ฤทธิ์และกลไกการออกฤทธิ์ของสารสกัดแห้ม ต่อระดับน้ำตาลในเลือด
ของหนูปกติและหนูเบาหวาน

นางสาววัลยา จิตตประสาทศีล

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

EFFECTS AND MECHANISM OF ACTION OF *COSCINIUM FENESTRATUM* EXTRACT ON BLOOD GLUCOSE LEVEL IN NORMAL AND DIABETIC RATS

Miss Wanlaya Jittaprasatsin

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วัลยา จิตตประสาทศีล : ฤทธิ์และกลไกการออกฤทธิ์ของสารสกัดแห้ม ต่อระดับน้ำตาลในเลือด ของหนูปกติและหนูเบาหวาน. (EFFECTS AND MECHANISM OF ACTION OF *COSCINIUM FENESTRATUM* EXTRACT ON BLOOD GLUCOSE LEVEL IN NORMAL AND DIABETIC RATS) อ.ที่ปรึกษา: รศ.สพ.ญ.ดร. ศิรินทร หยิบโชคอนันต์, 110 หน้า. ISBN 974-53-1804-3

แห้ม *(Coscinium fenestratum)* เป็นสมุนไพรที่มีสรรพคุณในการรักษาโรคเบาหวาน โดยมีผู้นิยมใช้กันอย่างแพร่หลาย ในขณะที่รายงานการศึกษาฤทธิ์ทางเภสัชวิทยาของแห้มยังมีไม่มากนัก จึงเป็นที่มาของการศึกษาวิจัยในครั้งนี้ โดยวัตถุประสงค์ของการวิจัยคือ การศึกษาฤทธิ์ของสารสกัดเอธานอลจากแห้ม ต่อการลดระดับน้ำตาลในเลือดของหนูปกติและหนูเบาหวาน และศึกษากลไกการออกฤทธิ์ต่อการกระตุ้นการหลั่งอินสุลินโดยตรงจากตับอ่อน และฤทธิ์ยับยั้งการทำงานของเอนไซม์แอลฟ่ากลูโคซิเดส โดยเฉพาะเอนไซม์มอลเตส และ ซูเครส

จากการศึกษาฤทธิ์ของสารสกัดแห้มต่อการลดระดับน้ำตาลในเลือดของหนูปกติและหนูเบาหวานด้วยวิธี oral glucose tolerance test (OGTT) โดยการป้อนน้ำตาล 3 ชนิด ได้แก่ กลูโคส, มอลโตส และซูโครส พบว่า สารสกัดแห้มขนาด 250 - 1000 มิลลิกรัม/กิโลกรัม สามารถยับยั้งระดับน้ำตาลที่เพิ่มขึ้นในหนูปกติกลุ่มที่ได้รับน้ำตาลทั้ง 3 ชนิด และการยับยั้งนี้จะผันแปรตามขนาดของสารสกัดที่เพิ่มขึ้น ส่วนในหนูเบาหวานพบว่า สารสกัดแห้มขนาด 500 มิลลิกรัม/กิโลกรัม สามารถลดระดับน้ำตาลได้อย่างมีนัยสำคัญทางสถิติ ในหนูกลุ่มที่ได้รับการป้อนน้ำตาล กลูโคส และ มอลโตส และไม่มีผลลดระดับน้ำตาลในหนูกลุ่มที่ได้รับการป้อนน้ำตาล ซูโครส

จากนั้นคณะผู้วิจัยได้ทำการทดสอบฤทธิ์ของสารสกัดแห้มต่อการกระตุ้นการหลั่งอินสุลินโดยตรงจากตับอ่อน และฤทธิ์ยับยั้งการทำงานของเอนไซม์แอลฟ่ากลูโคซิเดส จากลำไส้เล็กของหนูขาว โดยเฉพาะเอนไซม์มอลเตส และ ซูเครส พบว่าสารสกัดแห้มขนาด 10 ไมโครกรัม/มิลลิลิตร สามารถกระตุ้นการหลั่งอินสุลินได้ในลักษณะของ biphasic คือมีการเพิ่มขึ้นของระดับอินสุลินอย่างรวดเร็วจากนั้นระดับอินสุลินจะลดต่ำลงและคงที่อยู่ในระดับหนึ่ง ที่สูงกว่าระยะพักเล็กน้อย ในขณะที่ berberine ณ ความเข้มข้นเดียวกัน สามารถกระตุ้นการหลั่งอินสุลินได้อย่างช้าๆ และเพียงเล็กน้อย โดยรูปแบบการเพิ่มขึ้นเป็นแบบ monophasic ซึ่งผลจากการทดลองบ่งชี้ว่า ฤทธิ์ลดระดับน้ำตาลในกระแสเลือดของสารสกัดแห้ม น่าจะมีผลมาจากการกระตุ้นการหลั่งอินสุลินโดยตรงจากตับอ่อน นอกจากนี้สารสกัดแห้มยังสามารถยับยั้งการทำงานของเอนไซม์ มอลเตส ได้ดีกว่า ซูเครส ซึ่งผลที่ได้จะคล้ายคลึงกับ ยาอคาร์โบส ที่ใช้เป็นกลุ่มควบคุม

จากผลการทดลองดังกล่าวข้างต้นสรุปได้ว่า สารสกัดเอธานอลจากแห้ม มีฤทธิ์ลดระดับน้ำตาลในเลือดได้ทั้งในหนูปกติและหนูเบาหวาน โดยมีกลไกการออกฤทธิ์ผ่านทางการกระตุ้นการหลั่งอินสุลินจากตับอ่อน และการยับยั้งการทำงานของเอนไซม์ มอลเตส และ ซูเครส ดังนั้นจึงมีความเป็นไปได้ที่จะนำสารสกัดแห้มมาพัฒนาเป็นยารักษาโรคเบาหวานต่อไปในอนาคต

สาขาวิชา เภสัชวิทยา (สหสาขาวิชา) ลายมือชื่อนิสิต...

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WANLAYA JITTAPRASATSIN: EFFECTS AND MECHANISM OF ACTION OF *COSCINIUM FENESTRATUM* EXTRACT ON BLOOD GLUCOSE LEVEL IN NORMAL AND DIABETIC RATS. THESIS ADVISOR: ASSOC. PROF. SIRINTORN YIBCHOK-ANAN, D.V.M., Ph.D. 110 pp. ISBN 974-53-1804-3

Coscinium fenestratum (Gaertn.) Colebr. or "Hamm" has been widely used as a traditional medicine. The request to use this plant in diabetes is increasing. However, the scientific information in biological activities of *C. fenestratum* is limited, therefore, the study on pharmacological effects of this plant has been necessary. The objective of this study was to investigate 1) the effects of *C. fenestratum* extract (CFE) on blood glucose level in normal and diabetic rats 2) the stimulatory effects on insulin secretion from perfused rat pancreas and 3) the inhibitory effects of rat intestinal α-glucosidase, maltase and sucrase.

The anti-hyperglycemic effects of CFE on plasma glucose levels were studied in both normal and streptozotocin-induced diabetic rats by performing OGTT with several kinds of sugar, glucose, maltose and sucrose. In normal rats, the CFE at concentrations of 250-1000 mg/kg inhibited increases of plasma glucose levels in all three kinds of sugar-loaded rats in a dose-dependent manner. In diabetic rats, CFE (500 mg/kg) significantly decreased plasma glucose levels in glucose and maltose loaded rats, while it did not demonstrated the statistical hypoglycemic effect in sucrose-loaded rats.

