was dissolved in water to make 1 mg/ 20 µl , spotted 5 µl of Cf solution on TLC plate, developed and air-dried. Solvent systems were chosen to provide the best separation and identification.

Solvent system : 1. MeOH : H_2O : NH_3 solution 25% (8 :1:1)

2. $CHCl_3$: MeOH (8:2)

3. $CHCl_3$: MeOH (1:1)

4. $CHCl_3$: Acetone (6:4)

5. EtOAC : MeOH (7:3)

The solvent system in chamber was saturated. Chromatogram was obtained having a solvent front up to 8 cm above the origin, removed and air-dried at room temperature (30-35^{0}C).

Detection : using either of the following :

1. Fluorescence under UV 254 and 350 nm.
2. Spraying Dragendorff's reagent
3. Spraying 10% Sulfuric acid in ethanol reagent
4. Eye observation under visible light.

2.1.5 UV Spectra

The UV spectra were determined using Spectrophotometer (spectronic® GENESYS™). UV spectra were made in ethanol under neutral condition.

2.1.6 Melting point

Melting point of each pure compound (Cf_2 and Cf_3) was determined using a Melting Point Apparatus (Gallenkamb). The compound was packed in a capillary tube and melting temperature of the compound was observed.

2.1.7 Infrared absorption spectra

Infrared absorption spectra was determined using a Fourier Transform Infrared Spectrometry (FT-IR). The Cf_2-crystal or Cf_3 powder was directly examined using potassium bromide (KBr) disc, triturated approximately 1 part of the Cf_2-crystal or Cf_3 with 100 part of dried, fine powder KBr. The mixture was thoroughly ground in an agate mortar to obtain a uniform mixture, speed compressed in die, 7 mm in diameter, using the Qwik Handi-Press. The scanning range used was 400-4000 cm^{-1}.

2.1.8 Nuclear magnetic resonance

Nuclear Magnetic resonance (^{1}H-NMR and ^{13}C-NMR) Spectra was determined using JNM-A500 Spectrometer. The ^{1}H-NMR spectra was obtained using tetradeuteromethanol (CD_3OD) as operating solvent at 500 MHz. ^{13}C-NMR spectra was obtained using 1D ^{13}C correlation spectrometry. Chemical shifts were reported in ppm scale, the signals were assigned according to Siwon et al 1980 .

2.1.9 Mass spectrometry

Mass spectrometry of Cf_2 -crystal or Cf_3 powder was determined and identified using analytical condition for MS.

MALDI-TOF time of flight UV : 337 nm

Probe voltage : +20 kV

2.2 Animal model

Male Wistar rats, 80-100 grams, were obtained from National Laboratory Animal Centre, Mahidol University, Bangkok, Thailand. Animals were acclimatized for 1 week before being used, the experiment started under a constant 12 hr (light : dark), temperature of 22-25 ^{0}C and humidity 55 ± 5%.

2.2.1 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.

2.2.2 Effect of single-oral dose of *C. fenestratum* (Cf_2) fraction or crude water extract of *C. fenestratum* (CE) in oral glucose tolerance test (OGTT)

Normal male Wistar rats were randomly divided into 4 groups, 6 rats each group and fed single dose of Cf_2 fraction and CE as follows :

Treament 1 : 20 mg of Cf_2 / kg body weight.

Treament 2 : 60 mg of Cf_2 / kg body weight.

Treament 3 : 1 g of CE / kg body weight.

Control : 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 blood glucose concentration. Single dose each of Cf_2 fraction, CE or water were fed 30-min prior to feeding of glucose, 1 g/kg body weight. Blood was collected just before glucose feeding. Blood glucose concentration 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., 1999; Naik et al., 1991).

2.2.3 Effect of single-oral dose of *C. fenestratum* (Cf_3) fraction, Berberine hemisulfate and crude water extract of *C. fenestratum* (CE) in oral glucose tolerance test (OGTT)

Normal male Wistar rats were randomly divided into 8 groups, 6 rats each group and fed single dose of Cf_3 fraction , CE or berberine hemisulfate as follows:

Treament 1 : 20 mg of Cf_3 / kg body weight.

Treament 2 : 60 mg of Cf_3 / kg body weight.

Treament 3 : 180 mg of Cf_3 / kg body weight.

Treament 4 : 20 mg of berberine hemisulfate / kg body weight.

Treament 5 : 60 mg of berberine hemisulfate / kg body weight.

Treament 6 : 180 mg of berberine hemisulfate / kg body weight.

Treament 7 : 1 g of CE / kg body weight.

Control : 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 dose each of Cf_3 fraction, CE and water were fed 30-min prior to feeding of glucose at 1 g/kg body weight. Blood was collected just before glucose feeding. Blood glucose concentration 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).