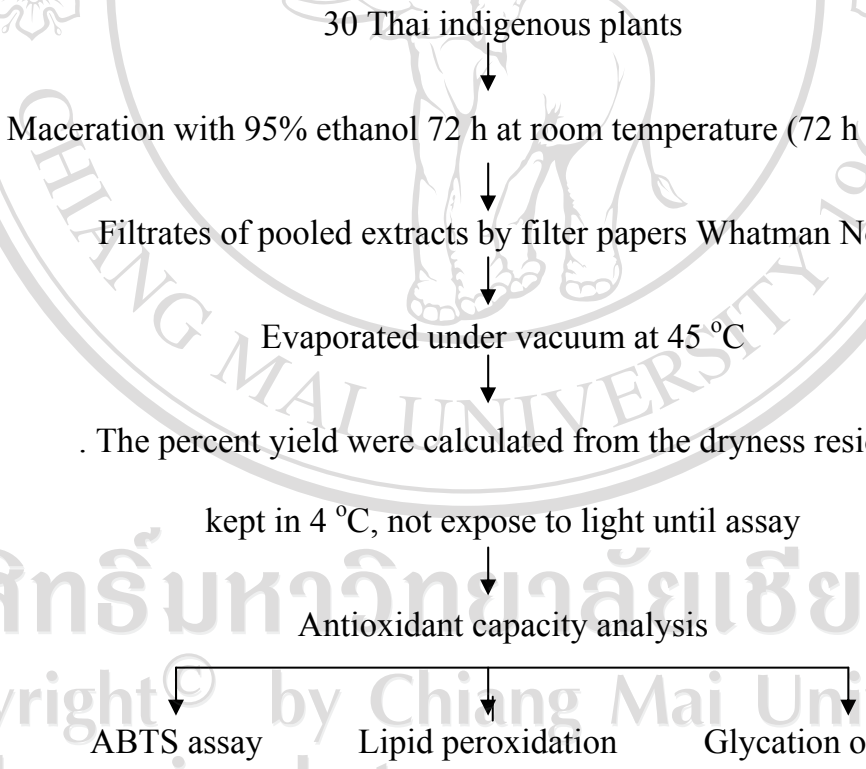
CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and instruments are in appendix A.

3.2 *In vitro* study: The effects of antioxidant activity from 30 Thai indigenous plants against diabetic stress in the *in vitro* study.



Scheme 3.1 Schematic diagram showing the experimental protocols for study of the effects of antioxidant activity of 30 Thai indigenous plants against diabetic stress in the *in vitro* study.

The antioxidant activities of the 30 indigenous plants (pictures in the appendix) were analyzed according to Scheme 3.1. Afterward, five plants with strong antioxidant activity were used as raw material to produce mixed-biologically fermented plant beverage product. Major active compounds of both plants and fermented product were identified.

3.2.1 Plant materials

Thirty fresh plants were purchased from an organic farm in Chiang Mai, Thailand, during July 2006-February 2007. They were identified botanically from voucher specimens of certain plants in the Herbarium of the Faculty of Pharmacy, Chiang Mai University, as shown in Table 3.2. The criteria for choosing plants in this study are as follows: 1) freshly indigenous plants which were traditionally used in diabetes 2) they could easily be grown in northern Thailand and consumed in daily life.

Table 3.1 List of 30 Thai indigenous plants used in the study

Scientific name	Common name	Thai name	Family name	Plant parts
1. *Allium sativum* Linn.	Garlic	กระเทียม	Alliaceae	Bulb
2. *Eryngium foetidum* Linn.	Puckcheefarang	ผักชีฝรั่ง	Apiaceae	Leaf
3. *Gymnema inodorum* Decne.	Puckchiangda	ผักเชียงดา	Asclepiadaceae	Leaf, Stem
4. *Terminalia chebula* Retz	Myrobalan	สมอไทย	Combretaceae	Fruit
5. *Ipomcea aquatica* Forsk.	Morning glory	ผักบุ้ง	Convolvulaceae	Leaf
6. *Costus speciosus* Koen. J.E. Smith	Crape ginger	เอื้องหมายนา	Costaceae	Rhizome
7. *Luffa acutangula* (Linn.) Roxb.	Angled Loofah	บวบเหลี่ยม	Cucurbitaceae	Fruit
8. *Coccinia grandis* (L.) Voigt. Syn.	Ivy Gourd	ตำลึง	Cucurbitaceae	Leaf
9. *Momordica charantia* Linn.	Balsam pear	มะระขี้นก	Cucurbitaceae	Fruit
10. Phyllanthus emblica Linn.	Emblic Myrobalan	มะขามป้อม	Euphorbiaceae	Fruit

