CHAPTER III

MATERIALS AND METHODS

Materials

A. Raw material

Gymnema inodorum tea was purchased from Jinda Rungrueng, Chantaburi, Thailand.

B. Glasswares

Borosilicate glass tubes (16 x 125 mm) with Teflon-lined screw caps were bought from Pyrex and regularly leak provided by re-weighting, the reduced weight tubes were avoid and/or discarded. All glasswares were washed with mild detergent and rinsed twice with distilled water and air dried before use in the experiment.

C. Chemicals

All chemicals were analytical grade and purchased from domestic distributors. The main chemicals in this study are listed below:

1. Glucose (Glucolin)
2. Glucose Oxidase (GLUCOSE liquicolor, GOD-PAP Method, Human Gesellschaft fur Biochemica and Diagnostica mbH)
3. Standard glucose 100 mg/dL (Human Gesellschaft fur Biochemica and Diagnostica mbH)
4. Control glucose, normal range 87-107 mg/dL (Human Gesellschaft fur Biochemica and Diagnostica mbH)
5. FlexiGene DNA Kit (QIAGEN)
6. PCR Master mix (Invitrogen)
7. 100 pM/μl primer sense for -374T/A polymorphism study (5'-GGGGGCAGTTCTCTCCTC-3')

8. 100 pM/μl primer anti-sense for -374T/A polymorphism study (5'-TCAGAGCCCCCGATCCTATTT-3')
9. 100 pM/μl primer sense for -429T/A polymorphism study (5'-GGGGGCAGTTCTCTCCTC-3')
10. 100 pM/μl primer anti-sense for -429T/A polymorphism study (5'-TCAGAGCCCCCGATCCTATTT-3')
11. *Alu* I enzyme (Promega)
12. *Tsp 5091* enzyme (Promega)
13. p-nitrophenyl-α-D-glucopyranoside (Sigma)
14. α-glucosidase enzyme (Sigma)
15. Acarbose
16. Insulin assay kit (Insulina IRMA kit, RADIM S.p.A.)
17. RPMI-1640 (Gibco BRL)
18. Ethanol (Sigma)
19. Methanol (Sigma)
20. 1-butanol (Sigma)
21. Ethyl acetate (Sigma)

D. Instruments

All experiments were carried out at Faculty of Allied Health Sciences, Chulalongkorn University. The main instruments employed in the experiment are listed below:

1. Microcentrifuge CS 150 GXL, Hitachi, Tokyo, Japan
2. Refrigerated centrifuge CF 7D2, Hitachi, Tokyo, Japan
3. Photometer Humalyzer 3000, Human, Germany
4. Rotary evaporator R-114, Buchi, Switzerland
5. Vacuum system, model B-169 Buchi, Switzerland
6. Gene Amp PCR System 2400, Perkin Elmer
7. Thermo Hybrid PCR, Bio-Active Co.,Ltd

8. Gel electrophoresis set, Bio-Active Co.,Ltd

Figure 3 Commercial *Gymnema inodorum* tea (purchased from Jinda Rungrueng, Chantaburi, Thailand.)

Methodology

A. Study of hypoglycemic effect of *G. inodorum* tea in human

This study has been approved by the Ethic Committee on Human Studies, Faculty of Medicine, Chulalongkorn University. Eighty healthy voluntary subjects of both sexes, aged between 18-25 years participated in this study. Blood samples were obtained with written informed consent. Before the study, all subjects were tested for fasting plasma glucose (FPG). The subjects who have normal FPG (60-110 mg%) will be included in this study, but the subjects who have higher FBG level will be excluded. Finally, with this criteria only 73 subjects took part in the study.

The study was designed as a before/after experiment and oral glucose tolerance test (OGTT) was used as a tool. The "before" or control group was the group of healthy subjects that was studied for OGTT without GI tea consumption (n=73) and the "after" or treatment groups were the groups of subjects that were studied for OGTT with GI tea consumption at various period of time after the oral glucose load in standard oral glucose tolerance test (0, 15, 30 min). Usually the GI tea was prepared by soaking one bag (1.5 g) of tea in 150 mL of boiling water and soaked for 5 minutes. The studied details are as following;

a. Forty subjects drank 1.5 g of GI tea in 150 ml boiling water immediately after oral glucose load (treatment I).
b. Seventy three subjects drank 1.5 g of GI tea in 150 ml boiling water, 15 minutes after oral glucose load (treatment II).
c. Twenty subjects drank 1.5 g of GI tea in 150 ml boiling water, 30 minutes after oral glucose load 9treatment III).
d. Nineteen subjects drank 3.0 g of GI tea in 150 ml boiling water (double concentration of GI tea), 15 minutes after oral glucose load (treatment IV).

