CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and equipment

2.2 Medicinal plants

2.3 Selected Thai and Nigerian hypoglycemic medicinal plants

Table 2.1 List of hypoglycemic medicinal plants of Thai origin

Sample No.	Name of Plant	Part Used
1.	*Anogeissus acuminata* (Roxb. ex. DC) Guill. & Perr. (Ta Khian nu)	Bark
2.	*Catunaregam tomentosa* (DC.) Tirveng (Nam Taeng)	Bark
3.	*Dioecrescis erythroclada* (Kurz) Tirveng (Ma Khang Daeng)	Leaf
4.	Yamed Boraped Pungchang® (*Stephania rotunda, Acanthus ilicifolius, Cyprus rotundus, Rhinaeanthus nasutus)*	Recipe
5.	Mai Tau Lusi (*Curcuma zedoria* (Berg) Roscoe, *Eugenia caryophyllum* Bullock &Harrison, *Piper chaba* Hunt, *Abroma malvaceae*, *Piper nigrum* Linn, *Myristica fragans* Houtt, *Amomum krevanh* Pierre, *Zingiber cassumunar* Roxb)	Recipe
6.	*Dendrophthoe pentandra* (L.) Miq (Ka Fak Ma Muang)	Leaf
7.	*Mimosa pudica Linn. var. hispida* Bren (Mai Ya Rab)	Leaf
8.	*Moringa oleifera* Linn. (Ma room)	Root
9.	*Pterocarpus macrocarpus* kurz (Pra Doo)	Bark
10.	*Rauwolfia serpentina* (L.) Bth. ex. Kurz (Rayom)	Leaf

Table 2.2 List of hypoglycemic medicinal plants of Nigerian origin

Sample No.	Name of Plant	Part Used
1.	*Anisopus mannii* N.E.Br (Kashe zaki)	Leaf
2.	*Anogeissus leiocarpus* (D.C) Guill & Perr. (Mareke)	Bark
3.	*Daniella Oliveri* (Rolfe) Hutch & Dalz. (Maje)	Leaf
4.	*Detarium macrocarpum* Harms (Taura)	Bark
5.	*Ficus Thonningii*. Blume, (chediya), *Raphia vinifera* P. Beauv, (kimba), *Leptedenia hastata* (Pers) Dec'ne (Yaa diya)	Recipe
6.	*Leptedenia hastata* (Pers) Dec'ne (Ya diya)	Leaf
7.	*Mimosa invisa* var. inermis Adelb (Idon zakara)	Leaf
8.	*Moringa oleifera* (Zogale)	Root
9.	*Pterocarpus erinaceus* (Madobiya)	Bark
10.	*Rauwolfia serpentina* (Ganyen Ghana)	Leaf

Part 1: Collection of the hypoglycemic medicinal plants

Thai hypoglycemic medicinal plants

A total of 8 hypoglycemic medicinal plants and 2 recipes (prepared in capsules) were collected considering their high traditional patronage for the treatment of diabetes mellitus.

a. *Anogeissus acuminata* (Roxb. ex. DC) Guill. & Perr.

The bark and leaves were collected were from Mae Fa Luang, Bu Na (Akha + Lahu) Village, Teut Tai sub district at an elevation of 975 m, Chiang Rai, Thailand in

February, 2009. A voucher specimen (Number 09-41) (Fig 2.1) was deposited in the CMU Herbarium, Faculty of Science. Leaves and green branches were removed bark was air dried in shade prior to the extraction.

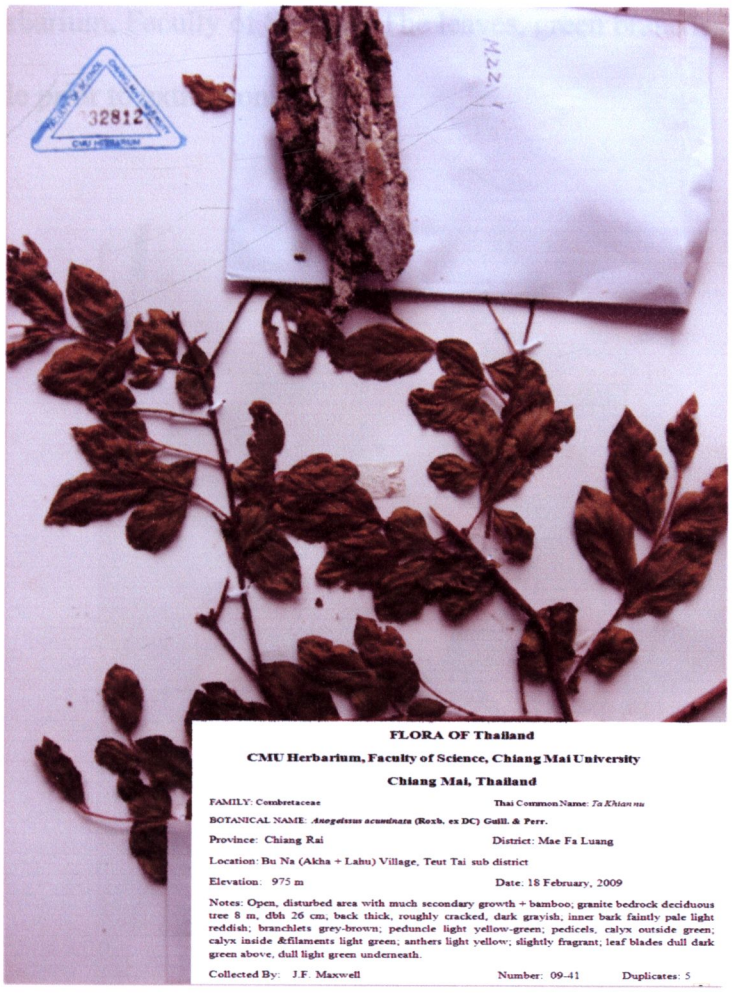

Figure 2.1 The herbarium specimen of *Anogeissus acuminata (Roxb. ex DC)* Guill. & Perr.

b. *Pterocarpus macrocarpus* kurz

The bark and the leaves were collected from Chiang Mai University Campus, Chiang Mai, Thailand. The tree is about 8 m tall with hard dark brown bark, pods were green turning brown. A voucher specimen (Number 8) (Fig 2.2) was deposited in the CMU Herbarium, Faculty of Science. The leaves, green branches and bark were air dried in shade prior to extraction.

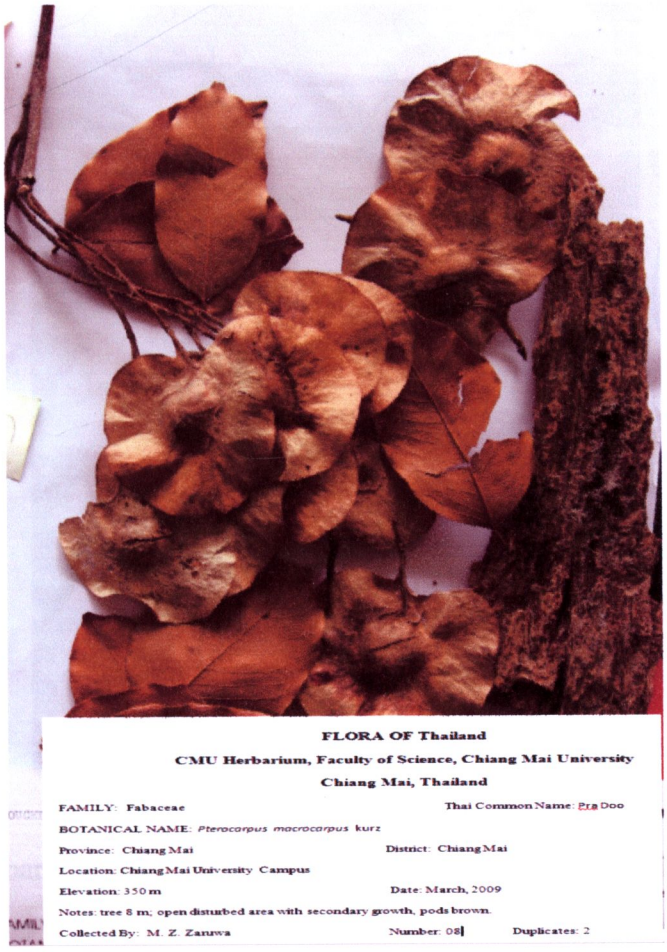

Figure 2.2 The herbarium specimen of *Pterocarpus macrocarpus* kurz

c. *Mimosa pudica* Linn. *var. hispida* Bren.

The leaves were collected from an open overgrown weedy area in a soy bean field in Ban Tah Mah Giang Sahnsai sub-district valley by J. F. Maxwell. A voucher specimen (Number 06-0) (Fig 2.3) was deposited in the CMU Herbarium, Faculty of Science, Leaves and green branches were air dried in shade prior to extraction.