We further investigated the stimulatory effect of CFE on insulin secretion from perfused rat pancrease and its inhibitory activity on rat intestinal α-glucosidase, maltase and sucrase. The result showed that CFE (10 μg/ml) significantly increased insulin secretion in a biphasic pattern, a peak follow by sustained phase and berberine at the same concentration slightly and gradually stimulated insulin secretion in a monophasic pattern, indicating that the *in vivo* hypoglycemic effect of CFE was due to stimulation of insulin secretion from pancreatic β-cells. In addition, CFE inhibited *in vitro* activities of maltase better than sucrase, which was similar to acarbose.

Taken together, it is concluded that the crude ethanol extract of *C. fenestratum* had anti-hyperglycemic action in both normal and STZ-induced diabetic rats. The mechanisms underlying hypoglycemic activity of CFE were at least partly due to stimulation of insulin secretion and inhibition of intestinal α-glucosidase, maltase and sucrase. Thus, this compound is worth to be developed as a new drug for treating diabetes in the future.

Field of study Pharmacology Student's signature……………………...

Academic year 2004 Advisor's signature……………………...

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List of Abbreviations

ADA	American Diabetes Association
AECF	Ethanol extract of *C. fenestratum*
BMI	Body mass index
CFE	*C. fenestratum* extract
EDTA	Ethylenediamine tetraacetic acid
FBG	Fasting blood glucose
FPG	Fasting plasma glucose
GDM	Gestational diabetes mellitus
GIP	Gastric inhibitory polypeptide
GLUT	Glucose transporter
GTTs	Glucose tolerance tests
Hb	Hemoglobin
HDL	High density lipoprotien
HNF	hepatocyte nuclear factor
IC_{50}	50% inhibit concentration
IDDM	Insulin dependent diabetes mellitus
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IVGTT	Intravenous glucose tolerance test
KRB	Krebs-ringer bicarbonate buffer
LD_{50}	Lethal dose 50%
LDL	Low density lipoprotien
µg	Microgram
ml	Millilitre
MODY	maturity-onset diabetes of the young
NDDG.	National Diabetes Data Group
NGDM	Non-gestational diabetes mellitus

List of Abbreviations (continued)

Abbreviation	Meaning
NIDDM	Non-insulin dependent diabetes mellitus
NMR	Nuclear magnetic resonance
NO	Nitric oxide
OGTT	Oral glucose tolerance test
PAI-1	Plasminogen activator inhibitor-1
PG	Postload glucose
RBCs	Red blood cells
SGLT	Na^+-dependent glucose transporters
STZ	Streptozotocin
TLC	Thin layer chromatography
WHO	World Health Organization

CHAPTER I

INTRODUCTION

Nowadays, Diabetes mellitus is a serious health problem of the people in the world. The prevalence of diabetes worldwide increases from 171 million people in 2000 to 366 million people in 2030. In addition, the total number of people in diabetes from WHO South-East Asia region division, especially in Thailand, is projected to rise from 1.536 million in 2000 to 2.739 million in 2030 [1].

Diabetes mellitus is a group of metabolic disease characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both [2]. The hyperglycemia is known to be associated with an increased incidence of microvascular complication to several organs such as retina, kidney and peripheral nerves in patients with type 2 diabetes [3-5].

There are two major types of diabetes mellitus. Type 1 diabetes (also known as insulin dependent diabetes mellitus or IDDM), the cause is an absolute deficiency of insulin secretion resulting from an autoimmune destruction of β-islet cells of the pancreas. The type 2 diabetes (also known as non-insulin dependent diabetes mellitus or NIDDM), the causes are a combination of resistance to insulin action and impaired regulation of insulin secretion [6].

Classic treatment of type 1 diabetes consists of dietary control, exercise and administration of insulin. Treatment of type 2 diabetes except dietary control and exercise is often combined with oral hyperglycemic agents and/or insulin. Insulin lowers the concentration of blood glucose levels by inhibiting hepatic glucose production and stimulating the uptake and metabolism of glucose by muscle and adipose tissue. The oral hyperglycemic agents had many categories such as sulfonylureas, biguanides, α-glucosidase inhibitors and thiazolidinediones. Sulfonylureas stimulate insulin secretion. Biguanides increase insulin action in peripheral tissue and reduce hepatic glucose uptake via inhibition of gluconeogenesis. α-Glucosidase inhibitors inhibit intestinal α-glucosidase enzyme to delay carbohydrate absorption, reducing the postprandial increase of blood glucose level.

Thiazolidinediones enhance insulin action and promoting glucose utilization in peripheral tissue [2, 7].

In Thailand, there are several kinds of traditional medicine that have been used in diabetes for a long times. Malee Banjob and Suthida Chairaj have collected the data in the researches of hypoglycemic plants in Thailand between 1982 to 1998 [8]. They found that the hypoglycemic effects often found in the plants of family Cucurbitaceae, Menispermaceae, Moraceae, Myrtaceae as well as Papillionaceae and the most often reported plant that exerted the hypoglycemic effect is “Nikort” or *Ficus bengalensis Linn*. In addition, there are several kinds of hypoglycemic plants in Thailand such as “Tamleung” or *Coccinia indica Wight & Arn*., “Mara” or *Momordica charantia Linn*., “Hom-Yai” or *Allium cepa Linn*., “Bua-Luang” *or Nelumbo nucifera Gaertn*., “Bor-ra-phet” or *Tinospora crispa Miers ex Hook.f.& Thoms*., “Ka-prao” or *Ocimum sanctum Linn*., and “Van-hang-jor-ra-kae” or *Aloe barbadensis Mill*.

At present, people in Thailand and other countries use many kinds of plants to treat diabetes. However, there are only few scientific reports that support the efficacy, safety and toxicity of these plants. One of the medicinal plants that we are interested in our study is “Hamm” or *Coscinium fenestratum (Gaertn.) Colebr*., family Menispermaceae. Hamm is widely used as a traditional medicine in the North-Eastern part of Thailand, particularly along the border of Lao People’s Democratic Republic. It has been claimed as a Laos’s traditional medicine and used for balancing blood pressure, detoxifying toxic agents, lowering blood glucose and cholesterol levels and others [9]. The request to use this plant as an effective remedy for anti-hyperglycemic activity is increasing; therefore, it is worth to study the antidiabetic effects of Hamm and its mechanisms. These data will provide values for further development of this compound to pharmaceutical dosage form.

สถาบันวิทยบริการ
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The objectives of this study were to investigate

1. The effects of ethanolic *Coscinium fenestratum* extract on blood glucose level in normal and diabetic rats.
2. The stimulatory effects of this extract on insulin secretion from perfused rat pancreas.
3. The inhibitory effects of this extract on rat intestinal α-glucosidase, maltase and sucrase.