Table 3.1 (continued)

Scientific name	Common name	Thai name	Family name	Plant parts
11. *Acacia penneata* Linn.	Chaom	ชะอม	Fabaceae	Leaf
12. *Glycine max* Merr.	Soybean	ถั่วเหลือง	Fabaceae	Seed
13. *Ocimum basilicum* Linn.	Hairy basil	แมงลัก	Labiatae	Leaf
14. *Pisum sativum* Linn.	Green pea	ถั่วลันเตา	Leguminosae	Pod
15. *Abelmoschus esculentus* Linn.	Okra	กระเจี๊ยบ	Malvaceae	Fruit
16. *Artocarpus heterophyllus* Lamk.	Jackfruit	ขนุน	Moraceae	Leaf
17. *Musa sapientum* Linn.	Banana	ปลีกล้วย	Musaceae	Flower
18. *Sesbania grandiflora* (L.) Desv.	Sesban	แคแดง	Papilionaceae	Flower
19. Piper samentosum Roxb.	Betel	ใบชะพลู	Pipereaceae	Leaf
20. *Piper retrofractum* Vahl.	Long pepper	ดีปลี	Piperaceae	Fruit

Table 3.1 (continued)

Scientific name	Common name	Thai name	Family name	Plant parts
21. *Morinda citrifolia* Linn	Indian mulberry	ยอ	Rubiaceae	Fruit
22. *Aegle marmelos* correa (L.)	Beal	ใบมะตูม	Rutaceae	Leaf
23. *Houttuynia cordata* Thunb.	Heart leaf	พลูคาว	Saururaceae	Leaf
24. *Capsicum frutescens* Linn.	Chili pepper	พริกขี้หนู	Solanaceae	Fruit
25. *Lycopersicon esculentum* Mill.	Tomato	มะเขือเทศ	Solanaceae	Fruit
26. *Solanum torvum* Sw.	Devil's Fig	มะเขือพวง	Solanaceae	Fruit
27. *Apium graveolens* Linn.	Celery	ผักคื่นไช่	Umbelliferae	Leaf
28. *Coriandrum sativum* Linn.	Coriander	ผักชี	Umbelliferae	Leaf
29. *Kaempferia parviflora* Wall.	Krachaidam	กระชายดำ	Zingiberaceae	Rhizome
30. *Zingiber officinale* Linn. Adrak	Ginger	ขิง	Zingiberaceae	Rhizome

3.2.2 Preparation of ethanol plant extracts

Thirty plants have been used as an alternative treatment in DM. The edible portion of each plant was cleaned and cut into small pieces before being air-dried in a sunless place (which comparable real time using). The plant portions were homogenized using a blender and macerated in 95% ethanol (200 g of each plant/600 ml of 95% ethanol). The macerations were done at room temperature and protected from light for 72 h. The plants were macerated three times consecutively. The extracts were pooled and filtered by filter papers whatman No.4 and evaporated under vacuum at 45 ^{o}C until all extraction solvents were completely removed. The dry residues were obtained and were calculated for the percent yield. They were kept in 4 ^{o}C not exposed to light for assay.

3.2.3 Measurements of antioxidant activity

1. Trolox equivalent antioxidant capacity (TEAC)
2. Thiobarbituric acid reactive substance (TBARS)
3. Glycation of protein

3.2.3.1 ABTS free radicals decolorization assay

The ABTS radical-scavenging activity was analyzed according to Re et al. [148]. This method is based on the ability of antioxidant molecules to

quench the long-lived ABTS radical cation, a blue-green chromophore with characteristic absorption at 734 nm, compared with the Trolox, a water-soluble vitamin E analog. A stable stock solution of ABTS radical cation was produced by reacting 7 mmol/L aqueous solution of ABTS with 2.45 mmol/L potassium persulfate (final concentration) and allowed the mixture to stand in the dark at room temperature for 12–16 hours before use. The solution was diluted with ethanol to obtain an absorbance of 0.70 ± 0.02 units at 734 nm. Each plant extract was dissolved in ethanol. Aliquot of each sample (20 µl) in ethanolic solution was added into 2.0 ml of ABTS free radical cation solution, the absorbance monitored for 3 minutes at 734 nm, verified by a UV/VIS spectrophometer Jasco model 7800. The antioxidant activity of each sample was expressed as trolox or ascorbic acid equivalent antioxidant capacity (TEAC or VEAC), defined as mg of trolox or ascorbic acid /g of dry weight of plant extract.