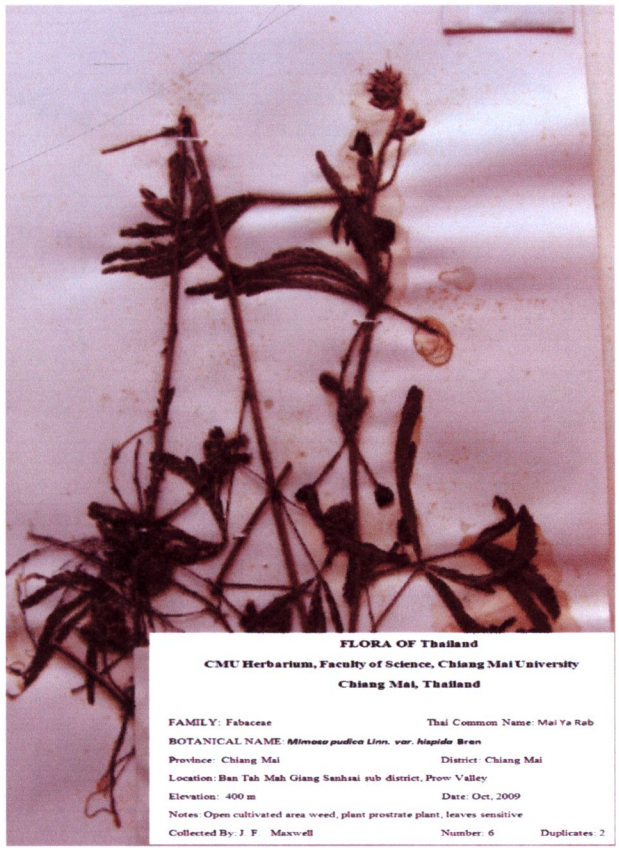

Figure 2.3 The herbarium specimen of *Mimosa pudica* Linn. *var. hispida.* Bren

d. *Rauwolfia serpentina* (L.) Bth. ex Kurz

The leaves were collected by J. F. Maxwell in Chiang Mai, Muang on the East side of Doi Suthep at an elevation of 350 m. The habitat is open disturbed, remnant secondary growth; grassy place. It has deciduous, perennial, tap rooted ground herb;

stem green, turning brown; branch lobes red in colour; corolla tube white with pink. A voucher specimen (Number 04-0) (Fig 2.4) was deposited in the CMU Herbarium, Faculty of Science, Leaves and green branches were air dried in shade prior to extraction.

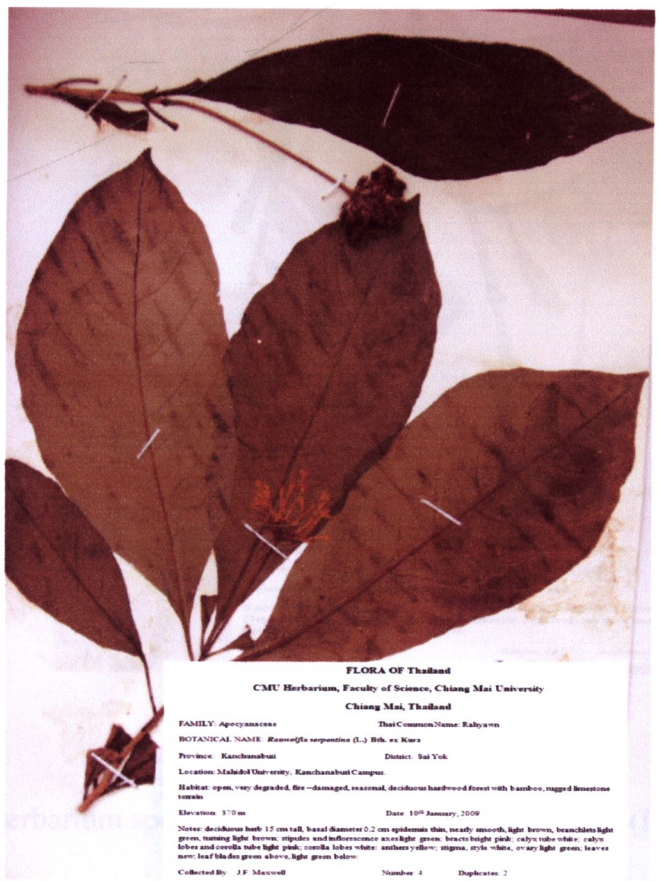

Figure 2.4 The herbarium specimen of *Rauwolfia serpentina* (L.) Bth. ex Kurz

e. *Catunaregam tomentosa* (DC.) Tirveng

The bark and the leaves were collected from Nung Bua, Chiang Mai, Thailand. The tree is about 3 m tall with hard dark brown bark, fruits were green

turning brown. A voucher specimen (Number 03-0) (Fig 2.5) was deposited in the CMU Herbarium, Faculty of Science. The bark was air dried in shade prior to extraction.

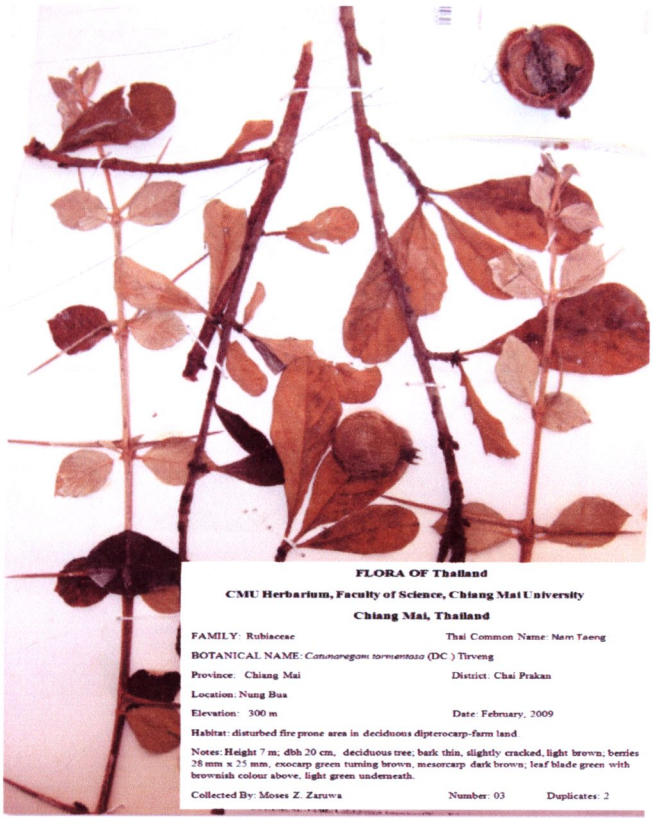

Figure 2.5 The herbarium specimen of *Catunaregam tormentosa* (DC.) Tirveng

f. *Dioecrescis erythroclada* (Kurz) Tirveng

The tree is perennial; deciduous with a height of 7.5 m tall, bark very crack, easily peeled off thin, dark grey; stem and branches dark grey; blades dark green above and light green underneath. The leaves were collected from a partly open area in a degraded fire damaged site at an elevation of 350 m at the foot of Doi Suthep in

March, 2010 by J. F. Maxwell. A voucher specimen (Number 06) (Fig 2.6) was deposited in the CMU Herbarium, Faculty of Science. The leaves were air dried in shade prior to extraction.

Fig 2.6 The herbarium specimen of *Dioecrescis erythroclada* (Kurz) Tirveng

g. *Moringa oleifera* Linn.

The leaves and root were collected from the Medicinal Plant Garden, Faculty of Pharmacy, Chiang Mai University, Suthep Road, Chiang Mai, Thailand in January, 2010. Partly open area in the garden, deciduous seasonal, hardwood forested area; granite bedrock. It is a cultivated tree 5 m tall, the bark is thin, other characteristics are rough, light brown, inflorescence axe, green calyx, white petals, cream turning,

yellow anthers, light green filaments, dark green leaflet segments above, light green underneath.

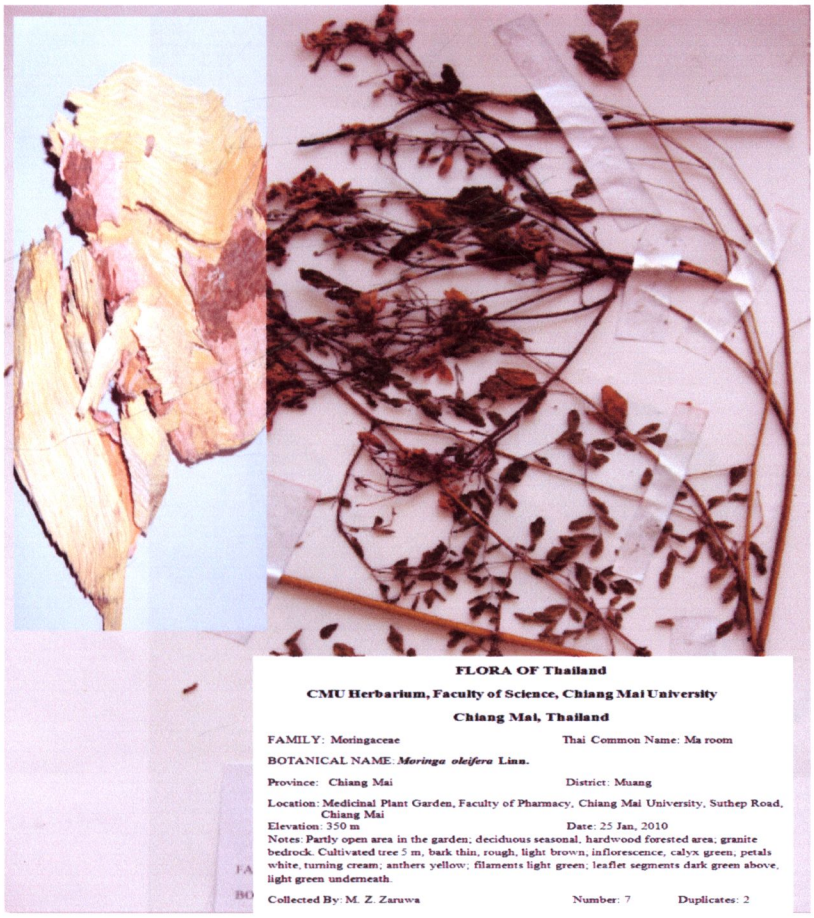

Fig 2.7 The herbarium specimen of *Moringa oleifera* Linn.

h. *Dendrophthoe pentandra* (L.) Miq

The leaves were collected from a partly open area. It is seasonal deciduous, with hardwoods found at the Faculty of Pharmacy frontage. It is an ever green hemi-parasite, shrub on the branches of *Mangifera indica* L. A voucher specimen (Number

5) (Fig 2.8) was deposited in the CMU Herbarium, Faculty of Science. The leaves were air dried in shade prior to extraction.