CHAPTER II

GENERAL BACKGROUND

1. BLOOD GLUCOSE REGULATIONS

In the fed state, carbohydrates, proteins and lipids were digested. Glucose, amino acids and fatty acids were absorbed into blood stream. These substances stimulate the pancreatic β-cells to release insulin and inhibit the pancreatic α-cells from secreting glucagon. The liver cells, fat cells and muscle cells are the major target cells of insulin. Insulin lowers blood glucose concentration by stimulating liver and muscle cells to store glucose in glycogen and make proteins from amino acids, stimulating fat cells to form fats from fatty acids and glycerol, and inhibiting the liver and kidney cells from making glucose from intermediate compounds of metabolic pathways (gluconeogenesis).

In the fast state, the lowering of blood glucose level stimulates glucagon secretion from the pancreatic α-cells and inhibits insulin secretion from the β-cells. Glucagon acts on the same cells as insulin, but has the opposite effects. Glucagon increases blood glucose concentration by stimulating the liver and muscles to break down stored glycogen (glycogenolysis) and release the glucose and stimulates gluconeogenesis in the liver and kidneys [10].

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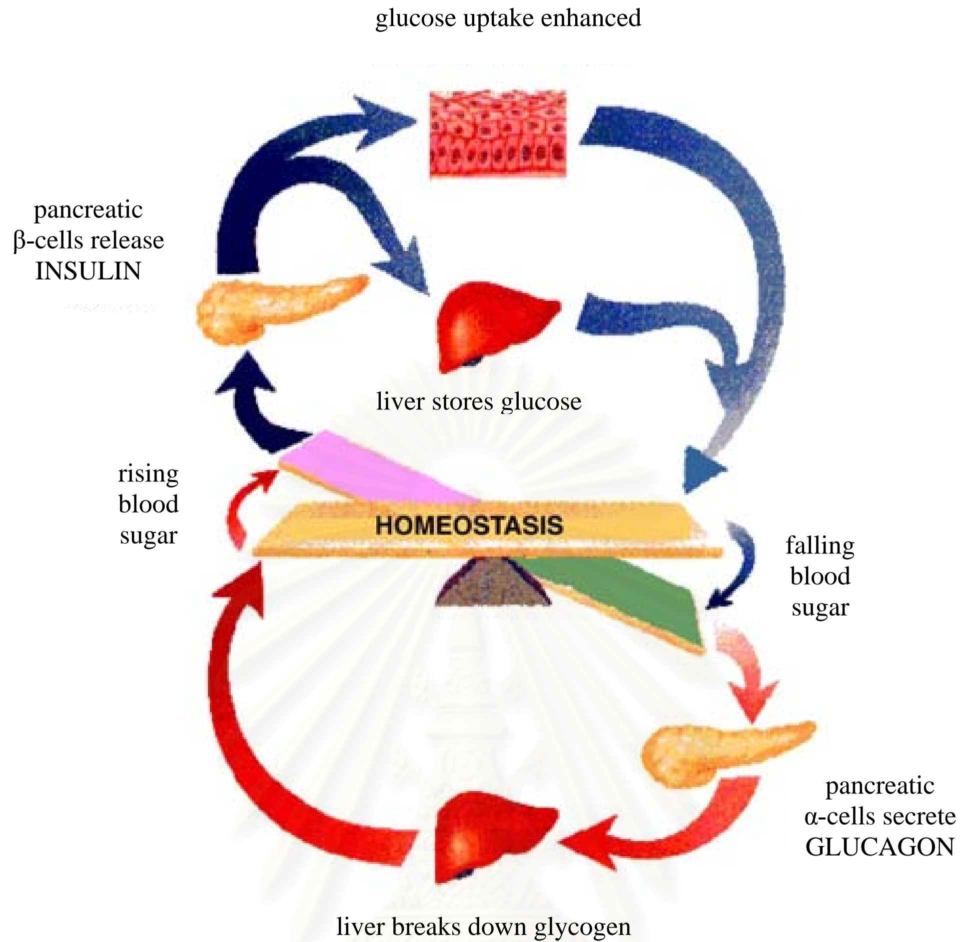

Figure 1 Regulation of blood glucose level [11]

Entry of glucose into most cells is modulated by a family of proteins called facilitative glucose transporters (GLUT 1 – GLUT 5). The tissue-specific expression of the various GLUT is an important feature in the metabolism and homeostasis of glucose.

Table 1 Properties of the GLUT1 – GLUT5 isoforms of glucose transport proteins [12]

Transporter	Tissue distribution	comments
GLUT 1	Human erythrocyte Blood-brain barrier Blood-retinal barrier Blood-placental barrier Blood-testis barrier	Expressed in cell types with barrier functions; a high-affinity glucose transport system
GLUT 2	Liver Kidney Pancreatic β–cell Serosal surface of intestinal mucosa cells	A high capacity, low affinity transport. May be used as the glucose sensor in the pancreas.
GLUT 3	Brain (neurons)	Major transporter in the central nervous system. A high affinity system.
GLUT 4	Adipose tissue Skeletal muscle Heart muscle	Insulin-sensitive transporter. In the presence of insulin the number of GLUT 4 transporters increases on the cell surface. A high-affinity system
GLUT 5	Intestinal epithelium Spermatozoa	This is actually a fructose transporter.

Genetic techniques have identified additional GLUT transporters (GLUT 7-12), but the role of these transporter has not yet been fully described.

2. DIABETES MELLITUS

Definition and description of diabetes mellitus

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction and failure of various organs, especially eyes, kidneys, nerves, heart, and blood vessels. Several pathogenic processes are involved in the development of diabetes. These include processes which destroy the beta cells of the pancreas with consequent insulin deficiency, and others that result in resistance to insulin action. The abnormalities of carbohydrate, fat and protein metabolism are due to deficient action of insulin on target tissues resulting from insensitivity or lack of insulin. Diabetes mellitus may

present with many symptoms such as thirst, polyuria, polydipsia, weight loss, sometimes with polyphagia, and blurred vision. Chronic hyperglycemia contributes to impairment of growth and susceptibility to infection. Ketoacidosis or a non–ketotic hyperosmolar state is a severe symptom of diabetes. The ineffective treatment lead to stupor, coma and death. The complications of diabetes mellitus are retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy with risk of foot ulcers, amputation, charcot joints, and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms and sexual dysfunction. The risks of atherosclerotic cardiovascular, peripheral vascular and cerebrovascular disease increase in people with diabetes [13-14].

Classification of Diabetes Mellitus and other categories of glucose regulation

An American Diabetes Association (ADA) and World Health Organization (WHO) expert group produced new diagnostic criteria and a new classification system for diabetes mellitus in 1997.