The Pattern of OGTT curves were analyzed and the peaks of glucose absorption were compared, using Student's t-test.

Plasma glucose

Plasma glucose was measured at 0 (baseline) and 30, 60 and 120 minutes after glucose load. Venous blood samples were used for measurement of plasma glucose, using glucose oxidase method (Human Gesellschaft fur Biochemica and Diagnostica mbH, Wiesbaden, Germany) (Table 3).

Table 3 Glucose oxidase method for determination of plasma glucose*

	Sample	Standard	Reagent Blank
Sample	20 µL	-	-
Standard	-	20µL	-
Distilled water	-	-	20 µL
Enzyme regent	2 ml	2ml	2ml
Mix well, and incubate for 15 minute at room temperature (20-25 °C). Measure absorbance at 505 nm within 60 minutes.			

*Glucose liquicolor manufacturer leaflet (2003): Human Gesellischaft Fuir Biochemica and Diagnostica mbH., Germany

B. Hypoglycemic effect of GI tea, using standard meal

Twenty healthy voluntary subjects of both sexes, aged between 18-22 years participated in this study. Blood samples were obtained with written informed consent. Before the study, all subjects were tested for fasting plasma glucose (FPG). The subjects who have normal FPG (60-110 mg/dL) will be included in this study, the subjects who have FBG exceed this limit will be excluded.

The standard meal was prepared from 66 g of baguette French bread (glycemic index = 95) and 15 g of strawberry jam (1 pack of Smuckers' strawberry jam, glycemic index = 49), total glycemic index equals to 88 with glycemic load = 72, and total calories of 225 kilocalories.

The subjects were assigned to eat standard meal and drink one cup of GI tea (1.5 g GI in 150 ml water) at 15 minutes after the first bite of the meal. Blood sample was drawn before taking the meal (0 min) and at 30 min, 60 min and 120 min after the first bite of the meal. Baseline or Control group is the same group of subjects that ate standard meal without drinking GI tea.

C. Effect of GI tea consumption on liver function test

Twenty healthy subjects with normal plasma glucose and liver function enzymes (aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT)) were included in this study. During the period of 28 days, the subjects will have daily consumption of 1 pack of GI tea (1.5 g in 150 ml hot water) and were asked for monitoring liver enzymes level and fasting plasma glucose from blood at day 0, 2, 4, 7, 14, 21, and 28.

D. Preparation of *G. inodorum* extract

Pretreatment / Decoloring step with and acid solution

Dried *Gymnema inodorum* leaves were crushed into 0.5-2 mm. To 1 kg of the dried crushed leaves, 10 liters of an aqueous citric acid solution (pH 2.5) was added and agitated at room temperature. After 1 hour, the resultant mixture was dehydrated using a 800 mesh filter cloth. Finally, the leaves were washed with 4 liters of water. Then,

the leaves were dried in a drier at 80°C for 16 hours and 752 g of acid-treated leaves was obtained.

Extraction step

To 400 g of the acid-treated leaves, 4 liters of aqueous 50% ethanol solution was added and agitated at room temperature. After 1 hour, the mixture was vacuum filtered using a filter paper No.2 (Whatman) to thereby obtain an extract. To the acid-treated leaf cake, 4 liters of aqueous 50% ethanol solution was added again, extracted under agitation for 1 hour at room temperature and then filtered similarly to thereby obtain an extract. Both extracts were mixed to obtain about 8 liters of extract.

Concentration step

The obtained extract was concentrated in a rotary evaporator under at 80°C until the smell of ethanol was eliminated. As a result, 2.2 liters of a concentrate was obtained. Of this concentrate, 200 ml was further concentrated and dried/caked to obtain 4.2 g of light brown, roughly purified *Gymnema inodorum* leaf extract. To the remaining 2 liters of the concentrate, water was added to make the total volume of 3 liters.