3.2.3.2 Lipid peroxidation assay

Thiobarbituric acid-reactive substances (TBARS) was used to evaluate lipid peroxidation [149] of blood plasma samples of diabetic patients. Briefly, plasma samples of type II diabetes patients were incubated with each of the 30 extracts (1µg/ml) in shaking water bath under 95% O_2 / 5% CO_2 atmosphere at 37 ^{o}C for 1 h. After incubation, it was centrifuged at 3,000 rpm for 10 minutes. The supernatant portion was removed to measure lipid

peroxidation by TBARS assay. The procedure, 40 µl of 0.2% butylated hydroxyl toluene was added into 375 µl of supernatant, divided the mixture into three equal aliquots (one was used as sample blank and the others were used as a duplicate). After 750 µl of phosphoric acid (0.44 M H_3PO_4) was added to each tubes, 250 µl of 0.6% (w/v) thiobarbituric acid (TBA) reagent was added to both assay tubes and 250 µl of deionized water added to blank tube. All mixtures were incubated at 90 ^oC for 30 minutes, cooled down at room temperature. The absorbance was read at 540 nm. The data are expressed as malonaldehyde (MDA) equivalents (µM) using 1, 1, 3, 3-tetramethoxypropane as a reference standard.

3.2.3.3 Glycation of protein analysis

The procedure followed the method previously described by Vinson & Howard [150]. Bovine serum albumin (BSA, 10 mg/ml), the extract, glucose (25 mM) and fructose (25 mM), all in 50 mM phosphate buffer; pH 7.4 (PBS), containing 0.02% (w/v) sodium azide. The 1.2 ml mixed solutions were incubated with each of 0.3 ml BSA, extracts at final concentration (µg/ml). Also glucose and fructose solutions were added to the reaction mixture in the end of process for 30 minutes at room temperature (25 ^oC). After incubating at 37 ^oC in 5% CO_2 in air for 1 week, the fluorescence intensity (FI) was measured at an excitation wavelength of 350 nm and an emission wavelength of 450 nm by microplate reader (Backman coulter, model DTX 880 multimode detector).

Results were expressed as percentage inhibition of the formation of glycated protein by the plant extracts, calculated according to the following formula: Inhibition of protein glycation (%) = 100 - [FI (sample) - FI (blank of sample)] x 100 / [FI (control) - FI (blank of control)], where FI (sample) is the FI in the presence of the sample extract in the mixture solution after incubated at 37 ^{o}C , FI (blank of sample) is the FI of sample extract in the mixture solution that not incubated to use for error correction arising from unequal colour of sample extract, FI (control) is the FI of mixture solution without sample extract after incubation and FI (blank of control) is the same solution of control that not incubation. The percentage of inhibition of glycation was plotted against the sample extract concentration (μg/ml) to obtain the IC_{50}, defined as the concentration of sample extract necessary to 50% inhibition of the control, calculated from linear regress equation.

3.2.4 Blood sample collections

Oxidative stress model; free radical in diabetes, obtained from the pooled plasma samples collected from the blood of type II diabetes mellitus patients (fasting plasma blood glucose ≥ 200 mg/dl, 15 males and 15 females, age over 35 years) at Maharaj Nakorn Chiang Mai, Faculty of Medicine, Chiang Mai University, Thailand. Blood samples were collected in EDTA tubes and centrifuged at 3,000 rpm, 4 ^{o}C for 10 minutes. Plasma was removed and kept frozen at -80 ^{o}C for lipid peroxidation analysis. The Faculty of

Medicine ethics Committee for Human Research approved the protocol for blood collections (Reference No.0515 (05).17/212).