Table 2 Etiologic classification of diabetes mellitus [13]

I. Type 1 diabetes* (β-cell destruction, usually leading to absolute insulin deficiency)
- A. Immune mediated
- B. Idiopathic

II. Type 2 diabetes* (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance)

III. Other specific types
- A. Genetic defects of β-cell function
    1. Chromosome 12, HNF-1α (MODY3)
    2. Chromosome 7, glucokinase (MODY2)
    3. Chromosome 20, HNF-4α (MODY1)
    4. Mitochondrial DNA
    5. Others
- B. Genetic defects in insulin action
    1. Type A insulin resistance
    2. Leprechaunism
    3. Rabson-Mendenhall syndrome
    4. Lipoatrophic diabetes
    5. Others
- C. Diseases of the exocrine pancreas
    1. Pancreatitis
    2. Trauma/pancreatectomy
    3. Neoplasia
    4. Cystic fibrosis

5. Hemochromatosis
6. Fibrocalculous pancreatopathy
7. Others

D. Endocrinopathies
1. Acromegaly
2. Cushing's syndrome
3. Glucagonoma
4. Pheochromocytoma
5. Hyperthyroidism
6. Somatostatinoma
7. Aldosteronoma
8. Others

E. Drug- or chemical-induced
1. Vacor
2. Pentamidine
3. Nicotinic acid
4. Glucocorticoids
5. Thyroid hormone
6. Diazoxide
7. β-adrenergic agonists
8. Thiazides
9. Dilantin
10. α-Interferon
11. Others

F. Infections
1. Congenital rubella
2. Cytomegalovirus
3. Others

G. Uncommon forms of immune-mediated diabetes
1. "Stiff-man" syndrome
2. Anti-insulin receptor antibodies
3. Others

H. Other genetic syndromes sometimes associated with diabetes
1. Down's syndrome
2. Klinefelter's syndrome
3. Turner's syndrome
4. Wolfram's syndrome
5. Friedreich's ataxia
6. Huntington's chorea
7. Laurence-Moon-Biedl syndrome
8. Myotonic dystrophy
9. Porphyria
10. Prader-Willi syndrome
11. Others

IV. Gestational diabetes mellitus (GDM)

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*Patients with any form of diabetes may require insulin treatment at some stage of their disease. Such use of insulin does not, of itself, classify the patient.

I. Type 1 diabetes (ß-cell destruction, usually leading to absolute insulin deficiency)

A. Immune-mediated diabetes.

Insulin-dependent diabetes (IDDM), type 1 diabetes, or juvenile-onset diabetes, results from a cellular-mediated autoimmune destruction of the ß-cells of the pancreas [15].

The rate of ß-cell destruction is variable, being rapid in infants and children and slow in adults [16]. In children and adolescents patients, ketoacidosis may be the first symptom of the disease. The hyperglycemia can rapidly change to severe hyperglycemia and/or ketoacidosis when the patients have an infection or stress. Adult patients may retain residual ß-cell function sufficient to prevent ketoacidosis. However, they eventually become dependent on insulin for survival and ketoacidosis will be developed for many years. At the last stage of the disease, there is little or no insulin secretion, as exhibited by low or undetectable levels of plasma C-peptide. Immune-mediated diabetes commonly occurs in childhood and adolescence, but it can occur at any age.

Autoimmune destruction of ß-cells has multiple genetic predispositions and is related to environmental factors that are still poorly defined. The patients in this type of diabetes are rarely obese. They are also prone to other autoimmune disorders such as Graves' disease, Hashimoto's thyroiditis, Addison's disease, vitiligo, and pernicious anemia.

B. Idiopathic diabetes.

Some of these patients have permanent insulinopenia and are prone to ketoacidosis. This form of diabetes is strongly inherited, lacks of immunological evidence for ß-cell autoimmunity, and is not HLA associated. The patients are absolutely required for insulin replacement therapy [17].

II. Type 2 diabetes (ranging from predominantly insulin resistance with relative to insulin deficiency to predominantly an insulin secretory defect with insulin resistance)

Non-insulin-dependent diabetes (NIDDM), type 2 diabetes, or adult-onset diabetes, is a term for patients who have insulin resistance and usually have insulin deficiency [18-21]. There are many causes of this form of diabetes.

Most patients with type 2 diabetes are obese. Obesity itself causes some degree of insulin resistance [22-23]. Patients who are not obese may have an increased percentage of body fat distributed predominantly in the abdominal region [24]. Ketoacidosis usually happens in association with the stress of another illness such as infection [25-26]. The first stage of type 2 diabetes is usually undiagnosed because the hyperglycemia develops but is often not severe enough for the patient to notice any symptoms of diabetes [27-29]. However, the patients are at risk for development of macrovascular and microvascular complications [29-33]. The patients may have higher blood glucose levels and expected to result in higher insulin levels that shows the ß-cell function has been normal [34]. Thus, insulin secretion is defective in these patients and insufficient to compensate for the insulin resistance. Insulin resistance may be improved by weight reduction and/or pharmacological treatment of hyperglycemia, but seldom recovers to normal [35-39]. The risk of developing this form of diabetes increases with age, obesity, and lack of physical exercise [28, 40]. It is more frequently found in women with prior GDM and in individuals with hypertension or dyslipidemia, and its frequency varies in different racial/ethnic subgroups [28-29, 40]. It is more often associated with a strong genetic predisposition, when compared to the autoimmune form of type 1 diabetes [41-42]. However, the genetics of this form of diabetes are complex and not clearly defined.

III. Other specific types of diabetes

A. Genetic defects of the ß-cell.

These types of diabetes are frequently characterized by onset of hyperglycemia at an early age (generally before age 25 years). They are referred to as maturity-onset diabetes of the young (MODY) and are characterized by impaired insulin secretion with minimal or no defects in insulin action [43-45]. They are inherited in an autosomal dominant pattern. To date, abnormalities at three genetic loci on different chromosomes have been identified. The first form, the most common found, is associated with mutations on chromosome 12 in a hepatic transcription factor or hepatocyte nuclear factor (HNF)-1α [46-47]. The second form is associated with

mutations in the glucokinase gene on chromosome 7p, resulting in a defective glucokinase molecule [48-49]. Glucokinase converts glucose to glucose-6-phosphate, the metabolism of which, in turn, stimulates insulin secretion by the β-cell. Thus, glucokinase serves as the "glucose senor" for the β-cell. The third form is associated with a mutation in the HNF-4 α gene on chromosome 20q [50-51]. HNF-4α is a transcription factor involved in the regulation of the expression of HNF-1α. The mutations in mitochondrial DNA have been found to be associated with diabetes mellitus and deafness [52-54].

B. Genetic defects in insulin action.

The metabolic abnormalities associated with mutations of the insulin receptor may range from hyperinsulinemia and slightly hyperglycemia to severe diabetes [55-56]. The patients with these mutations may have acanthosis nigricans. Women may be virilized and have enlarged, cystic ovaries [57-58]. In the past, this syndrome was termed type A insulin resistance [55]. Leprechaunism and the Rabson-Mendenhall syndrome are the syndromes in pediatric. They have mutations in the insulin receptor gene with subsequent alterations in insulin receptor function and extreme insulin resistance [56]. The former has characteristic facial features and is usually fatal in infant, while the latter is associated with abnormalities of teeth and nails and pineal gland hyperplasia.

Alterations in the structure and function of the insulin receptor cannot be demonstrated in patients with insulin-resistant lipoatrophic diabetes. Therefore, it is assumed that the lesion(s) must be in the postreceptor signal transduction pathways.

C. Diseases of the exocrine pancreas.

The damage to the pancreas such as pancreatitis, trauma, infection, pancreatectomy, and pancreatic carcinoma are causes of diabetes [59-61]. However, adenocarcinomas that involve only a small portion of the pancreas have been associated with diabetes. This implies a mechanism other than simple reduction in ß-cell mass. If extensive enough, cystic fibrosis and hemochromatosis will also damage ß-cells and impair insulin secretion [62-63].