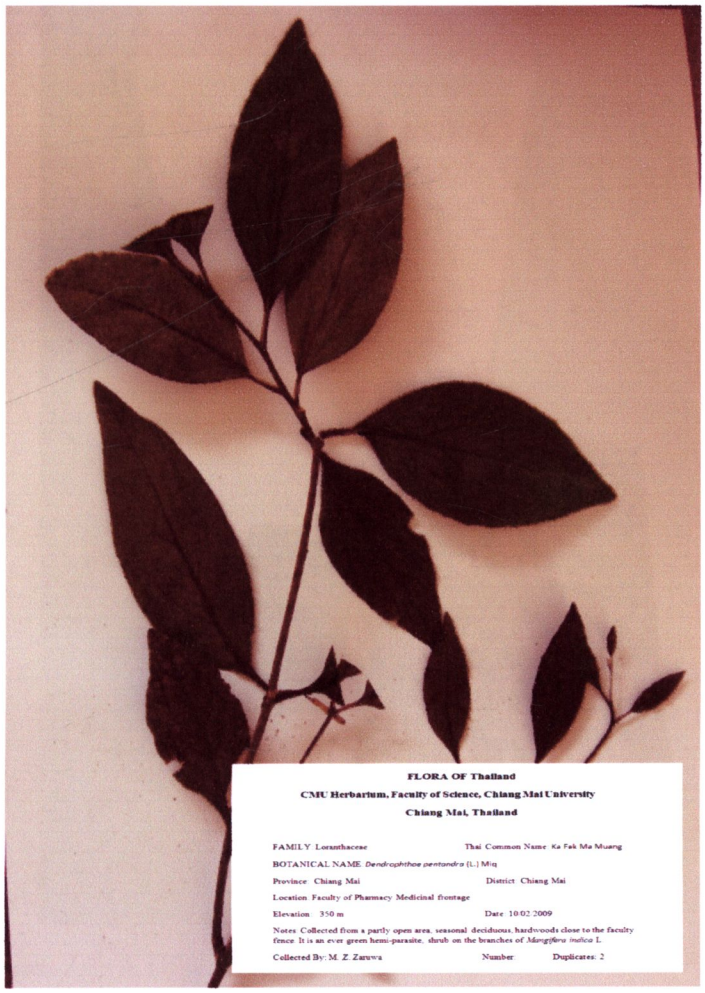

Fig 2.8 The herbarium specimen of *Dendrophthoe pentandra* (L.) Miq

i. Yamed Boraped Pungchang (YBP) recipe

This recipe presented in capsules, is a combination of *Stephania rotunda, Acanthus ilicifolius, Cyprus rotundus, Rhinacanthus nasutus* and was supplied by

Mr. Somnuek of the Department for the Development of Thai Traditional Medicine, Pratoomthani, Thailand. Sample of YBP is shown below (Fig 2.9).

Fig 2.9 Yamed Boraped Pungchang recipe
Picture by Moses Zaruwa

j. Mai Tau Lusi (MTL) recipe

This recipe contained in capsules (Fig 2.10) is a combination of *Curcuma zedoria* (Berg) Roscoe, *Eugenia caryophyllum* Bullock &Harrison, *Piper chaba* Hunt, *Abroma malvaceae*, *Piper nigrum* Linn, *Myristica fragans* Houtt, *Amomum krevanh* Pierre, *Zingiber cassumunar* Roxb in capsules. It was supplied by Mr. Somnuek of the

Department for the Development of Thai Traditional Medicine, Pratoomthani, Thailand.

Fig 2.10 Mai Tau Lusi recipe

Picture by Moses Zaruwa

Nigerian hypoglycemic medicinal plants and recipe

A total of 9 medicinal plants and 1 recipe (made up of three plants) were collected considering their traditional usage for the treatment of diabetes mellitus.

a. *Anisopus mannii* N.E.Br

The medicinal plant specimen (Fig 2.11) was collected from Yola North, Adamawa State, Nigeria at an elevation of 176 m by Mr. Luka Danladi. The leaves were air dried in shade prior to extraction. The voucher number was: ADSU/BIOSCI/002/08.

Fig 2.11 Herbarium specimen of *Anisopus mannii* N.E.Br

Picture by Prof. Jeff Barminas

b. *Anogeissus leiocarpus* (D.C) Guill & Perr.

The medicinal plant specimen was provided by Alh. Jungudo Shuganban Maganin Gargajiya, in Jalingo, Taraba State at an elevation of 233 m. The leaves were air dried in shade prior to extraction. The voucher number was ADSU/BIOSCI/010/08.

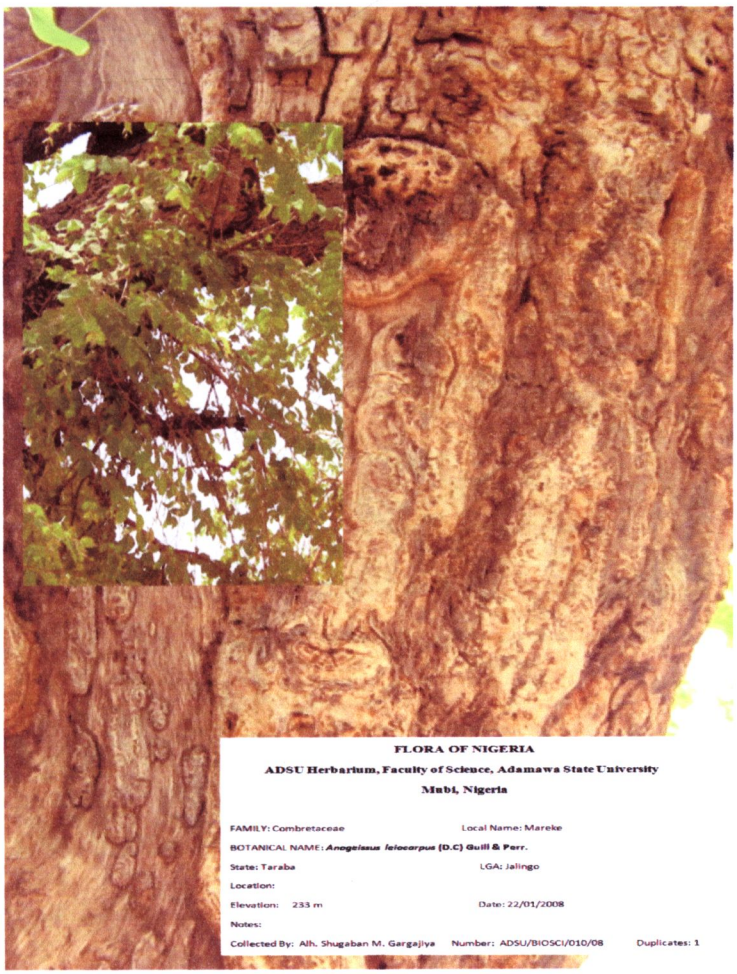

Fig 2.12 *Anogeissus leiocarpus* (D.C.) Guill & Perr.
Picture by Daniel Ali

c. *Daniella oliveri* (Rolfe) Hutch & Dalz.

The D. oliveri sample was provided my Florence Tarfa in Yola, Adamawa State at an elevation of 176 m. The leaves were air dried in shade prior to extraction. The voucher number was ADSU/BIOSCI/014/08.

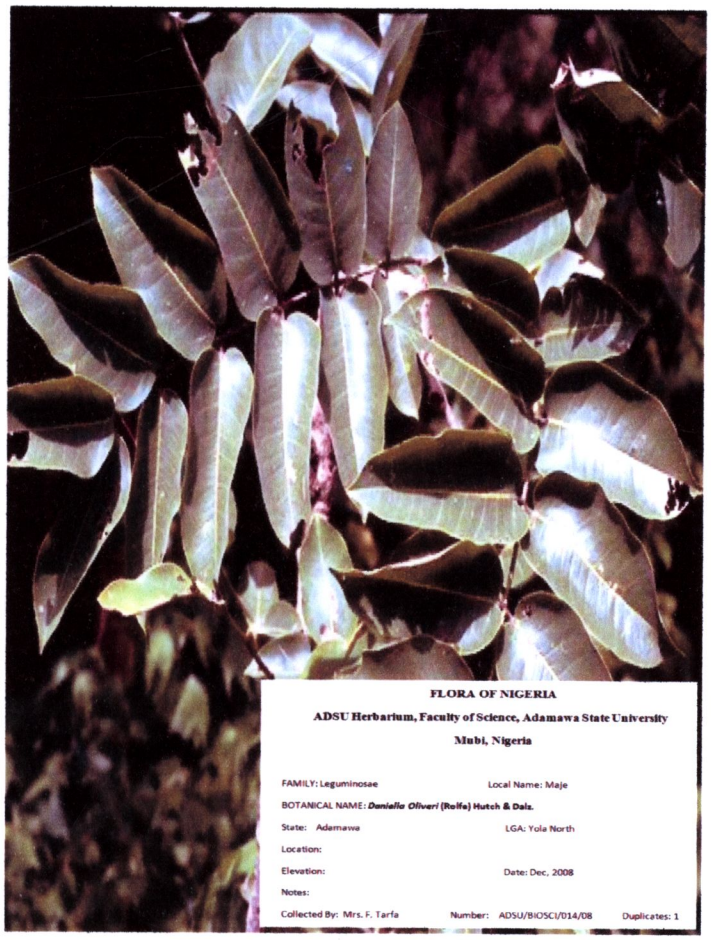

Fig 2.13 *Daniella Oliveri* (Rolfe) Hutch & Dalz.