3.2.5 Determination of total phenolic content

Total phenolic content was determined by Folin-Ciocalteu reagent [151]. A dilute extract of each plant extracts (0.5 ml of 1:10 g ml^{-1}) or gallic acid (standard phenolic compound) was mixed with Folin-Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 ml, 1 M). The mixtures were allowed to stand for 15 minutes and the total phenolic content was determined by colorimetry at 765 nm. The standard curve was prepared using gallic acid in methanol: water (50:50, v/v) and expressed as gallic acid equivalent (mg GAE/g dry weight of plant extract).

3.2.6 Determination of flavonoids content

The flavonoids content was determined by aluminum chloride colorimetric method [162]. Each plant extracts (0.5 ml of 1:10 g ml^{-1}) in methanol was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It remained at room temperature for 30 minutes; the absorbance was measured at 415 nm. The standard curve was made by preparing various

concentrations of quercetin in methanol and expressed as quercetin equivalent (mg QE/g dry weight of plant extract).

3.2.7 Determination of total tannins content

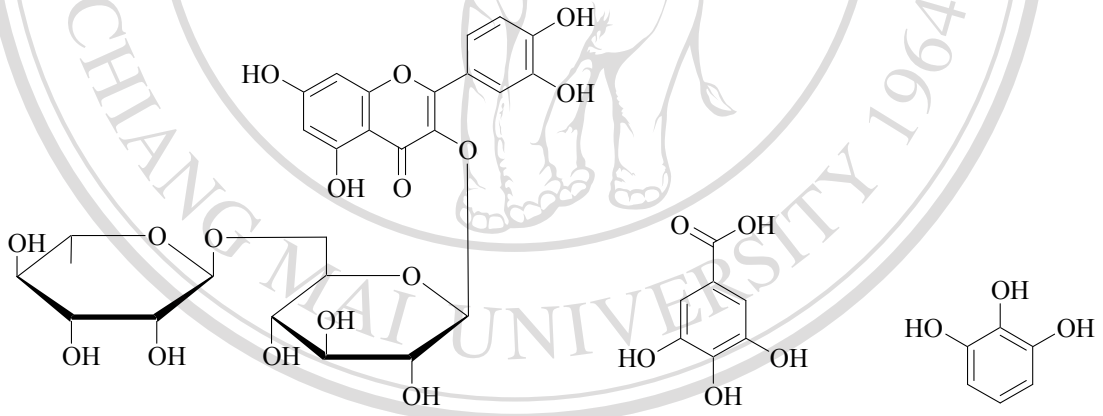
The total tannins content in the lyophilized extracts were determined by modified method of Polshettiwar et al. [153]. Each sample (0.1 ml) was mixed with 0.5 ml Folin- Denis reagent followed by 1 ml of Na_2CO_3 (0.5% v/v) solution and distilled water (up to 5 ml). The absorbance was measured at 775 nm within 30 minutes of the reaction against the reagent blank. Results were expressed as tannic acid equivalents (mg tannic acid/ g dried extract).

3.2.8 Preparation and plant extracts components identification by HPLC

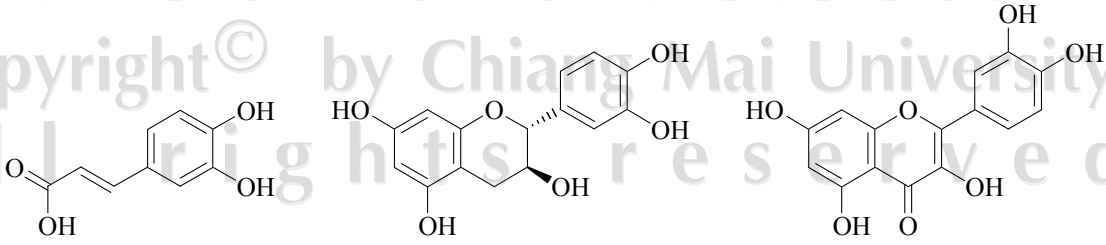
The isolation of polyphenol from five ethanolic plant extracts with the highest antioxidant activity was performed by solid phase extraction (SPE) following the method of Chiou et al. [154] slightly modified. Briefly, 0.1 gram of each extract was dissolved with distilled water and 1-2 drops of HCl concentration were added and boiled 20 minutes. The SPE, C18 Cartridge column was washed with ethyl acetate 3 ml, methanol 3 ml and mili Q water 5 ml, followed by the deposition of the sample, 10 ml. The solvent was allowed to dry under reduced pressure and polyphenols, retained in the dissolved with methanol 1 ml. Preparation of standard solution using reference of standard

compounds, such as rutin, gallic acid, pyrolgallol, catechin and caffeic acid (Figure 3.1) were dissolved in methanol.