D. Endocrinopathies.

Several hormones such as growth hormone, cortisol, glucagon, epinephrine antagonize insulin action. Excess amounts of these hormones (e.g., acromegaly, Cushing's syndrome, glucagonoma, pheochromocytoma) can cause diabetes [64-67]. This generally occurs in patients with preexisting defects in insulin secretion, and hyperglycemia can be corrected when the hormone excess is removed.

Somatostatinoma- and aldosteronoma-induced hypokalemia causes diabetes by suppressing insulin secretion [67-68]. Hyperglycemia generally resolves after successful removal of the tumor.

E. Drug- or chemical-induced diabetes.

Many drugs can impair insulin secretion. These drugs may not cause diabetes by themselves, but they may precipitate diabetes in individuals with insulin resistance [69-70]. Certain toxins such as Vacor (a rat poison) and intravenous pentamidine can permanently destroy pancreatic ß-cells [71-74]. There are also many drugs and hormones that can impair insulin action, for examples, nicotinic acid and glucocorticoids [69-70]. Patients receiving α-interferon have been reported to develop diabetes associated with islet cell antibodies and severe insulin deficiency [75-76]. The list shown in table 2 is not all, but shows the more commonly recognized drug-, hormone-, or toxin-induced forms of diabetes.

F. Infections.

Certain viruses have been associated with ß-cell destruction. Diabetes occurs in patients with congenital rubella [77], although most of these patients have HLA and immune markers characteristic of type 1 diabetes. In addition, coxsackievirus B, cytomegalovirus, adenovirus, and mumps have been implicated in inducing certain cases of the disease [78-80].

G. Uncommon forms of immune-mediated diabetes.

In this category, there are two known conditions, and others are likely to occur. The stiff-man syndrome is an autoimmune disorder of the central nervous system characterized by stiffness of the axial muscles with painful spasms [81].

Anti-insulin receptor antibodies can cause diabetes by binding to the insulin receptor, thereby blocking the binding of insulin to its receptor in target tissues [56]. However, in some cases, these antibodies can act as an insulin agonist after binding to the receptor and can cause hypoglycemia. Anti-insulin receptor antibodies are sometimes found in patients with systemic lupus erythematosus and other autoimmune diseases [56]. As in other states of extreme insulin resistance, patients with anti-insulin receptor antibodies often have acanthosis nigricans. In the past, this syndrome was termed type B insulin resistance.

H. Other genetic syndromes sometimes associated with diabetes.

Many genetic syndromes are accompanied by an increased incidence of diabetes mellitus [82]. These include the chromosomal abnormalities of Down's syndrome, Kline-felter's syndrome, and Turner's syndrome. Wolfram's syndrome is an autosomal recessive disorder characterized by insulin-deficient diabetes and the absence of ß-cells at autopsy [83]. Additional manifestations include diabetes insipidus, hypogonadism, optic atrophy, and neural deafness. Other syndromes are listed in table 2.

IV. Gestational diabetes mellitus (GDM)

GDM is defined as any degree of glucose intolerance with onset during pregnancy. Insulin or only diet modification is used for treatment or the condition recovers after pregnancy. It does not exclude the possibility that unrecognized glucose intolerance may have antedated or begun concomitantly with the pregnancy [84]. Six weeks or more after pregnancy ends, the woman should be reclassified into one of the following categories: 1) diabetes, 2) impaired fasting glucose (IFG), 3) impaired glucose tolerance (IGT), or 4) normoglycemia. In the majority of cases of GDM, glucose regulation will return to normal after delivery.

Diagnostic criteria for diabetes mellitus

The diagnostic criteria for diabetes mellitus have been modified from those previously recommended by the National Diabetes Data Group (NDDG) [85] or WHO [86]. The revised criteria for the diagnosis of diabetes are shown in table 3. There are three ways to diagnose diabetes, and each must be confirmed, on a subsequent day, by any one of the three methods given in table 3. For example, one symptoms with casual

plasma glucose ≥200 mg/dl (11.1 mmol/l), confirmed *on a subsequent day* by 1) fasting plasma glucose (FPG) ≥126 mg/dl (7.0 mmol/l), 2) an OGTT with the 2-h postload value ≥200 mg/dl (11.1 mmol/l), or 3) symptoms with a casual plasma glucose ≥200 mg/dl (11.1 mmol/l), warrants the diagnosis of diabetes.

Table 3 Criteria for the diagnosis of diabetes mellitus [13]

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1. Symptoms of diabetes plus casual plasma glucose concentration ≥200 mg/dl (11.1 mmol/l). Casual is defined as any time of day without regard to time since last meal. The classic symptoms of diabetes include polyuria, polydipsia, and unexplained weight loss.

or

2. FPG≥126 mg/dl (7.0 mmol/l). Fasting is defined as no caloric intake for at least 8 h.

or

3. 2-h PG ≥200 mg/dl (11.1 mmol/l) during an OGTT. The test should be performed as described by WHO (2), using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.

In the absence of hyperglycemia with acute metabolic decompensation, these criteria should be confirmed by repeat testing on a different day. The third measure (OGTT) is not recommended for routine clinical use.

The Expert Committee recognizes an intermediate group of subjects whose glucose levels are too high to be considered as normal, but they are not meet criteria for diabetes. This group is defined as having FPG levels ≥110 mg/dl (6.1 mmol/l) but <126 mg/dl (7.0 mmol/l) *or* 2-h values in the OGTT of ≥140 mg/dl (7.8 mmol/l) but <200 mg/dl (11.1 mmol/l). Thus, the categories of FPG values are as follows:

- FPG <110 mg/dl (6.1 mmol/l) = normal fasting glucose;
- FPG ≥110 (6.1 mmol/l) and <126 mg/dl (7.0 mmol/l) = impaired fasting glucose;
- FPG ≥126 mg/dl (7.0 mmol/l) = provisional diagnosis of diabetes (the diagnosis must be confirmed, as described above).

The corresponding categories when the OGTT is used are the following:

- 2-h postload glucose (2-h PG) <140 mg/dl (7.8 mmol/l) = normal glucose tolerance;
- 2-h PG ≥140 (7.8 mmol/l) and <200 mg/dl (11.1 mmol/l) = impaired glucose tolerance;
- 2-h PG ≥200 mg/dl (11.1 mmol/l) = provisional diagnosis of diabetes (the diagnosis must be confirmed, as described above).

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Because the 2-h OGTT cutoff of 140 mg/dl (7.8 mmol/l) will identify more people as having impaired glucose homeostasis than will the fasting cutoff of 110 mg/d (6.1 mmol/l), it is essential that investigator always report which test was used.

Testing for diabetes

The hyperglycemia in type 2 diabetes causes microvascular disease and may provide to macrovascular disease. Patients with undiagnosed type 2 diabetes are at significantly increased risk for coronary heart disease, stroke, and peripheral vascular disease. In addition, they are probable to have dyslipidemia, hypertension, and obesity [87].

The early detection and treatment might help to reduce the complication of type 2 diabetes. However, to increase the cost-effectiveness of testing undiagnosed, testing should be considered in high-risk populations. Suggested criterias for testing are given in table 4. Although the OGTT and FPG are both suitable tests, the FPG is strongly recommended because it is easier and faster to perform, more convenient and acceptable to patients, more reproducible, and less expensive.