Source: http://www.prota4u.org

d. *Detarium macrocarpum* Harms

D. macrocarpum was provided by Mr. Daniel Ali and Umar Buba of Adamawa State University, Mubi. It was collected from Wuro Harde village at an elevation of 580 m. The leaves were air dried in shade prior to extraction. The voucher number was ADSU/BIOSCI/021/08.

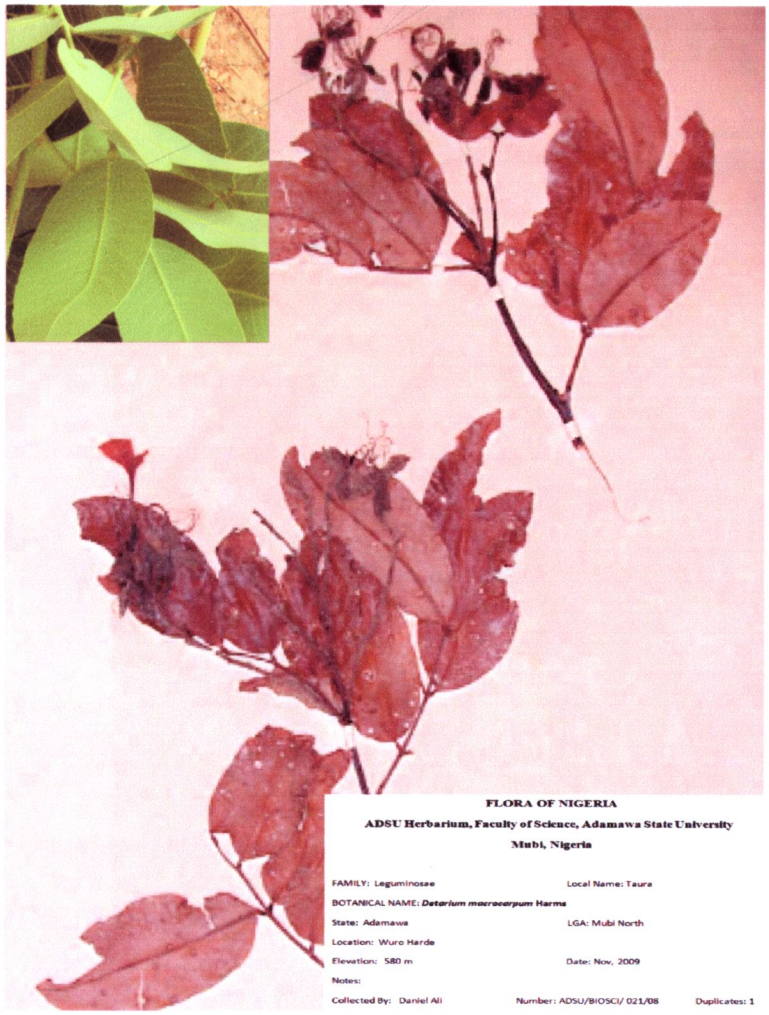

Fig 2.14 *Detarium macrocarpum* Harms

Source: *http://phyto-afri.ird.fr* (Picture by Daniel Ali)

e. *Leptadenia hastata* (Pers) Dec'ne

Collected by M. Z. Zaruwa along a semi tarred road to Kuzum, Bazza, at Mararaba Vimtim, Mubi North Local Government Area, Mubi, Adamawa State (Fig 2.15). Elevation 580 m. The leaves were air dried in shade prior to extraction. The voucher number was ADSU/BIOSCI/140/08.

Fig 2.15 Herbarium specimen of *Leptedenia hastata* (Pers) Dec'ne
Picture by Daniel Ali

f. *Mimosa invisa* var. inermis Adelb

Collected at Mararaba Mubi, Mubi South Local Government Area, from a house used commercially for selling drinks where it is used for ornamental purposes. Elevation 580 m. The leaves were air dried in shade prior to extraction. The voucher number was ADSU/BIOSCI/008/08.

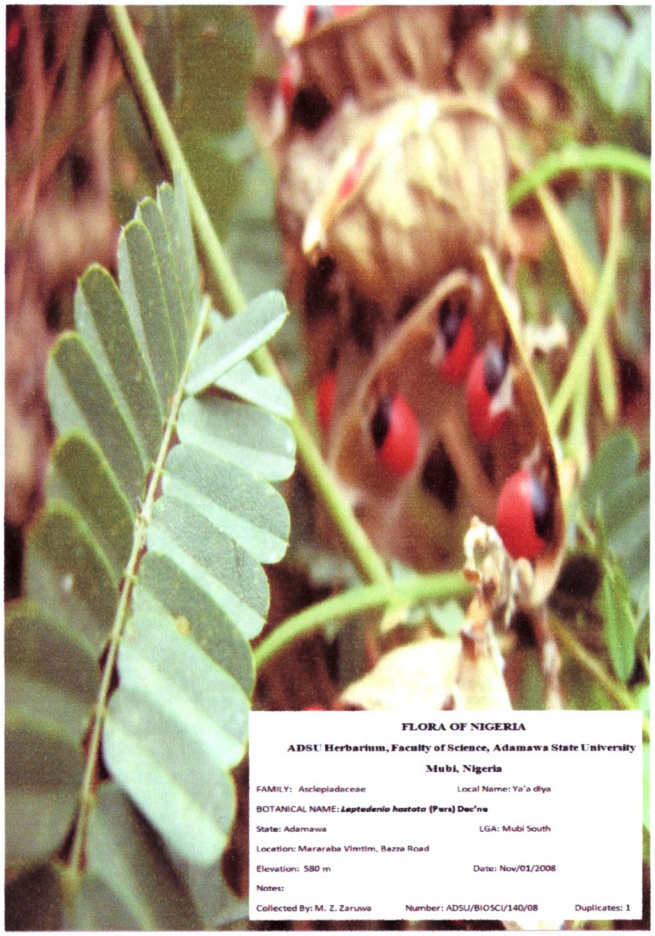

Fig 2.16 *Mimosa invisa var. inermis* Adelb

Picture by Daniel Ali

g. *Moringa oleifera Linn*

The roots were provided by Mr. Adamu Marghi of Mubi North Local Government Area, from a vegetable garden at Kolere ward, Mubi, Adamawa State. Elevation: 580 m. The leaves were air dried in shade prior to extraction. The voucher number was ADSU/BIOSCI/090/08.

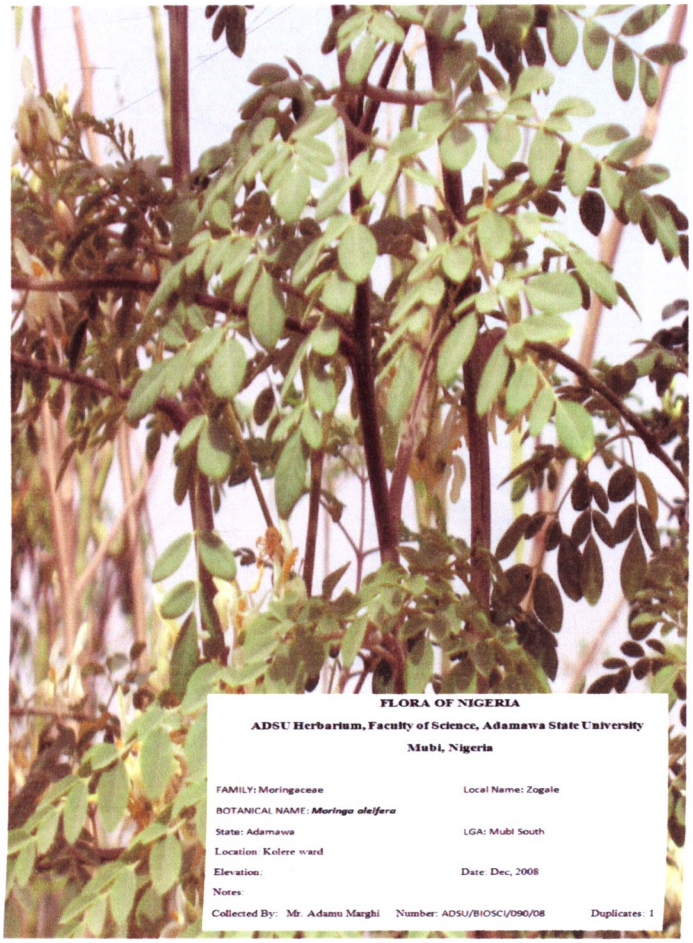

Fig 2.17 *Moringa oleifera Linn*

Picture by Daniel Ali

h. *Rauwolfia serpentina*

Provided from bushes behind Federal University of Technology, Yola (FUTY) campus by Mr. Danladi Luka of the Department of Chemistry, Federal University of Technology, Yola, Adamawa State, Nigeria. Elevation: 180 m. The leaves were air dried in shade prior to extraction. The voucher number was ADSU/BIOSCI/007/08.