The analytical HPLC system condition was the Agilent 1100 Series diode-array detector high-performance liquid chromatograph. Polyphenolic compounds were identified with the diode array detector wavelengths at 200 nm and separation was done by ODS Hypersil (250 x 4 mm), 5µm, column oven was set up at 25 °C. Isocratic elution was employed with mobile phase consisting of water: 0.4% acetic acid: methanol: acetronitrile (70:20:5:5), flow rate of the mobile phase was 0.7 ml/min and the injection volume was 6 µl.



Rutin Pyrogallol Gallic acid



Caffeic acid Catechin Quercetin

Figure 3.1 Chemical structures of some polyphenols found in the samples

3.2.9 Quantitative analysis of polyphenol compounds by thin layer chromatography

Thin layer chromatography (TLC) was carried out following the method of Mabinya et al. [155] on Merck silica gel 60 F254 plates (20 cm x 20 cm). Aliquots of a standard rutin, quercetin, pyrolgallol, gallic acid, caffeic acid and cathechin solution in methanol ranging from 500-1000 µg were applied as spots at the origin on a plate and developed with n-hexane: ethyl acetate: acetic acid (20:19:1) and dichloromethane: methanol: acetic acid (28:18:1) in pre-saturated chromatographic chambers. Developed plates were dried in a stream of hot air (hair dryer) and visualized at 254 nm and 366 nm UV light. The spots were carefully circled with a pencil and sprayed with 2, 6-dichlorchinon-4-chlorimid followed ammonium solution concentration.

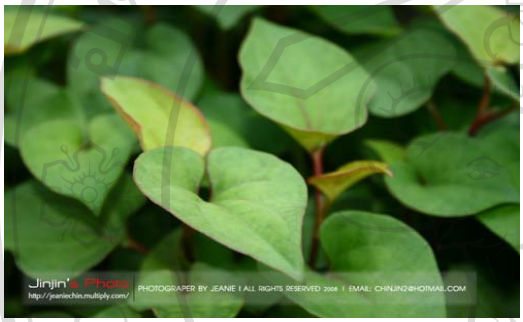
3.3 *In vivo* study: The effects of biologically fermented Thai indigenous plants against diabetic oxidative stress in streptozotocin-induced rats.

3.3.1 Preparation and identification of biologically fermented plant beverage (BFPB)

Biologically fermented plant beverage (BFPB) is the product from the Research and Development of Health Product Unit, Department of Pharmaceutical Science, Faculty of Pharmacy, Chiang Mai University, Thailand. The composition of BFPB are five Thai indigenous plants as follows: Malacea tree (*Phyllanthus emblica* Linn.), Indian mulberry (*Morinda citrifolia* Linn.), Heart leaf (*Houttuynia cordata* Thunb.), Myrobalan (*Terminalia chebula* Retz.) and Krachai-Dam (*Kaempferia parviflora* Wall.) (Figure 3.2). The major compounds of BFPB were analyzed for polyphenolic compounds and were identified for active ingredients by Chromatography and HPLC.

Phyllanthus emblica Linn.

Morinda citrifolia Linn.



Houttuynia cordata Thunb.

Terminalia chebula Retz.

Kaempferia parviflora Wall.

Biologically fermented plant beverages

Figure 3.2 Five Thai indigenous plants with strong antioxidant activity and their mixed biologically fermented plant beverages product.

3.3.1.1 Preparation of mixed BFPB [110]

Each fresh plant of *Phyllanthus emblica* Linn., *Morinda citrifolia* Linn., *Terminalia chebula* Retz., *Kaempferia parviflora* Wall. and *Houttuynia cordata* Thunb. was washed and cut into small pieces and crushed with the crusher. After that, each plant was mixed with cane sugar and reverse osmosis water. The mixture was 3 parts of each crushed plant (3.47 kg), 10 parts of reverse osmosis water (11.57 kg) and 1 part of raw cane sugar (1.16 kg) inoculated with the starter culture in concentration of 10 % (w/w) (1.8 kg). BFPB containing five plants were prepared with the following formulas:

A: Fermentation of each crushed plant, reverse osmosis water, raw cane sugar and inoculated with the starter culture in concentration of 10% (w/w).