Table 4 Criteria for testing for diabetes in asymptomatic, undiagnosed individuals [13]

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1. Testing for diabetes should be considered in individuals at age 45 years and above, particularly in those with a BMI ≥25 kg/m^2*; if normal, it should be repeated at 3-year intervals.
2. Testing should be considered at a younger age or be carried out more frequently in individuals who are overweight (BMI ≥25 kg/m^2*) and have additional risk factors:
   - have a first-degree relative with diabetes
   - are habitually physically inactive
   - are members of a high-risk ethnic population (e.g., African-American, Hispanic American, Native American, Asian American, Pacific Islander)
   - have delivered a baby weighing ≥9 lb or have been diagnosed with GDM
   - are hypertensive (≥140/90)
   - have an HDL cholesterol level≤35 mg/dl (0.90 mmol/l) and/or a triglyceride level ≥250 mg/dl (2.82 mmol/l)
   - have polycystic ovary syndrome
   - on previous testing, had IGT or IFG
   - have a history of vascular disease

The OGTT or FPG test may be used to diagnose diabetes; however, in clinical settings the FPG test is greatly preferred because of ease of administration, convenience, acceptability to patients, and lower cost. *May not be correct for all ethnic groups.

Impaired glucose tolerance (IGT) and impaired fasting glucose (IFG)

The terms IGT and IFG refer to a metabolic stage intermediate between normal glucose homeostasis and diabetes. This stage includes individuals who have IGT and individuals with fasting glucose levels ≥110 mg/dl (6.1 mmol/l) but <126 mg/dl (7.0 mmol/l). The term IFG refers to a fasting plasma glucose (FPG) level ≥110 mg/dl (6.1 mmol/l) but <140 mg/dl (7.8 mmol/l).

Many individuals with IGT are euglycemic in their daily lives [88] and may have normal or near normal glycated hemoglobin levels [89]. Patients with IGT often show hyperglycemia only when challenged with the oral glucose load used in the standardized OGTT.

In the absence of pregnancy, IFG and IGT are the risk factors for future diabetes and cardiovascular diseases [90]. IFG and IGT are associated with the insulin resistance syndrome (also known as syndrome X or the metabolic syndrome), which consists of insulin resistance, compensatory hyperinsulinemia to maintain glucose homeostasis, obesity (especially abdominal or visceral obesity), dyslipidemia of the high-triglyceride and/or low-HDL type, and hypertension [91]. Insulin resistance is directly caused in the pathogenesis of type 2 diabetes. IFG and IGT are associate with the risk factors for cardiovascular disease. The insulin resistance syndrome includes well-recognized cardiovascular risk factors such as low HDL levels and hypertension. In addition, it includes hypertriglyceridemia, which is highly correlated with small dense LDL and increased plasminogen activator inhibitor-1 (PAI-1) levels. These factors enhanced atherogenicity, as a result of its weakness to oxidation than normal LDL. PAI-1 is a cardiovascular risk factor probably because it inhibits fibrinoloysis. Thus, the insulin resistance syndrome contains many features that increase cardiovascular risk. IFG and IGT may not be directly involved in the pathogenesis of cardiovascular disease, but rather may serve as statistical risk factors by association because they correlate with those factors of the insulin resistance syndrome that are cardiovascular risk factors.

Laboratory test for diabetes [92]

Glucose tolerance test

The diagnosis of diabetes is made by demonstrating abnormally increased blood glucose values under controlled conditions. If insulin deficiency is small, abnormality is remarkable when heavy carbohydrate loads is placed on the system. In uncompensated insulin deficiency, fasting glucose is abnormal. In compensated insulin deficiency, a variety of carbohydrate tolerance test procedures are available to unmask the defect.

Glucose tolerance tests (GTTs) are tests in which a relatively large dose of glucose challenges the body homeostatic mechanisms. If all other variable are normal, it is assumed that the subsequent rise and fall the blood glucose is mainly due to production of insulin in response to hyperglycemia. The degree of insulin response is reflected in the action of the blood glucose.

Oral glucose tolerance test (OGTT)

The OGTT is more reliable when the patient is in the hospital and does not have other severe acute or chronic illnesses. The test should be preceded by at least 3 days of adequate carbohydrate diet and should be performed in the morning after the patient has fasted at least 10 hours (but no longer than 16 hours). The test dose has been standardized by the NDDG at 75 gm of glucose or dextrose for nonpregnant persons and 100 gm for pregnant woman. The dose may be calculated from body weight. The dose can be given up to 300 ml of water and should be eaten up by the patient within 5 minutes. The test starts when the patient begins to drink. The NDDG recommends that the patient should remain seated during the test and should not smoke. One should also beware of medication that could affect test results, such as oral contraceptives, steroids, diuretic and anticonvulsants.

Oral glucose tolerance test interpretation

The NDDG criteria are listed in table 5. All values will be given in milligrams per 100 ml, using true glucose methods.

Table 5 National Diabetes Data Group criteria for diagnosis of diabetes mellitus in nonpregnant and pregnant adults* [92]

	Plasma or serum (mg/100ml)			Venous whole blood (mg/100ml)+		
OGTT	Normal	NGDM#	GDM$	Normal	NGDM#	GDM$
Fating	70-115	≥140	≥150	60-100	≥120	≥90
Peak (0.5-1.5 hr.)	<120	≥200	≥190	<180	≥180	≥170
2 hr	<140	≥200	≥165	<120	≥180	≥145
3 hr	70-115	-	≥145	60-100	-	≥125

* NGDM = nongestrational diabetes mellitus; GDM = gestrational diabetes mellitus; Nonpregnant, 75-gm glucose dose; Pregnant, 100-gm glucose dose.

The serum FBG level is ≥140/100ml. If the serum FBG level is <140/100ml, both the peak and the 2-hr value must ≥200/100ml (75-gm glucose dose).

$ Two GTT curve values must be diabetes (100-gm glucose dose).

+ Capillary whole blood cutoff values same as venous whold blood when fasting; thereafter, same as venous plasma.

The NDDG has qualified a diagnosis of diabetes mellitus to be made in any of three difference ways:

1. Classical symptoms of diabetes mellitus (e.g., polydipsia, polyuria, ketonuria, and weight loss) plus either an elevation of the FBG level or an elevation of the non-FBG level greater than 200 mg/100ml.

2. Elevation of the FBG level (venous serum or plasma) greater than 140 mg/100ml (7.8mmol/L) on more than one occasion (assuming on condition is present that falsely increase blood glucose values).

3. A normal FBG level but OGTT peak and 2-hour values both greater than 200 mg/100ml (1.1 mmol/L) on more than one occasion.

Three points should be noted. First, the diagnosis of diabetes can be made in nonpregnant adults if classic clinical symptoms are present plus a nonfasting serum specimen more than 200 gm/100ml. Second, the diagnosis can be made without requiring a GTT if the FBG level is sufficiently elevated. Third, when the diagnosis is based predominantly on blood glucose measurement, either the FBG level or the OGTT, diagnosis requires sufficient abnormality on 2 different days rather then only one occasion.