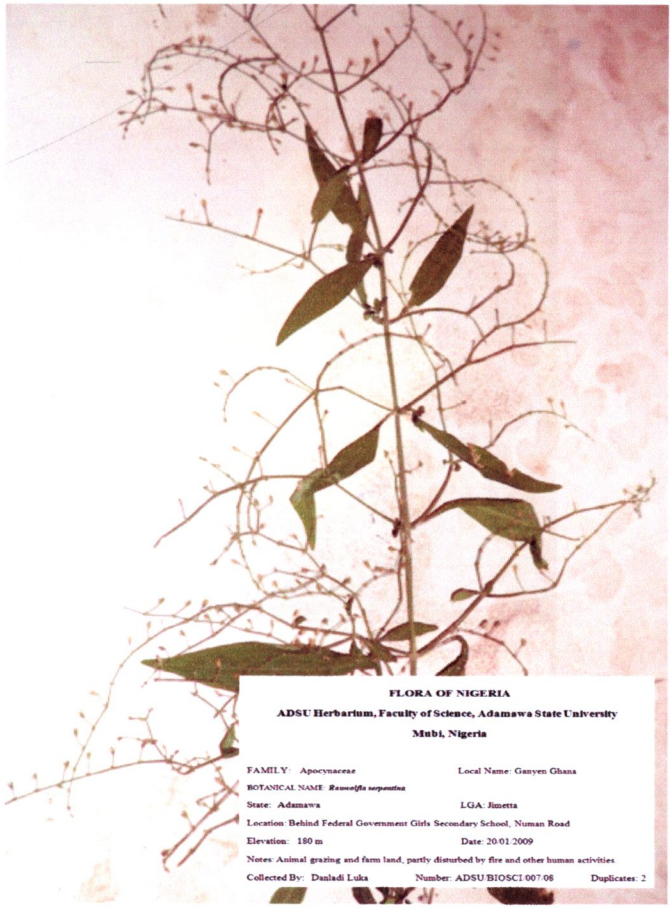

Fig 2.18 *Rauwolfia serpentina*
Picture by Prof. Jeff Barminas

i. *Pterocarpus erinaceous* kurz

Provided by Mr. Simon Aboki of the Forestry Department, Mubi North Local Government Area, at an elevation of 580 m. The leaves were air dried in shade prior to extraction. The voucher number was ADSU/BIOSCI/030/08.

Fig 2.19 *Pterocarpus erinaceous* kurz.
Source: http://*universaldata.sytes.net*

j. Maganin Ciwon Suga recipe

This is a combination of three medicinal plants in equal quantities namely; *Ficus thonningii, Raphia vinifera* and *Leptedenia hastata.*

k. *Ficus Thonningii.* Blume

The medicinal recipes were provided by Dr. Musa Waila. It was obtained from Maduguva village of Mubi North Local Government Area, Adamawa State, Nigeria. Elevation: 580 m. The leaves were air dried in shade prior to extraction. The voucher number was ADSU/BIOSCI/111/08.

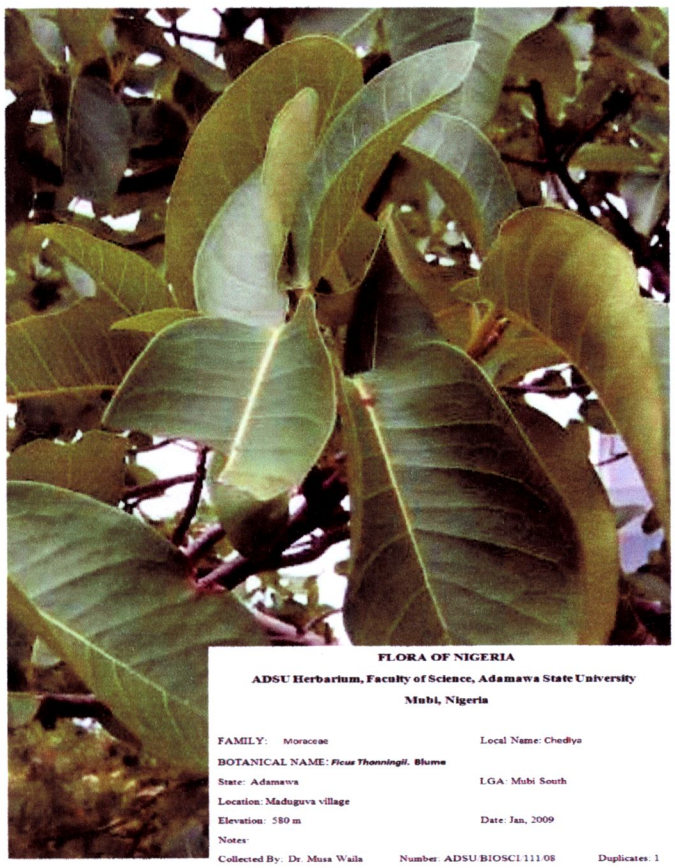

Fig 2.20 *Ficus thonningii*. Blume

Source: http://*plants.usda.gov*

l. *Raphia vinifera* P. Beauv

The leaves (Fig 2.21) were provided by Dr. Musa Waila. It was obtained from Maduguva village of Mubi North Local Government Area, Adamawa State, Nigeria. Elevation: 580 m. The voucher number was ADSU/BIOSCI/111/08.

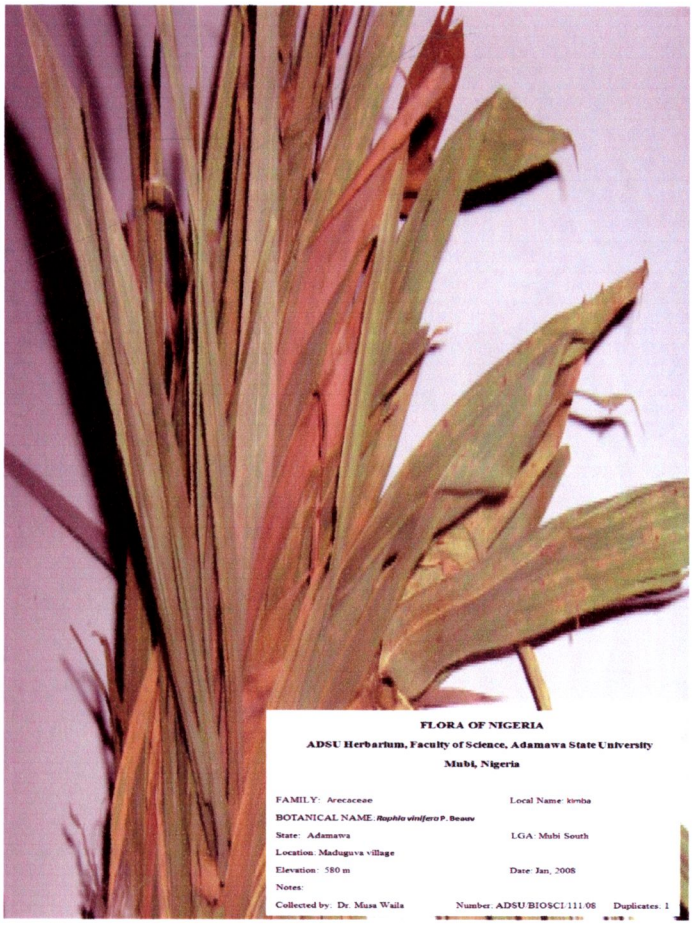

Fig 2.21 Herbarium specimen *Raphia vinifera* P. Beauv

Picture by Umar Buba

m. *Leptedenia hastata* (Pers) Dec'ne

The leaves (Fig 2.22) were provided by Dr. Musa Waila. It was obtained from Maduguva village of Mubi North Local Government Area, Adamawa State, Nigeria. Elevation: 580 m. The voucher number was ADSU/BIOSCI/111/08.

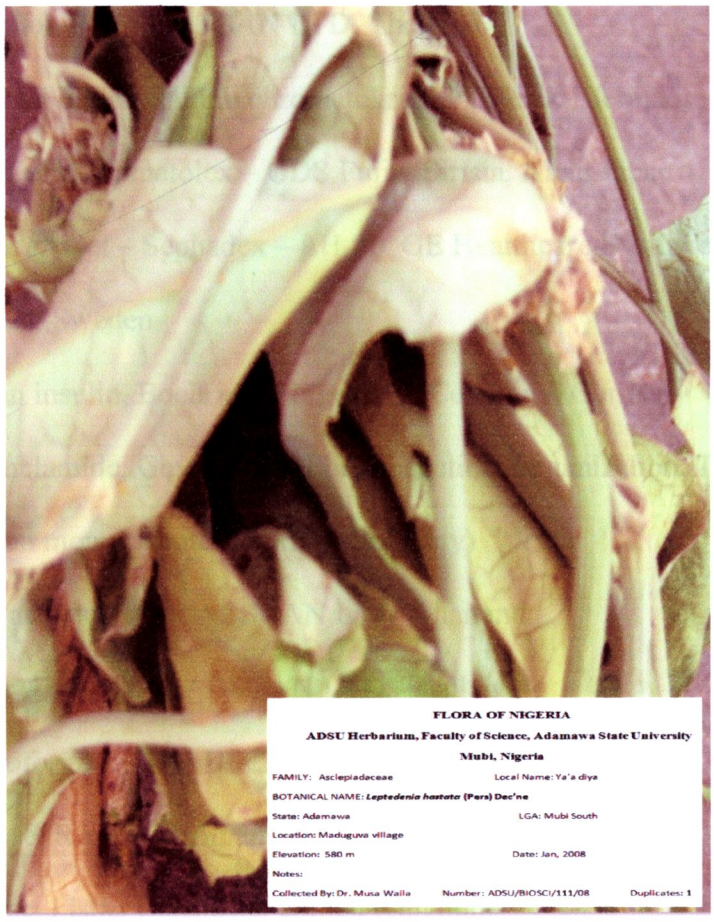

Fig 2.22 Herbarium specimen of *Leptedenia hastata* (Pers) Dec'ne
Picture by Daniel Ali