Partition extraction step

The prepared 3 liters concentration was divided into six 1 liter separating funnels. To each of these funnels, 300 ml of 1-butanol was added, shaken for 30 minutes using a shaker and then left stationary. About 1.8 liters of 1-butanol solution was obtained by collecting the separated fraction from each funnel. The aqueous phase was concentrated to thereby obtain a concentrated residue from partition.

Defatting / Decoloring step

To the resultant 1-butanol solution, 80 g of diatomaceous earth was added, and then concentrated and dried at 80°C using a rotary evaporator. The resultant cake was further dried in a drier at 80°C for 16 hours and crushed, to obtain 121.2 g of fine powder of which excipient was diatomaceous earth, then one liter of petroleum ether was added. The mixture was defatted at room temperature for 10 minutes using an ultrasonic cleaner and vacuum filtered with a filter paper No. 2 (Whatman). The residue was defatted again by the above-mentioned procedures and filtered. The resultant residue was further defatted using 1 liter of ethyl acetate and

filtered. The resultant residue was dried in a drier at 80°C for 1 hour to obtain 105.6 g of fine powder. All of the filtrates obtained from individual filtration steps were collected and concentrated to thereby obtain a concentrated residue from defatted solution.

Purification with a highly polar solvent

To the defatted powder (105.6 g), 1 liter of methanol was added, treated in an ultrasonic cleaner for 10 minutes at room temperature, and vacuum filtered with a filter paper No.2 (Whatman). The residue was treated again by the same procedures as described above and then filtered. Both methanol solutions were mixed together, and then concentrated and dried at 80°C using a rotary evaporator. The resultant cake was further dried in a drier at 80°C for 16 hours and then crushed, to thereby obtain 22.2 g of light yellow-green, purified *Gymnema inodorum* leaf extract.