Intravenous glucose tolerance test (IVGTT)

The intravenous glucose tolerance test (IVGTT) was devised to eliminate some of the objections to the OGTT. Standard procedure for the IVGTT is as follows: The patient has a 3-day high-carbohydrate diet. After the FBG level is measured, a standard solution of 50% glucose is injected intravenously over 3- to 4- minute periods at 0.33 gm/kg ideal body weight. Blood is corrected at 0.5, 1, 2, and 3 hours. The curve reaches a peak immediately after injection (300-400 mg/100ml [16.2-22.2 mmol/L], accompanied by glucosuria), then falls steadily but not linearly toward fasting levels. Criteria for interpretation are not uniform. However, a normal response usually occurs by return to fasting levels by 1-1.25 hours. The height of the curve has no significance. Most agree that the IVGTT response is adequately reproducible. In diabetes, fasting levels are not reached in 2 hours and often not even by 3 hours. The curve in liver disease mostly returns to normal in 1.25-2 hours; however, some patients with cirrhosis have a diabetic-type curve. Many of the same factors that produce a diabetogenic effect on the OGTT are similar to IVGTT; these include carbohydrate deprivation, inactivity, old age, fever, uremia, stress, neoplasms, and the various steroid-producing endocrine disease. There are, however, several differences from the OGTT. The IVGTT is said to be normal in pregnancy and also in hyperthyroidism, although one report found occasional abnormality in thyrotoxicosis. The IVGTT is less sensitive than the OGTT, however it is a little more specific.

Plasma (or serum) insulin assay

Insulin was the first hormone measured successfully by radioisotope immunoassay. Insulin assay is now available in many reference laboratories. Insulin is excreted primarily through the kidneys. In general, juvenile diabetics have low fasting insulin levels, and an OGTT using insulin determinations usually produces a flat curve. Mild diabetes have normal fasting insulin levels and display an insulin GTT curve that has a delayed rise, either to normal height or to a point moderately above normal; in either case the curve thereafter falls in a normal fashion. Decreased tolerance due to many other causes produces similar curves; an insulin OGTT has not been more efficient in uncovering subclinical diabetes than blood glucose OGTT. The ratio of insulin values to glucose levels obtained on the same specimen during the OGTT is more reliable than insulin values alone. Thus, plasma insulin levels should not be used for diagnosis of diabetes mellitus.

Plasma anticoagulated with ethylenediamine tetraacetic acid (EDTA) is reported to produce plasma insulin values equal to serum, but heparin is associated with plasma insulin values greater than serum.

Patients being treated with insulin frequently develope antibodies after approximately 6 weeks. These antibodies interfere with insulin RIA measurement by competing with insulin antibodies used in the test. Endogenous antibodies do not interfere with tolerance test, since the quantity of endogenous antibody remains uncharged throughout the test; only the baseline value is affected.

Glycosylated hemoglobin (glycoHb) assay

In adults, hemoglobin A (Hb A) constitutes about 97%-98% of normal hemoglobin; the remainder includes about 2.5% hemoglobin A_2 and about 0.5% of hemoglobin F (Hb F). About 6%-7% of Hb A consist of Hb A molecules that have been partially modified by attachment of a glucose molecules to terminal valine amino acid of the globin beta chain. This process is called “glycosylation,” and this particular glycosylated hemoglobin is called “hemoglobin A_1” (Hb A_1). Although Hb A_1 comprises the great majority of glycosylated hemoglobin under normal conditions, glycosylation to some degree may occur at other locations in the globin chain and in

other hemoglobins beside Hb A. The some of the various glycosylation activities occurring in all hemoglobins (normal or abnormal) in the patient is known as total glycosylated hemoglobin.

Glycosylation of hemoglobin occurs during exposure of red blood cells (RBCs) to plasma glucose; hemoglobin and glucose can form a bond that initially is labile, but then becomes stable. Once stable bond occurs, it is very slowly and poorly reversible. In Hb A_1, the labile bonding fraction normally constitutes about 10% of total glucose bonding. Formation of Hb A_1 occurs very slowly during the 120-day life span of the RBC, and the Hb A_1 molecules affected by glycosylation depend on the degree and duration of RBC exposure to glucose. HbA_1 consists of three hemoglobins: A_{1A}, A_{1B}, and A_{1C}. Hb A_{1C} is about 70% glycosylated, whereas the other two are less than 20% glycosylated. In addition, A_{1C} constitutes about 60%-70% of total Hb A_1. Because Hb A_1 comprises the majority of predominant glycosylate Hb A fraction, under normal conditions Hb A_{1C} therefore represents the majority of glycosylated hemoglobin. Because of this relationship the term glycosylated hemoglobin (or glycoHb) has been used for both Hb A_1 and its major component Hb A_{1C}.

An increase in glycoHb quantity can be produced by very high short-term increases in blood glucose (in which case labile bonding is primary affected), but is most often caused either by relative continual elevation of blood glucose or by intermittent elevations that are frequent enough to produce abnormally high average glucose levels (in both of cases stable glycosylation is primary affected). A measurable increase in glycosylate (stable) hemoglobin begins about 2-3 weeks after a sustained increase in the average blood glucose level and takes at least 4 weeks to begin decreasing after a sustained decrease in the average blood glucose level. GlycoHb assay represents the average blood glucose levels during the preceding 2-3 months. In contrast, blood glucose increase or decreases of “moderate” (100 mg/100ml; 5.55 mmol/L) degree that occur within the 3 days just before Hb A_1 measurement add sufficient labile component so as to constitute as much as 15% (range, 12%-19%) of the glycol Hb result. Spontaneous sudden decreases in blood glucose of this magnitude are not common, so that under most circumstance a normal glycoHb level is good evidence of relatively normal average blood glucose during at least the preceding 4 weeks. Most of the clinical problems with labile bonding

component occur when it produces false increases in glycoHb levels. In summary, an elevated glycoHb level is most often due to long-term average blood glucose elevation over the preceding 2-3 months, but the possibility exists for elevation due to marked short-term blood glucose increase if an assay method is used that is not specific for stable bonding.

GlycoHb measurement has been used to monitor effectiveness of (long-term) diabetic therapy, to monitor patient compliance with therapy, and to differentiate between short-term stress-related glucose tolerance abnormality (e.g., myocardial infraction) and diabetes. The most widely accepted indications are monitoring of diabetic therapy effectiveness and monitoring of patient compliance. GlycoHb assay has also been used to diagnose diabetes mellitus, but this is controversial.

Serum fructosamine assay

Besides Hb A, albumin and various globulins are nonenzymatic glycosylation. In contrast to hemoglobin, which has a serum half-life of about 60 days, albumin has a half-life of about 17-20 days, and total protein (about one half albumin and one half globulin) has a half-life of about 30 days. Either glycosylated albumin or glycosylated total protein can be assayed, but most laboratories assay total protein using the fructosamine procedure. This does not involve the sugar fructose and is based on biochemical reaction with glucose bond to protein with a ketoamine linkage, most often using nitro blue tetrazolium as the reagent. Serum fructosamine assay results suggest average glycosylation within the preceding 2-week time period (range, 1-3 weeks). This time period is shorter than that of glycoHb but longer than that for labile hemoglobin glycosylation. Disadvantage of fructosamine assay include changes in serum level due to changes in albumin rather than blood glucose. The changes in albumin affect fructosamine levels significantly only if decreased albumin levels are due to increased catabolism (decreased half-life) or increased albumin loss, but not when there is decreased metabolism of protein. Reducing substances in serum may interfere with the assay in some methods.