2.4 Chemicals

- α- Tocopherol (Sigma Co, St. Louis, MO, USA)
- Ascorbic acid (Carlo Erba, Italy)
- 2, 2-Diphenyl-1-picryhydrazyl (DPPH, Sigma Co, St. Louis, MO, USA)
- Methanol, RCI LABSCAN LTD, Thailand
- Silica gel 60 for column chromatography (Merck, Germany)
- ODS Gel: Chromatolex – ODS Fuji – Sirisia, Aichi Japan
- Sephadex Gel – Sephadex – LH-20. GE Healthcare Bio-Science AB. Uppsala, Sweden
- Human insulin, Boehringer Ingelheim Company, Germany
- Glibenclamide, Government Pharmaceutical Organization, Thailand
- Alloxan monohydrate (Aldrich, Germany)
- Ethyl acetate, RCI LABSCAN LTD, Thailand
- Acetic Acid, RCI LABSCAN LTD, Thailand
- Chloroform, RCI LABSCAN LTD, Thailand
- Glucose, Rungid Company Ltd, Bangkok, Thailand
- Sucrose, Fisher Scientific, United Kingdom
- Lactose, Cambridge Commodities Ltd, United Kingdom
- Corn starch, Thai Nakorn Pitana, Thailand
- Isosorbide dinitrate (10 mg), Berlin Pharmaceutical Industry Ltd, Thailand
- Nifedipine (20 mg), Berlin Pharmaceutical Industry Ltd, Thailand
- Anthraquinones, Sigma-Aldrich Company, Germany

- Flavone, Sigma-Aldrich Company, Germany
- Tannin, Sigma-Aldrich Company, Germany
- Xanthones, Sigma-Aldrich Company, Germany
- Quinine sulphate, Sigma-Aldrich Company, Germany

2.5 Equipment

- Centrifuge tube 50 ml (TPP, Switzerland)
- Rotary evaporator (Buchi, Switzerland).
- Single Channel Pipette (Buchi, Switzerland)
- Soxhlet Apparatus, Colepalmer, U.S.A
- Lyophilizer (Martin Christ - ALPHA 1-2, Germany)
- 96 well plates, Nunc, USA
- Thin Layer Chromatographic Plates, MERCK, Germany
- Finetest Glucometer (Infopia Co., Ltd. Korea)
- High Performance Liquid Chromatography (HPLC), RESTEK, U.S.A
- Well reader (Seikagaku SK601, Japan)
- Ultracentrifuge (Avanti^{TM} 30 Centrifuge, Beckman, USA)
- UV- visible spectrophotometer (Cary 1 E) Shimadzu, Japan
- Evaporative Light Scattering Detector (ELSD), Agilent Technologies, Japan
- Refractive Index (RI) Detector, Senshu Scientific Co. Ltd, Japan
- Nuclear Magnetic Resonance Spectroscopy (NMR), JEOL JNM LA-500
- Spectrometer. JEOL Model JNM – ECX400 Fourier-Transform NMR

System, Japan

- Mass Spectroscopy (MS): TOF: Agilent 1100 LC/MSD TOF System, Japan

2.6 Methods

The scope of the study was designed as in the below figure 2.1.

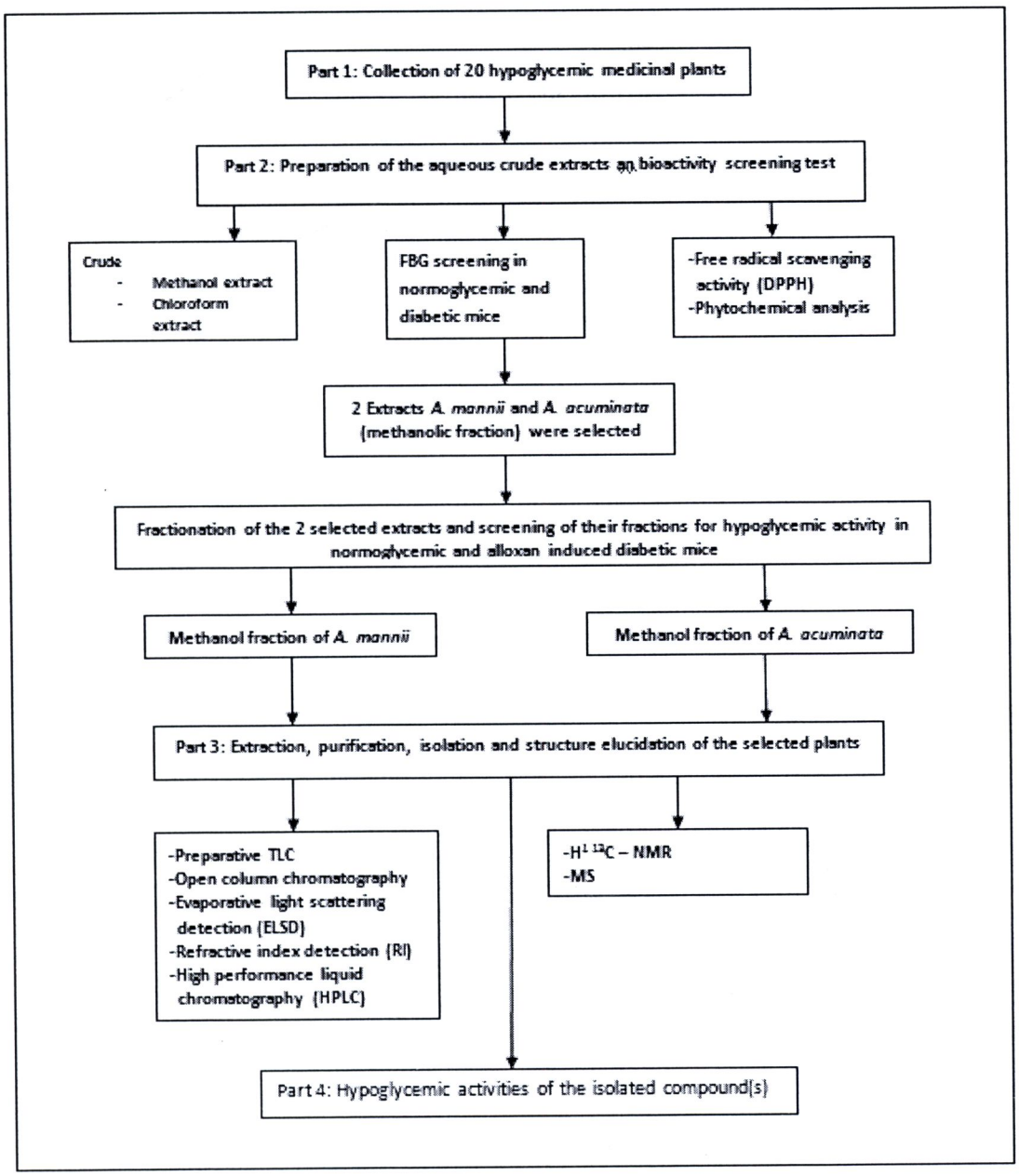

Fig 2.23 Scope of the study of Parts 1 to 4

Part 2: Preparation of the crude extracts and bioactivities screening test

2.6.1 Preparation of the crude extracts

The wood barks and leaves of the selected nineteen medicinal (eighteen single plants and three others mixed in equal proportion) plants were separately milled into powder after drying at controlled temperature (40 – 45 ^{o}c) in a hot air oven. Three other recipes provided in ready to use form (capsules) was weighed after losing the capsule to collect their contents. The different powder samples (150 – 300 g) were macerated in distilled water and the solution was filtered and evaporated under reduced pressure using a rotary evaporator. The aqueous extracts were lyophilized and weighed. The extracts were screened for their hypoglycemic effects in normoglycemic and alloxan induced diabetic mice over a 4 h period and 2 of the extracts with the highest hypoglycemic effects were chosen (Fig 2.24).

The two selected hypoglycemic medicinal plants powder (200 g) were extracted in 85% methanol using a Soxhlet extractor for 72 hours. The solvent was evaporated at reduced pressure in a rotary evaporator. The residues were re-extracted with chloroform and concentrated by evaporation with a rotary evaporator under reduced pressure. The percentage yields were calculated and dosage to be used for screening.