B: Fermentation of each crushed plant, water and raw cane sugar.

C: Fermentation of each crushed plant, water and raw cane sugar in solid state fermentation during the first period (added water on the 15th day of fermentation period)

D: Fermentation of each crushed plant, water and raw cane sugar. Potassium metabisulfite (KMS) was added, about 250 mg/L, before being inoculated with the starter culture in a concentration of 10% (w/w).

The mixture of each plant was contained in an 18.9 liter-plastic container (polypropylene tank). They were closed by air locked rubber to limit aeration. After that, the tanks were kept in the incubator room to maintain

temperature (30±2 ^{o}C) throughout the fermentation for 1 month. Next, the fermented juice of five plants were mixed to be 10 liters of mixed BFPB. The formula consists of *Phyllanthus emblica* Linn. 1 liter (10%), Linn., *Terminalia chebula* Retz. 1 liter (10%), *Kaempferia parviflora* Wall. 0.5 liter (5%), *Morinda citrifolia* Linn 2 liters (20%).and *Houttuynia cordata* Thunb. 5.5 liters (55%). This mixed BFPB was used in this experiment.

3.3.1.2 Preparation of sample solution

The isolation of polyphenol from BFPB was performed by solid phase extraction (SPE) [154]. Briefly, the SPE, C18 Cartridge column was washed with ethyl acetate 3 ml, methanol 3 ml and mili Q water 5 ml, followed by the deposition of sample 10 ml. Solvent was allowed to dry under reduced pressure and polyphenols, retained in the column, were eluted with ethyl acetate 3 ml. Solvent was evaporated at room temperature and the residue was dissolved with methanol 1 ml. Preparation of standard solution using reference of standard compounds, such as rutin, gallic acid, pyrolgallol, catachin and caffeic acid were prepared and dissolved in methanol.

3.3.1.3 Analysis of active ingredients in BFPB crude extract by chromatography and HPLC

Active ingredients of the BFPB were analyzed by using a gas chromatography/mass spectrometry (GC-MS) technique at the Science and Technology Service Center, Chiang Mai University, Thailand. A gas chromatography machine (GC6890, Agilent Technologies, U.S.A.) was included a column (HP-5MS, 30m x 0.25mm ID. x 0.25µm film thick) and conditioned with inlet of 270 °C, 1.0-µl split ratio (10:1), oven temperature of 50 °C (12°C/min) to 260°C (42.5 min), a flow rate of helium gas carrier at 1.0 min/min. A mass spectrometry detector (MSD5973 EI Hewlett Packard, MS Quadrupole 150 °C, MS source 230°C) was on-line connected to the GC machine. Data of analysis was acquired using ChemStation program. Mass spectra of analyzed samples were compared with those of standard compounds as stored in a database library.

The analytical HPLC system condition was Agilent 1100 Series diode-array detector high-performance liquid chromatograph. Polyphenolic compounds were identified with the diode array detector wavelengths at 200 nm and separation was done by ODS Hypersil (250 x 4 mm), 5µm, column oven was set up at 25 °C. Isocratic elution was employed with mobile phase consisting of water: 0.4% acetic acid: methanol: acetronitrile (70:20:5:5), flow rate of the mobile phase was 0.7 ml/min and the injection volume was 6 µl.

3.3.2 Animals

Male Wistar rats (180-200 g) were purchased from the National Animal Laboratory Center, Mahidol University at Salaya Campus, Bangkok, Thailand. The rats were housed in stainless-steel cages, with constantly controlled room temperature (23±2 ^{o}C), humidity temperature (65±10%), 12/12 h light / dark cycle and were given chow and water ad libitum. The rats were acclimated one week before being used for the experiment. During monitoring period, blood was collected from tail vein aseptically under light anesthesia (ether) at one week after induction of experimental diabetes and every 2 weeks after treatment with plant product. At the end of study in the 8th week, rats were anesthetized under pentobabetal sodium (50 mg/Kg BW) by intraperitoneal (IP) injection and sacrificed. All procedures were approved by the Ethics Committee for the use of experimental animals in the Faculty of Medicine, Chiang Mai University (Protocol No.10/2549).