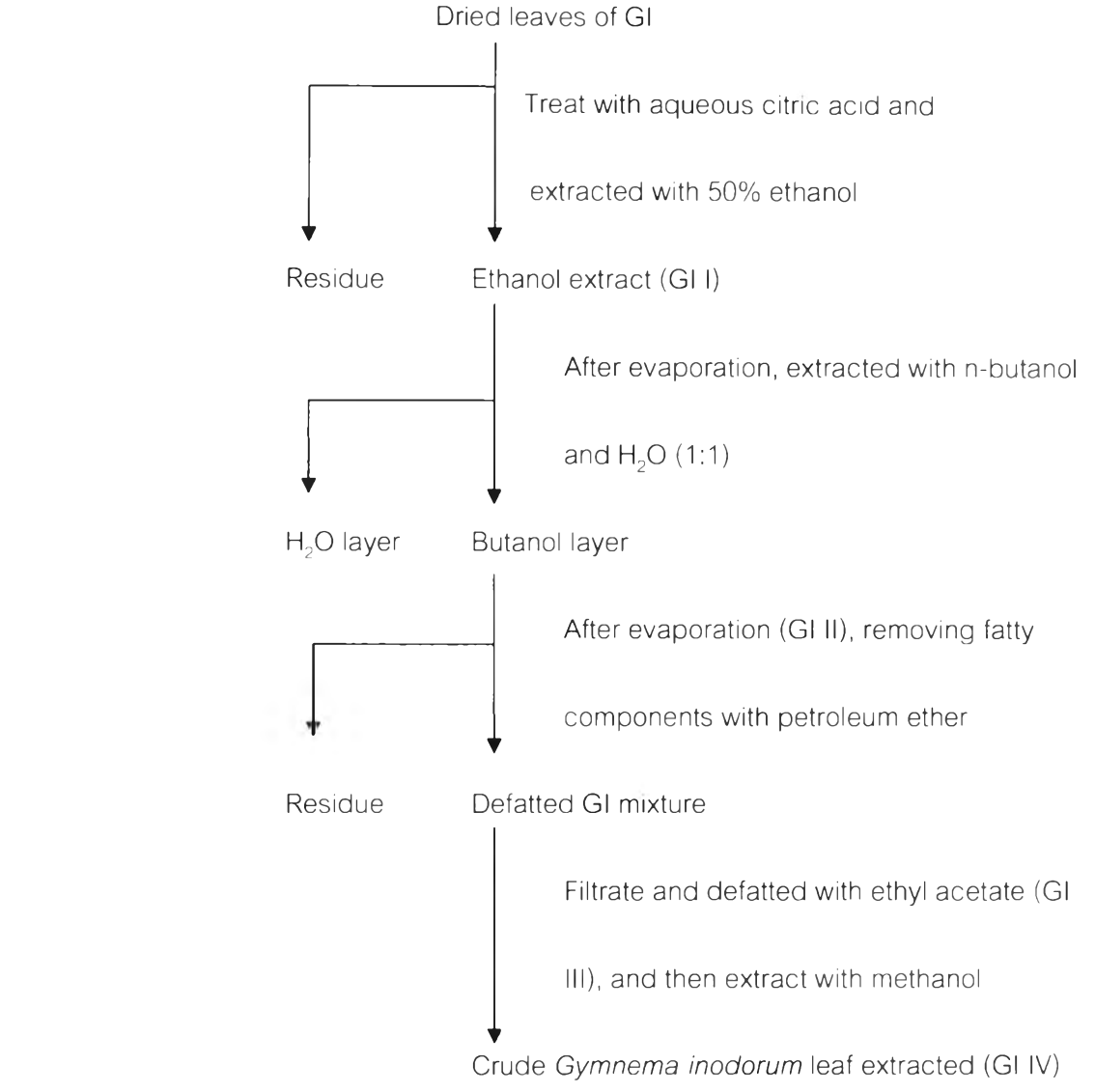


Figure 4 Isolation diagram of triterpenoid extracted from *G. inodorum* leaves

E. Culture of INS-1 cells

The INS-1 rat insulinoma cell line was cultured under 5% CO_2/ 95% air at 37°C in the RPMI-1640 (Gibco BRL, Green Island, NY) containing 11.2 mmol/L glucose and 2 mmol/L L-glutamine. The medium was supplemented with 10% fetal bovine serum (FBS), 1 mmol/L pyruvate, 10 mmol/L HEPES, 50 μmol/L 2-mercaptoethanol, 100 units/mL penicillin, and 100 μg/mL streptomycin. All experiments were performed using INS-1 cells between the 20^{th} and 30^{th} passage. The cells were passaged 2-4 days before each experiment and plated in 24-well multiwell plates at a density of $5x10^{5}$ cells/well with glucose (100 mg/dL). Five milligram of each GI extract; GI I-IV fractions were dissolved with 1 ml DMSO before incubated with INS-1 cells for 1 hour before insulin secretion measurement. DMSO was used as negative control.

F. Measurement of insulin secretion

The insulina IRMA kit, purchased from RADIM S.p.A., Italy, is an immunoradiometric assay based on coated-tube separation. Mabs1, the capture antibodies, are attached to the lower and inner surface of the plastic tube. Calibrators or samples added to the tubes will at first show low affinity for Mabs1. Addition of Mab2, the signal antibody labeled with ^{125}I, will complete the system and trigger the immunological reaction. After washing, the remaining radioactivity bound to the tube reflects the antigen concentration.

Procedure

1. Label coated tubes in duplicate for each calibrator, sample, control. For the detection of total counts, label 2 normal tubes.
2. Briefly vortex calibrators, samples and controls and dispense 50 μl of each into respective tubes.
3. Dispense 50 μl of conjugate radioactive into each tube.

4. Shake the rack containing the tubes gently by hand to liberate any tapped air bubbles.
5. Incubate for 2 hours at room temperature.
6. Aspire (or decant) the content of each tube (except total counts). Be sure that the plastic tip of the aspirator reaches the bottom of the coated tube in order to remove all the liquid.
7. Wash tubes with 2 mL of Washing solution (except total counts.) Avoid foaming during the addition of the Working Wash solution.
8. Aspired (or decant) the contain of each tube (except total counts.)
9. Wash tubes again with 2 mL Washing solution (except total counts) and aspirate (or decant)
10. After the last washing, let the tube stand upright for two minutes and aspirate the remaining drop of liquid
11. Count tubes in a gamma counter for 60 seconds