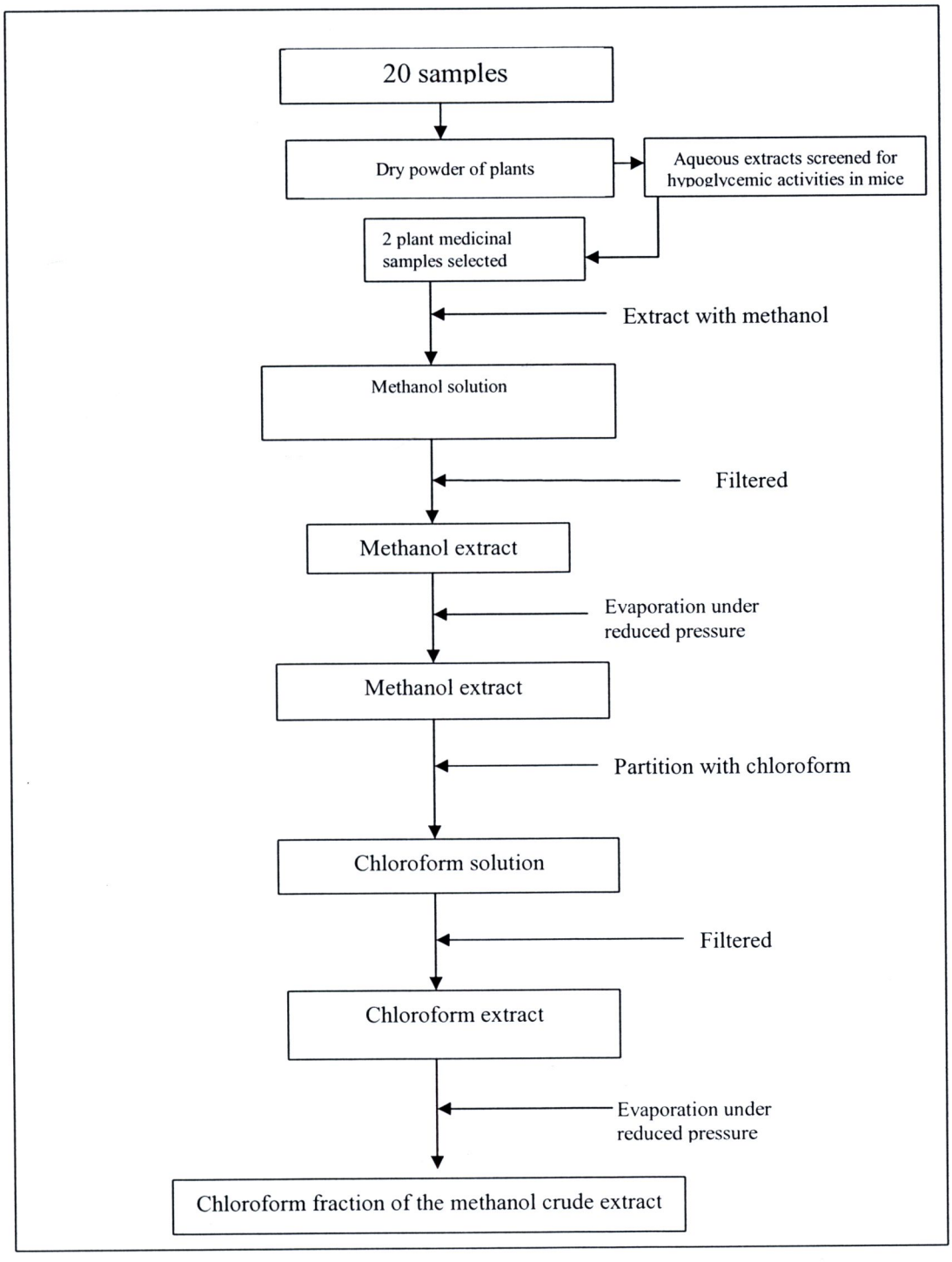

Fig 2.24 Diagram of the preparation of crude extract

2.6.2 Partitioning of the crude extracts

The chloroform and methanol crude extracts were subjected to TLC (ethyl acetate-butanol-methanol – 7:2:2 and chloroform-methanol – 8:2 for *A. acuminata* and ethyl acetate-acetic acid-methanol – 8:2:2 and chloroform-methanol-acetic acid – 9:1:0.1) and subsequent liquid phase partitioning to obtain various fractions as shown in the figure below (Fig 2.25).

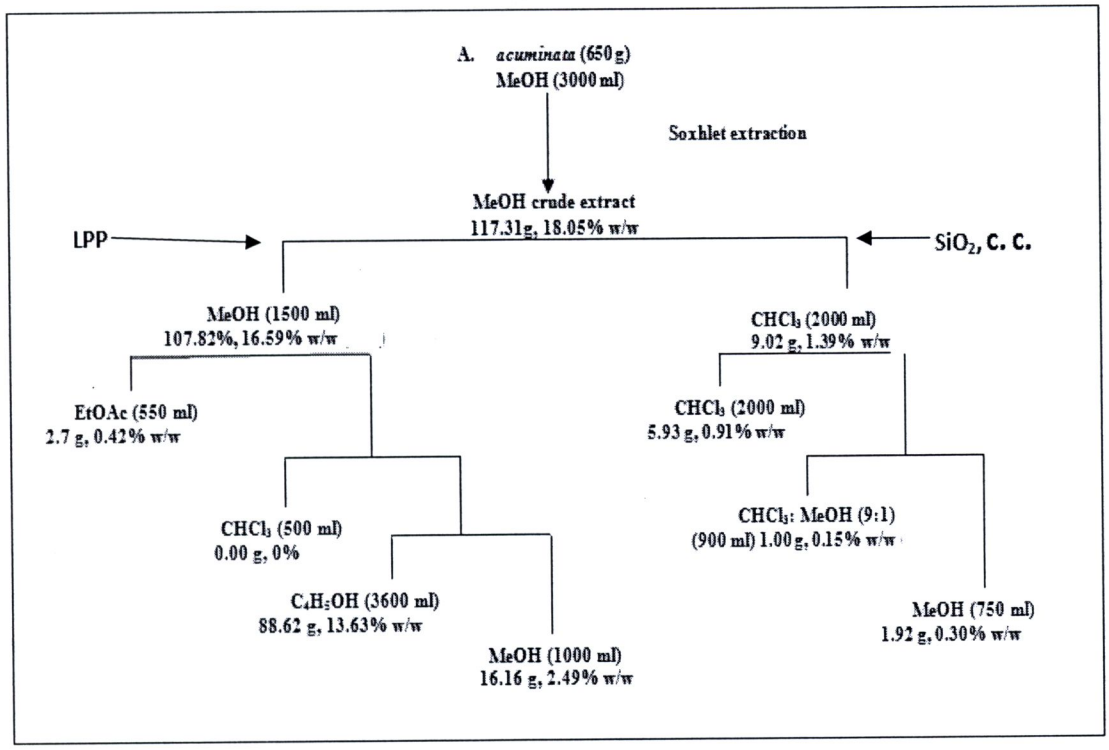

Fig 2.25 Diagram of the partitioning of the *A. acuminata* crude extract

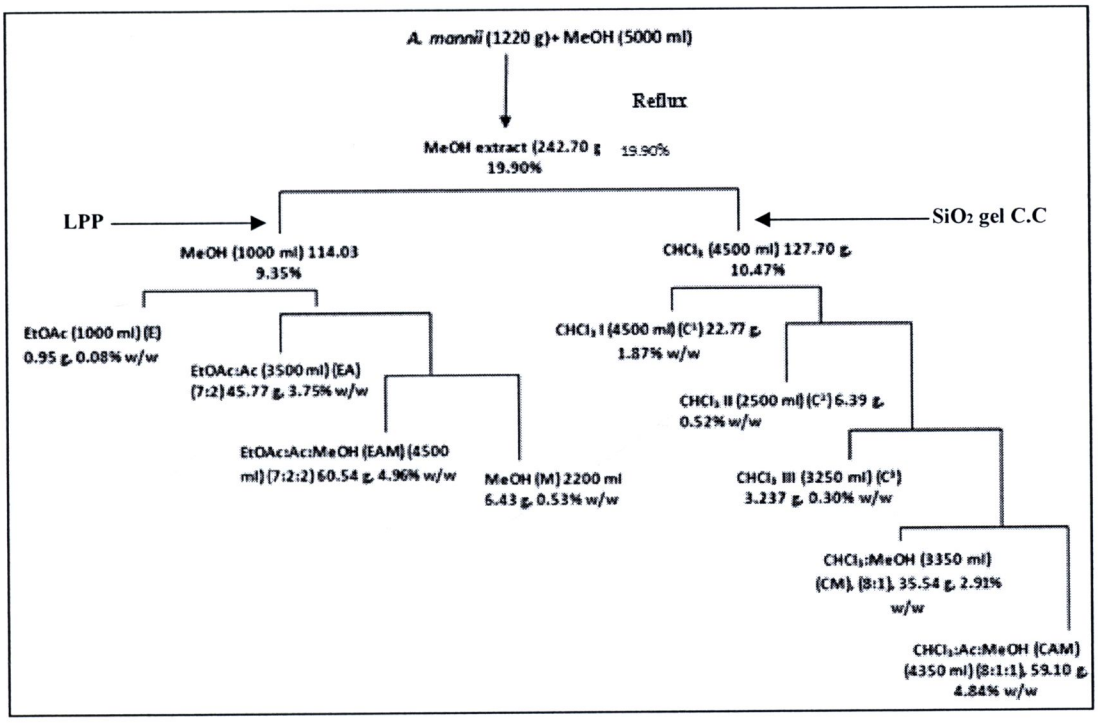

Fig 2.26 Diagram of the partitioning of *A. mannii* crude extract

2.6.3 Phytochemical analysis of 20 medicinal plant crude extracts

The phytochemicals including flavones, glycosides, saponins, tannins, alkaloids, anthraquinones and xanthones were analyzed by the standard methods. Shinoda test: the concentrated HCl with Mg ribbon was added to the extract. The pink red colour of the solution indicated the presence of flavonoids.

Glycosides: the extract was hydrolyzed with dilute HCl and neutralized with NaOH solution and added with drops of Fehling's solution. The red precipitate indicated the presence of glycosides.

Froth Test: an amount of 5ml of distilled water was added to 0.5g of the extract, shake and heat to boiling. Frothing indicated the presence of saponins.

Van Middlesworth and Cannell Test: the bromine water was added to the extract. The formation of a yellow gelatinous precipitate indicated the presence of tannins.

Dragendorff's Test: addition of Dragendorff's reagent to the extract leading to the formation of orange precipitate indicated of the presence of alkaloids. Quinine sulphate (Sigma-Aldrich, Germany) was used as standard.

Borntrager's test: an amount of 0.1g of the extract was boiled with 4 ml of ethanolic KOH for 2-3 mins, diluted with 4ml of H_2O and filtered. Filtrate was acidified with dilute HCl, filtered, cooled and mixed with benzene with shaking. The benzene was transferred to a clean test tube containing 2ml of dilute NH_3 solution. Orange or deep red colour of the mixture indicated the presence of anthraquinones. Anthraquinones (Sigma Chemicals, Mo, USA) was used as the standard.

Test for xanthones: an amount of 5% KOH was added to the extract. Yellowish coloration indicated the presence of xanthones (Trease and Evans, 2002).