3.3.3 Induction of diabetic rats

All Wistar rats were acclimatized for a one-week period. Afterwards, they were weight-matched into 2 groups: healthy and diabetic rat group. A single dose of STZ 45 mg/kg body weight (BW) dissolved in citrate buffer (45 mg/kg BW) was IP injected to induce diabetes in the diabetic rat group [138] and the others received vehicle only. One week after treatment, all rats were

fasted overnight, blood samples were taken from tail veins under ether anesthesia, and plasma glucose levels were measured. Rats with a fasting plasma glucose level above 250 mg/dl were regarded as diabetes.

3.3.4 Experimental protocols

The experimental protocol for studying the effects of BFPB on diabetic oxidative stress in rats was randomly dividing them into 4 groups by matching weight and plasma blood glucose levels, 9 rats in each group. BFPB were administrated in the experiment by oral gavage feeding once a day. Treatment was given to each group as follows:

Group 1: a healthy-control group, Wistar rats were given distilled water in the same volume as those given to the treated group.

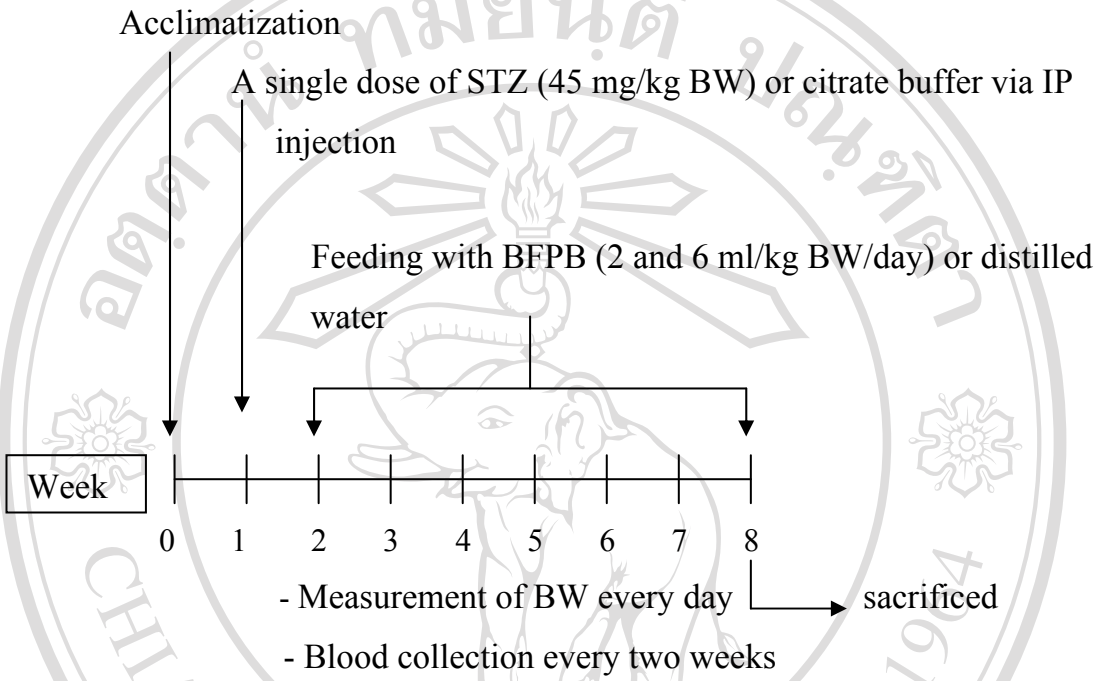
Group 2: a diabetic-control group, Wistar rats were given distilled water in the same volume as those given to the treated group.

Group 3: a diabetic-treated group, Wistar rats were given BFPB at a dose of 2 ml/kg BW/day.

Group 4: a diabetic-treated group, Wistar rats were given BFPB at a dose of 6 ml/kg BW/day.

Blood samples were taken every 2 weeks. The rats were fasted overnight (14-16 h) for blood collection for analysis. They had been

administered treatment for 6 weeks and then sacrificed. The experimental protocol details are in Scheme 3.2.



