CHAPTER III

RESEARCH METHODOLOGY

Instruments and materials

1. **Instruments**

   Autocentrifuger (Biofuge fresco)

   Microplate reader (Labsystems iEMS Reader MF)

   Tissue embedding machine (Leica EG 1160)

   Tissue processing machine (Leica TP 1020)

   Microtome (Leica RM 2235)

   Light microscope (Nikon Eclipse 80i, Nikon)

   PowerLab A/D converter (Chart v5; ADInstruments, Bella Vista NSW 2153, Australia)

   Mouse restrainer

   Finger pulse oximeter

   Tail cuff

   Accu-Chek glucometer (Accu-CHEX Performa, USA)

2. **Materials**

   40% formalin (RCI Labscan)

   96 well plate (Greiner bio-one)

   Sodium chloride (MERCK)

   Cover slips (Menzel-glaser)

   Distilled water

   Tween 80 (Biotect)

   Embedding cassette

   Permount (Fisher)

   Liquid nitrogen

   Eosin (C.V. Labolatories CO., Ltd)

   Hematoxyline (C.V. Labolatories CO., Ltd)

   Alcohol

Pentobarbital (Nembutal)

Cholesterol, triglycerides and high-density lipoprotein-cholesterol enzymatic colorimetric Test Kits (Human Gesellschaft für Biochemical und Diagnostica mbH, Wiesbaden, Germany)

Mounting media (Fisher scientific, New Jersey, USA)

K. parviflora crude extract preparation

K. parviflora Wall.ex Baker rhizomes were collected from cultivable sources in Phitsanulok province of Thailand. A voucher specimen of the rhizome was kept at Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok, Thailand. The plant was identified by Associate Professor Dr. Kornkanok Ingkaninan (Pharmaceutical Sciences, Naresuan University).

The rhizomes were washed in tap water and cut into small pieces. Before they were mashed to powder, must be shade-dried in hot air oven at 45 ° C for 24 hours. The *K. parviflora* dried rhizome powder was macerated by hexane at room temperature for 3 days. Subsequently, using the 95% ethanol macerated the residue for 3 days, then filtered and extracted again. The ethanolic extraction of *K. parviflora* was stored at -20 °C until use in the investigation. Before *K. parviflora* extracts (KPE) used in the investigation, it was dissolved with 50 µl of Tween-80 then adding distilled water in a dose of 10 and 100 mg/kg body weight (BW).

Animals

Sixty male C57BL/6Mlac mice (3 weeks old) weighing 15-20 g were used in the study. The mice were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakhon Pathom, Thailand. Before starting the trials, all mice were acclimatized for least one week. They were divided into five animals per cage and maintained in housed at temperature 24 ± 2 °C, humidity 50 ± 10 °C, under a standard 12 h light/dark cycle. Animals were receiving freely standard pellet chow and water *ad libitum*. The protocols of this experiment were approved by the Animal Ethics Committee of Naresuan University, Phitsanulok, Thailand.

Methods

1. Animal groups

The sixty male mice were divided into 2 groups as following: 30 animals of the control (C) group fed normal (standard pellet chow) diet and 30 animals of the high fat high sucrose (HFHS) group received high fat and high sucrose diet for 20 weeks. They were allowed free access to diet and water *ad libitum* for induced a state of metabolic syndrome.

Twenty weeks after induction, each group of control and HFHS group were subdivided into 3 groups as following:

1. C group (n=10), the mice fed with normal diet were orally administered with vehicle
2. C+KPE10 group (n=10), the mice fed with normal diet were orally administered with KPE suspension 10 mg/kg BW
3. C+KPE100 group (n=10), the mice fed with normal diet were orally administered with KPE suspension 100 mg/kg BW
4. HFHS-C group (n=7), the mice fed with high fat high sucrose diet were orally administered with vehicle
5. HFHS+KPE10 group (n=10), the mice fed with high fat high sucrose diet were orally administered with KPE suspension 10 mg/kg BW
6. HFHS+KPE100 group (n=13), the mice fed with high fat high sucrose diet were orally administered with KPE suspension 100 mg/kg BW

The vehicle (distilled water plus Tween-80 50 µl) or KPE suspension was orally gavaged to the mice during 9:00-10:00 am once daily for 8 weeks.

2. Composition of the diets

Mice were fed difference diets during 20 weeks period. The composition of the fed in high fat and sucrose are as follow 58% of calories from fat and 26% of calories from carbohydrates (Parekh, et al., 1998) were used in the experiment (Table 1).