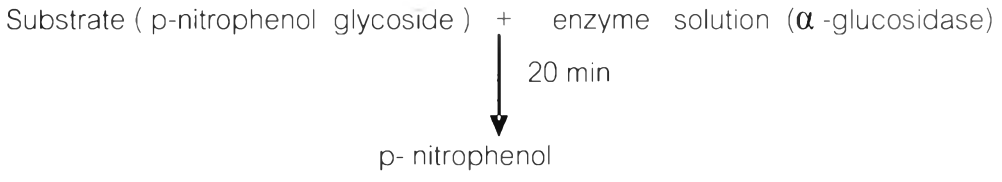
Calculation of results

1. Calculate the mean of duplicate determinations.
2. On semilogarithmic or linear graph paper plot the c.p.m. (originate) for each calibrator against the corresponding concentration of insulin and draw a calibration curve through the calibrator points, reject the obvious outliers.
3. Read the concentration for each control and sample by interpolation on the calibration curve.
4. Computer assisted data reduction will simplify these calculations.

G. Measurement of alpha-glucosidase inhibitory activity

Dried GI leaves were extracted with boiling deionized water and 70% methanol and lyophilized before use for determination for inhibitory activity on digestive

enzyme; α-glucosidase (Sigma), Acarbose was used as positive control. The α-glucosidase inhibitory assay was done by the chromogenic method (Sigma).



In brief, 10 µl of GI extract powders were dissolved in 1 M PBS (5 mg/mL and subsequent dilutions) and were incubated for 5 min with 5 µL of yeast α-glucosidase enzyme prepared in 100 mM phosphate buffer (pH 7.00). After 5 minutes of incubation, 50 mL of 5 mM substrate (p-nitrophenyl-α-D-glucopyranoside prepared in the same buffer) were added. After 20 minute, 8 mL of Na_2CO_3 were added. The reaction in tubes were recorded at 405 nm spectrophotometrically. The increases in absorbance from pre-substrate addition to post substrate reaction were obtained. Percent inhibition was calculated by (1-Absorbance test/Absorbance control)×100 and inhibitory concentration 50% (IC50) was calculated by applying suitable regression analysis.

H. Data Analysis

Results are expressed as mean± SD throughout the study. The effects of GI extract on hypoglycemia to OGTT and insulin were analyzed by one-way and two-way analyses of variance (ANOVA), and paired Student's t-test. All other clinical and parameters were analyzed by one-way ANOVA, followed by paired Student's *t*-test, as appropriate. Significance was accepted when $p< 0.05$

I. RAGE polymorphism study

Study population

For the study of -374 T/A polymorphism, the subjects were 161 donors from National Blood Service Center, Thai Red Cross and 203 type 2 diabetic patients

from the DM Clinic at King Chulalongkorn Memorial Hospital. Blood samples were obtained with written informed consent.

For the study of -429 T/C polymorphism, the subjects were 190 donors from National Blood Service Center, Thai Red Cross and 160 type 2 diabetic patients from DM Clinic at King Chulalongkorn Memorial Hospital. Blood samples were obtained with written informed consent.

PCR-RFLP

1. Genomic DNA was extracted from 100 μl of whole blood by FlexiGene DNA Kit (Qiagen)
2. The -374 T/A polymorphism was detected by PCR and RFLP using *Tsp 5091*
3. The – 429 T/C polymorphism was detected by PCR and RFLP using *Alu1*
4. Primers 5'-GGGGGCAGTTCTCTCCTC-3' and 5'-TCAGAGCCCCCGATCCTATTT-3' were used as forward and reverse primers accordingly for -374 T/A polymorphism.
5. Primer 5'-GGGGGCAGTTCTCTCCTC-3' and 5'-TCAGAGCCCCCGATCCTATTT-3' were used as forward and reverse primers accordingly for -429 T/C polymorphism.
6. For -374 T/A polymorphism, on 3% agarose gel electrophoresis, DNA fragments of 29, 70, 110, and 130 bp were detected for the wild-type allele, TT ; and 29, 70, and 240 bp for the mutated allele, AA.
7. For -429 T/C polymorphism, on 3% agarose gel electrophoresis, DNA fragments of 344 bp were detected for the wild-type allele, TT ; and 183 and 161 bp for the mutated allele, CC. Fisher's exact test was used to test the statistical significance of difference in allele frequency between groups.