Scheme 3.2 Schematic diagram show the protocol to study the effects of biologically fermented Thai indigenous plants against diabetic oxidative stress in streptozotocin-induced rats.

3.3.5 Measurement of oxidative stress markers

1. Plasma thiobarbituric acid reactive substance (TBARS)
2. Red blood cells (RBCs) oxidative stress by flow cytometry
3. Plasma superoxide ($O_2^{\cdot-}$) levels
4. Plasma nitric oxide (NO) levels
5. Degree of diabetes using fasting blood glucose (PBG)

3.3.5.1 Determination of plasma glucose levels

The blood was collected in a sodium fluoride tube then centrifuged. Plasma was removed and glucose levels were determined by using a commercially available enzymatic kit (purchased from BIOTECHNICAL Company, Bangkok, Thailand). The assay procedure was followed according to the method described [156] in the appendix.

3.3.5.2 Plasma lipid peroxidation assay

Thiobarbituric acid-reactive substances (TBARS) method was used to evaluate lipid peroxidation [149] with a slight modification. Briefly, blood samples of healthy and diabetic rats were collected in heparinized tubes and centrifuged at 3,000 rpm, 4 ^{o}C for 10 minutes. The plasma was removed to measure lipid peroxidation by TBARS assay. The procedure, 40 µl of 0.2% butylated hydroxyl toluene was added into 375 µl of plasma, divided the mixture into three equal aliquots (one was used as sample blank and the others were used as a duplicate). After 750 µl of phosphoric acid (0.44 M H_3PO_4) was added to each tubes, 250 µl of 0.6% (w/v) TBA reagent was added to both assay tubes and 250 µl of deionized water added to a blank tube. All mixtures were incubated at 90 ^{o}C for 30 minutes, cooled down at room temperature and the absorbance was read at 540 nm. The data are expressed as MDA equivalents (µM) using 1,1,3,3 - tetramethoxypropane as a reference standard.

3.3.5.3 Determination of oxidative stress status in red blood cells

Red blood cells (RBCs) oxidative stress was analyzed by the method established by Amer et al. [157]. Briefly, each whole blood (5 µl) sample was diluted with phosphate buffered saline (PBS) to a concentration of 1×10^6 RBCs /ml. Then, 20 µl of 0.4 mM DCFH-DA (dissolved in methanol) was added and incubated at 37 ^oC for 15 minutes, room temperature. The RBC suspension was centrifuged at 6,000 rpm, 10 ^oC for 10 minutes and washed with PBS (resuspended in PBS and centrifuged 2 times). 250 µl of RBC suspension was added to 500 µl of PBS and measured ROS by flow cytometry analysis. The RBCs were passed at a rate of about 1000 per second, using saline as the sheath fluid. The fluorescence intensity (FI) was measured at an excitation wavelength of 350 nm and an emission wavelength of 450 nm with the Fluorescence Activated Cell Sorter Scan.

3.3.5.4 Superoxide anion (O_2^-) scavenging activity assay

Superoxide level was measured using method based on that of Ewing & Janero [158]. The O_2^- scavenging activity was determined by measuring the decrease in ratio of the reduction of NBT. The plasma samples were added to the reaction buffer (the mixture in 50 mM PBS; pH 7.4 with 125 µM EDTA, 62 µM NBT and 98 µM NADH) containing 33 µM PMS. After

incubation at 37 ^{o}C for 30 minutes, the absorbance was measured at 560 nm, as an index of NBT reduction, using a Backman coulter, model DTX 880 multimode detector.

3.3.5.5 Nitric Oxide (NO) scavenging activity assay

Nitric oxide radical inhibition was estimated by the nitrite and nitrate levels, following the method of Misko et al. [159]. Briefly, nitrate in plasma was converted to nitrite by incubation with nitrate reductase in PBS (pH 7.4). Nitrite and nitrate level was measured by a microplate assay method based on the Griess reaction. A pink coloured chromophore is formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions. Sodium nitrite was used as a standard.

3.4 Statistical analysis

Experimental data were expressed as means $\pm$ standard deviation (SD) in the *in vitro* study and means $\pm$ standard error of means (SEM) in the *in vivo* study. Results were analyzed the difference between groups by one-way analysis of variance (ANOVA) and the time-series comparison within group using repeated measure ANOVA (two-sided). A *p*-value of less than <0.05 was considered to be statistically significant.