Table 1 Composition of the standard chow and high fat and high sucrose diet

Unit	Diets	
	Standard chow	**High fat and high sucrose**
Sucrose	-	26%
Lard	-	58%
Fat	4.5%	4.5%
Crude protein	24%	24%
Moisture	12%	12%
Fiber	5%	5%
Calcium	1.0%	1.0%
Phosphorus	0.9%	0.9%
Sodium	0.20%	0.20%
Potassium	1.17%	1.17%
Magnesium	0.23%	0.23%
Manganese (p.p.m.)	171	171
Copper (p.p.m.)	22	22
Zinc (p.p.m.)	100	100
Iron (p.p.m.)	180	180
Cobalt (p.p.m.)	1.82	1.82
Potassium iodide (p.p.m.)	1	1
Selenium (p.p.m.)	0.1	0.1
Energy content (Kcal/g)	3.040	15.91
* Vitamin mixtures		

Note: The C.P. mice feed (No. 082) were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakhon Pathom, Thailand.

3. The measurement of body weight and food intake

The weight of each mouse was recorded one time per week (1 pm) by weighing for 28 weeks. Throughout the experiment, the amount of food intake was measured by weighing three times a weeks (3 pm). The average of food intake can be calculated from the total amount of food intake per day subtracted by the amount of food remaining. The mean value of food intake was divided by the number of mice per cage (g).

4. The measurement of glucose tolerance test

In this experiment, all mice were performed the glucose tolerance test (OGTT) 2 times at before and after receiving the *K. parviflora* extract. After 12 h of fasting, the mice were oral administered with glucose 2 g/kg BW. Blood samples were collected from the lateral tail vein before and 15, 45, 90 and 120 minutes after administration of the glucose (Figure 8). The fasting blood glucose level was evaluated by glucose oxidase method (Accu-CHEX Performa, USA).

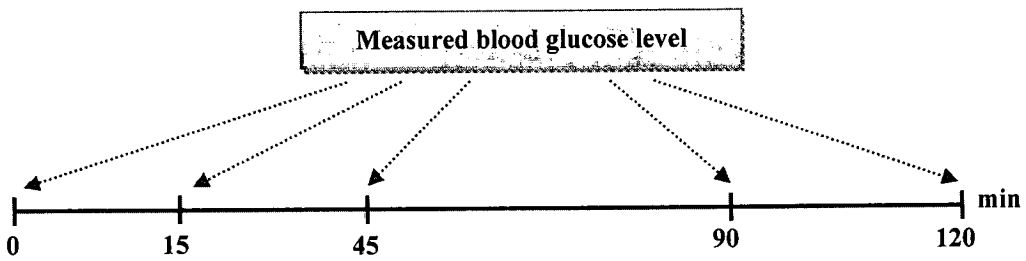

Figure 8 Oral glucose tolerance test measurement

5. The measurement of fasting blood glucose

The fasting blood glucose level was evaluated by glucose oxidase method (Accu-CHEX Performa, USA). It was measured three times as shown in figure 8; at the first week of induction (before fed with high fat high sucrose diet) which was a baseline, at the end of twentieth weeks of induction (before received KPE suspension) and at the end of eighth weeks of KPE treatment. After the mice were fasted for 12 h, each mouse was restricted into the restrainer. Afterwards the blood sample (4-5μl) was collected from the lateral tail vein and then the fasting blood glucose was measured immediately.

6. The measurement of systolic blood pressure

The systolic blood pressure was evaluated by tail cuff method (normal consciousness or without anesthetized condition). In this study, they were measured 2 times, before and after KPE administration. The mouse was restricted into the restrainer after that inserted the tail into the tail cuff and attached finger pulse transducer on the tail. The tail cuff and the finger pulse connected with non-invasive blood pressure pulse transducer system and the pressure output which were connected to the PowerLab system (Chart v5; ADInstruments, Bella Vista NSW 2153, Australia) for recording. Measurement of systolic blood pressure was recorded immediately after cardiovascular parameters stabilization (Figure 9).