2.6.4 Free radical scavenging activity of the crude extracts of 20 medicinal plants (DPPH assay)

The DPPH radical scavenging activity of the extracts was determined by a modified method previously described by Tachibana *et al.*, (2001). Briefly, a 100 µL of the five serial concentrations of the extracts in distilled water ranging from 0.001 – 10 mg/mL was added to each well of 96 well plate. A 100 µL volume of 1% DPPH in ethanol were transferred into each well of a 96-well microplate (Corning Costar, USA). The reaction mixture was allowed to stand for 30 mins at $27^{o} \pm 2^{o}C$ and measured the absorbance at 515 nm by a well reader (Bio-Rad, Model 680,

Philadelphia, PA19102-1737, USA). Ascorbic acid (Carlo Erba, Italy) was used as positive control. The experiments were performed in triplicate. The IC_{50} value, the concentration of the sample that scavenged 50% of DPPH radical was determined by probit-graph interpolation of six concentrations. The percentages of the DPPH radical scavenging activity were calculated by the formula shown below.

$$\text{Scavenging activity (\%)} = (Abs_{control} - Abs_{sample}/Abs_{control}) \times 100$$

2.6.5 Experimental animals

Adult male and female ICR mice weighing between 24 – 27 g purchased from the Faculty of Medicine, Chiang Mai University and the National Laboratory Animal Center-Mahidol University, Thailand, were used. The mice were fed with the standard diet, water ad libitum and were maintained under the standard conditions of temperature, humidity and light (23 ± 1°C; 70% RH and 12 h light/dark). The experiments was complied with the Organization for Economic Co-operation and Development (OECD/OCDE: 425) Guidelines for Testing of Chemicals (2008).

2.6.6 Production of the diabetic mice

Diabetic mice were produced as previously described by Zhou *et al.,* (2009). Briefly, the mice were injected at the tail vein with alloxan monohydrate in sterile normal saline solution at the dose of 75 mg/kg *bw*. Diabetes was confirmed on the third day after alloxan administration. The mice having blood glucose levels greater than 200 mg/dl were considered diabetic and selected for further study (Cunha *et al.,* 2008). Fasting Blood Glucose (FBG) was assayed from the tail vein blood of the mice using Finetest Glucometer (Infopia Co., Ltd. Korea). Three doses of the plants extract

(100, 200 and 400 mg kg^{-1} *bw*) (Tanko *et al.*, 2008) were orally administered to the 18 h fasted normal/diabetic mice (n = 5) using feeding tube (Moufid, 2009). Noticeable irritation or restlessness should not be observed after administration of the extracts. Blood glucose was measured hourly for 4 hrs. Control mice were fed with distilled water (oral), whereas insulin 0.5 IU/kg (injection/*ip*) and glibenclamide 1 mg/kg (oral) were used as reference hypoglycemic drugs.

2.6.7 Hypoglycemic studies

All fractions obtained from the partitioning were prepared into three doses of 100, 200 and 400 mg/kg *bw* based on the various concentrations obtained. The different doses were administered to groups (n =5) of normoglycemic and alloxan induced diabetic ICR mice after an 18 h fast and monitored over a 4 h period. The effect of each extract was compared with standard hypoglycemic drugs, insulin and glibenclamide.

2.6.8 Oral glucose tolerance test (OGTT)

OGTT was performed by using the modified methods as described by Wu *et al.*, (2011). Briefly, normoglycemic mice were divided into five groups of 5 mice each. The mice were fasted for 18 h, and then treated with the agent orally using a feeding tube. After two hours, DW or different carbohydrates were administered as indicated; glucose (2.5 g/kg, o.p.), Sucrose (2.5 g/kg, o.p.), Corn starch (6 g/kg, o.p.) and lactose (6 g/kg, o.p). The blood glucose were tested at 0, 1, 2, 3 and 4 h. Glibenclamide (Glb - 1.0 mg/kg *bw*) and Insulin (Ins - 0.5 iu/kg bw) served as control (1 mg/kg bw, o.p.).

2.6.9 Co-treatment with Ca^+ and K^+ ion channel regulators

Normoglycemic mice were divided into four groups (n=5) with three other groups treated with Ca^+ (Nifedipine) or K^+ (Isosorbate dinitrate) channel regulators (Wu *et al.*, 2010). The NC group was treated with normal saline (*i.p.*), AMS (250 mg/kg *bw* + normal saline, *i.p.*), AMN (250 mg/kg *bw* + normal saline + Nifedipine 13.6 mg/kg, *o.p.*) and AMI (250 mg/kg *bw* + normal saline + Isosorbide dinitrate 6.8 mg/kg bw) respectively. Blood glucose was tested at 0, 2, 4 and 6 h post treatment.

2.6.10 Intraperitoneal injection and oral administration studies

Mice were kept under standard conditions of temperature and humidity. The animals were fasted for 18 hours and 75 mg/kg *bw* alloxan monohydrate administered via the tail vein. Diabetic mice (< 200 mg/kg *bw*) were selected and grouped into three (n =5). To the control group was administered distilled water (DW), group 1 received AM (62.5 mg/kg *bw,* i.p.) and group 2 received AM (250 mg/kg *bw*, o.p.). Blood glucose was monitored at 0, 1, 2, 3 and 4 h post treatment.

2.6.11 Acute oral toxicity studies

The acute oral toxicity study was done with modified protocol as described by Adeneye *et al.,* (2006). Sub-fractions of AM were prepared using 2 ml normal saline solution and administered sequentially over a 12 h period using a feeding tube. Studies were conducted using the limit test of up and down procedure according to the OECD/OCDE Test Guidelines (2008). A total of five female mice were selected at random. The mice were fasted for 18 h. Body weight of each mouse was determined and dosed with equivalent of 2000 mg/kg *bw* (because no lethal effect was expected

since the medicine has been in use among humans) of the MeOH sub-fraction dissolved in distilled water using a feeding tube. Mortalities, clinical signs, body weight changes and gross findings were monitored for 14 days (1 – 15) (Ha *et al.*, 2010).

Each animal was observed each time for the first 10 min post dosing for signs of regurgitation and thereafter for every 30 min for the successive 6 h and daily for 14 days for possible lethal outcome. Behavioral manifestations of acute oral toxicity were also noted for each mouse. The same procedure was repeated with 5000 mg/kg *bw* of the AM methanol fraction on different groups of mice.

2.6.12 Experimental execution/histopathological examination

All mice were observed for 14 days and their body weights taken every 72 h. On the 14^{th} day, each mouse was decapitated with a shape surgical blade and blood collected in clean appendorf tubes and centrifuged at 3000 rpm for 10 minutes. Sera were collected for each mouse in an appendorf tube, labeled and stored at $4^{O}C$, to be used for blood urea nitrogen (BUN), Creatinine, AST, ALT and total bilirubin analyzes. The kidney and liver were removed, washed in normal saline, wiped and weighed on a digital balance and preserved in 10% formalin for histopathology examination.

In the histopathological examination, the kidney and liver of mice from the three groups were embedded in paraffin, cut into 6 and 12 μm thick, respectively. They were later stained with hematoxylin and eosin and examined under a light microscope (Huo *et al.*, 2003).

2.6.13 TLC/UV Spectrometric/HPLC analysis

Preliminary analysis of the MeOH fraction of AM with 10% H_2SO_4 spray was done alongside chemical constituent of phytochemical contents of *A. mannii* by HPLC using saponin purum standard in EtOH, Sigma-Aldrich Company, Germany.

One mg of *A. mannii* (MeOH,) fraction was dissolved in distilled water (1 ml) and filtered with a 0.45 μm and 0.75 ml was analyzed on a UV visible spectrophotometer (Shimadzu, Japan) to obtain a suitable spectral wave length for further analysis.

One ml of the filtered solution was transferred into an amber bottle for HPLC analysis and compared with a standard. A Gemini – Nx 5 μ C_{18} 110 A, 250 x 4.60 mm column (Thermo Finnigan- Auto sampler, Thailand). The mobile phase was 40% MeOH and using an isocratic program for 45 minutes. The flow rate was 0.8 ml/min and the wavelength set at 218 nm.

2.6.14 Ethical clearance

The animal experimental methods were approved by Chiang Mai University Animal Ethics Committee, Protocol Number: 40/2552.

2.6.15 Statistical analysis

The data are expressed as mean ± SEM or mean ± SD calculated from Microsoft excel 2003. Differences between 2 means were compared using student's t-test. Values were considered statistically significant at $p < 0.05$.

2.6.16 Extraction, isolation, purification and structure elucidation of selected (*A. acuminata* and *A. mannii*) fractions by ODS gel chromatography

The dried powder of *A. acuminata* and *A. mannii* were extracted with methanol and the extracts partitioned with chloroform, and evaporated to dryness. The methanol extracts were separated using liquid phase partitioning as described on pages 121 and 122 (Figures 2.25 and 2.26). ODS, Sephadex column chromatography, ELSD and HPLC were used to purify the methanol extract. Analytical TLC was used to compare the bands of all the fractions obtained and those with very similar characteristics were combined. The structures of the isolated fractions were elucidated by ^{1}H-, ^{13}C-NMR, DEPT 90, DEPT 135 and MS.

Part 3: Hypoglycemic activities of the isolated compounds

All fractions obtained from the partitioning were prepared into three doses of 100, 200 and 400 mg/kg *bw* based on the various concentrations obtained. The different doses were administered to groups (n = 5) of normoglycemic and alloxan induced diabetic ICR mice after an 18 h fast and monitored over a 4 h period. The effect of each extract was compared with the standard hypoglycemic drugs, insulin and glibenclamide.