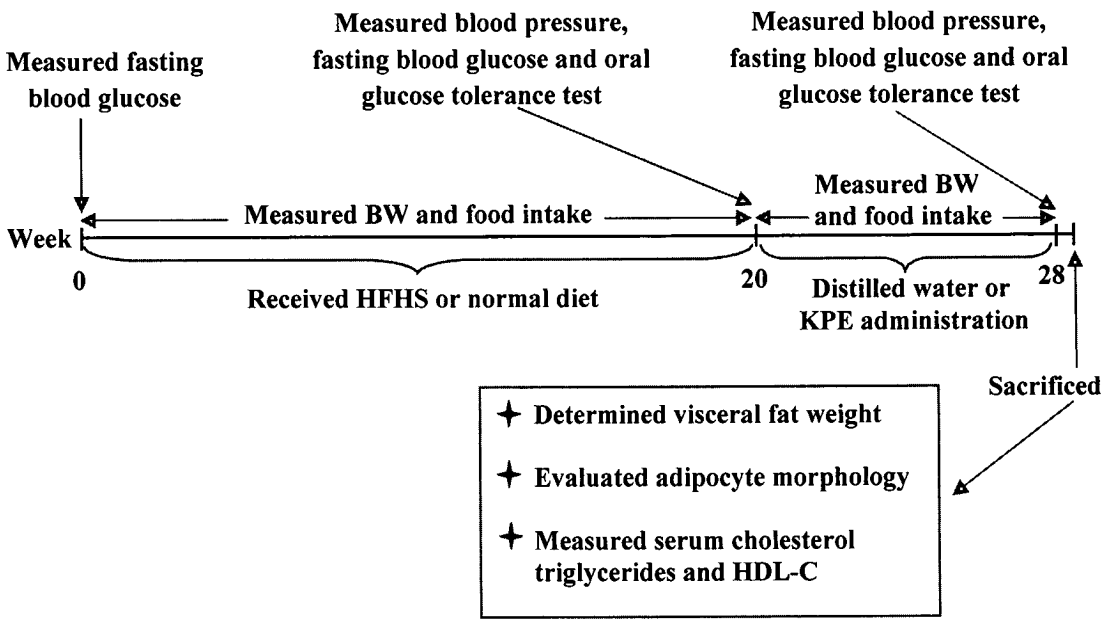

Figure 9 Experimental schedule

7. The collection of blood and tissues

Animals were euthanized with overdose of pentobarbital (50 mg/kg BW) by intraperitoneal (i.p.) injection. Then, blood was collected by cardiac puncture method. The serum was separated by centrifugation at 4000 rpm for 10 minutes at room temperature and stored at -20 °C for further analysis. The amount of visceral fat was instantly took off from the body after blood collection, after that it was blotted to dry and weighing. The visceral fat pads that mentioned were the fat in the abdominal cavity which consisted of retroperitoneal fat, mesenteric fat (fat contained in the lesser curvature of the stomach and sigmoid colon) and epididymal fat. After weighing, visceral fat was immersed in 10% formaldehyde solution to tissue structure preservation until used for analysis.

8. Serum cholesterol, triglycerides and HDL-C concentration evaluation

The serum cholesterol, triglycerides and HDL-C were evaluated using the enzymatic colorimetric method, according to the instructions of manufacturer (Human Gesellschaft für Biochemical und Diagnostica mbH, Wiesbaden, Germany). Briefly, for the cholesterol and triglycerides concentration measurement, the serum was mixed and incubated with the enzyme reagents (enzymatic colorimetric test for cholesterol or triglycerides) for 10 minutes at 25 °C. In this study using a microplate reader (Labsystems iEMS Reader MF) for measured the absorbance of every parameter at wavelength 500 nm. For the high-density lipoprotein cholesterol concentration assessment, the serum was first mixed and incubated gently with the HDL-C enzyme for 5 minutes at 37 °C. After that, the substrate was added and incubated at 37 °C for 5 minutes. Finally, the sample was read the absorbance at wavelength 593 nm.

9. Histological analysis

Visceral fat was fixed overnight in 4% paraformaldehyde/0.1 M phosphate buffer. After that, tissues were processed by dehydration with gradual series of alcohol concentration (70%, 80%, 90%, 95%, 100%, respectively) to remove water. Alcohol was removed from visceral fat by xylene. Then tissues were infiltrated and embedded in paraffin wax. In the each block of tissue was sectioned coronally using rotary microtome with thickness of 5 μm. Each tissues section was floated on warm water at 45 °C before mounting on glass slides. Then the section was mounted on slides and allowed to dry at room temperature overnight. Paraffin tissue sections were

deparaffinized and rehydrated by xylene and gradual series of alcohol dilution (100%, 95%, 90%, 80%, 70%, respectively) to distilled water. After that, the tissue sections were stained by dipped in hematoxylin and eosin (H&E) and rinsed with tap water. The tissue sections were dehydrated by gradual series of alcohol concentration (95% I, 95% II, absolute I and absolute II, respectively) and then removed alcohol by xylene. Lastly, the slide was mounted with mounting media (Fisher scientific, New Jersey, USA) and covered with cover slip slides. Each tissue section was determined by using light microscope (Nikon Eclipse 80i, Nikon) for histological change.

10. Statistical analysis

The data were shown as mean ± SEM (standard error of the mean). Statistical Package for the Social Sciences (SPSS) software version 17.0 was used for the statistical analysis. In this study, One-way analysis of variance (ANOVA) with post hoc LSD test (compared mean of more groups) was used to analyze. The statistically significant was determined as P values less than 0.05.