Treatment of diabetes mellitus [2]

Type 1 diabetes mellitus

Type 1 diabetes is a chronic disease that requires major modifications in life-style. The successful management of the disease include antithperglycemic treatment and regular checks of glucose control, combining both self blood glucose monitoring (SBGM) by the patient and laboratory measurement by the physician (glycated hemoglobin or HbA1c, whose level reflects mean glycemia for the last 6-8 weeks).

Classic treatment of type 1 diabetes comprises dietary control, exercise, and administration of insulin. The diet in type 1 diabetes is a dynamic component of the insulin management regimen, and the importance of individualizing the nutrition plan base on desired medical outcomes has been emphasized. Recommendations about exercise for patients with type 1 diabetes should be made on an individual basis, taking into consideration the patient's personal attitudes and desires about exercise, his/her knowledge and skills in blood glucose management, and the presence or absence of diabetes complications that might post risk or limitations to exercise. Insulin administration has been the pivotal treatment of type 1 diabetes since its discovery in 1921. The improvement in the purity of insulin preparations, interference with the duration of action of insulin, improvement of the mode of insulin administration (disposable syringes, pens, pumps), and even more important, the ability to control insulin's efficacy (SBMG), so as to rapidly adjust insulin treatment. An intensive diabetes treatment program that resulted in a significant improvement in glycemic control (decrease in HbA1c levels from 9 to 7.2%; normal values 3-6%) for up to 8 to 9 years could significantly delay the development and slow down the progressive of the microvascular complication of disease.

Type 2 diabetes mellitus

In contrast to type 1 diabetes, in which the metabolic abnormality is generally restricted to insulin deficiency and secondary hyperglycemia, type 2 diabetes appears to be a much more complex metabolic disease. Consequently, therapeutic guideline for type 2 diabetes should focus not only on correction of hyperglycemia, but also should encourage consideration of the patient globally and treatment of all risk factors

found in each individual, most particularly weight excess and abdominal adiposity, arterial hypotension, and dyslipidemias.

The initial treatment of choice in patients with type 2 diabetes is optimization of the meal plan and enhancement of physical activity. Reduction of excessive body weight should be main target in most patients. Unfortunately, traditional dietary strategies, and even very low calorie diets, usually have not been effective in achieving long-term weight loss. An appropriate exercise program should be an adjunct to diet and/or drug therapy to improve glycemic control, reduce certain cardiovascular risk factor, and increase psychologic well-being in individuals with type 2 diabetes mellitus. If progress toward glycemic goals is not apparent within a 3-month period after initiation of diet and exercise therapy, then the use of a pharmacologic agent is appropriate. Insulin also can be used in type 2 diabetes, as initial therapy or most often after secondary failure of oral drug treatment.

First choice: oral monotherapy It is regconized that metformin should be preferred in insulin-resistant, hyperinsulinemic, obese patients, whereas sulfonylurea should be prescribed in nonobese or only modestly overweight, insulin-deficient patient. Acarbose appears to be preferable as monotherapy in diabetic patients with only modest fasting hyperglycemia, but rather high postprandial glucose concentrations.

Second choice: combined oral therapy The most common combined therapy associates a sulfonylurea compound and metformin. Both compounds have an at least additive antihyperglycemic effect, without increasing the side effects of either pharmacologic class. Other combinations of oral drugs also may be used, such as acarbose combine with sulfonylurea and acarbose plus metformin.

Third choice: insulin therapy Insulin therapy may be used as an alternative to oral drugs after diet failure, following secondary failure of maximal oral treatment, and when oral agents are contraindicated or become temporarily ineffective. Insulin can be combined with various oral antidiabetic drugs in type 2 diabetes such as insulin and sulfonylurea, metformin combined insulin, glitazones with insulin, and acarbose added to insulin.

Oral hypoglycemic agents

Sulfonylureas

Sulfonylureas correct the main defects in NIDDM by enhancing insulin release, reducing hepatic glucose output, and improving insulin sensitivity. However, enhancing insulin release is the major pharmacological effect of sulfonylureas [93]. The sulfonylureas bind to a receptor on the β cell that causes a proximal potassium channel to close, reducing the inflow of K^+ and resulting in cell depolarization [94]. Subsequently an opening of the calcium channels in β cell occurs. As calcium enters the β cell, it stimulates insulin release. Glucose also inhibits this K^+ channel and thereby stimulating insulin secretion [95]. Long-term hypoglycemia may desensitize the β cell to glucose and to oral agents (glucose toxicity [96-97]).

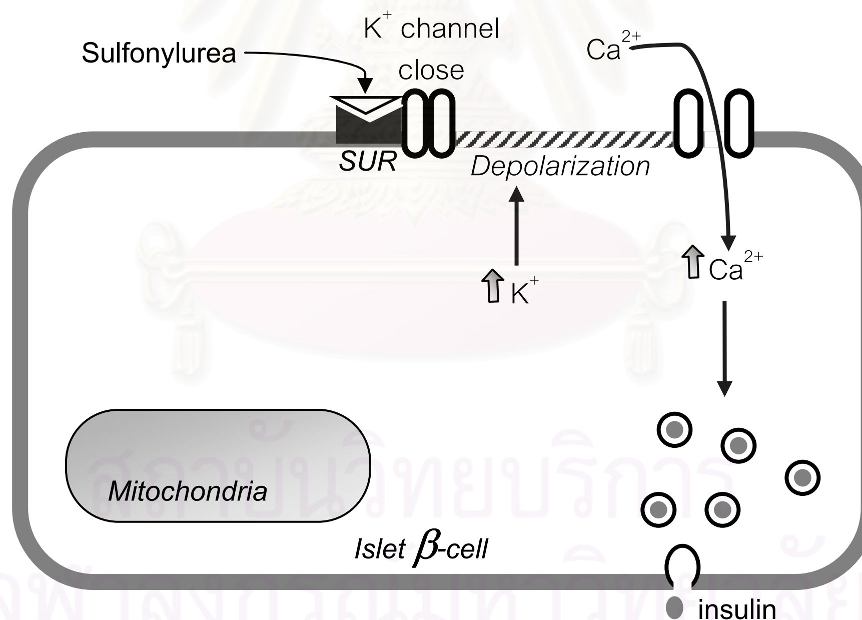

Figure 2 The mechanism underlying sulfonylureas stimulated insulin secretion [98]

Non-sulfonylureas

This relatively new class of medications is currently represented by nateglinide and repaglinide. Repaglinide is a benzoic acid derivative, and nateglinide is a phenylalanine derivative. The mechanism of action of these drugs is similar to that of the sulfonylureas (closure of the potassium–adenosine triphosphate channel, leading to calcium-dependent insulin secretion). However, they bind to the sulfonylurea receptor at a different site and with different kinetics than the sulfonylureas. Thus, the onset of action is faster and the half-life is shorter, which results in a brief stimulation of insulin release. These compounds are metabolized in the liver through the cytochrome p450 system into inactive biliary products [99].

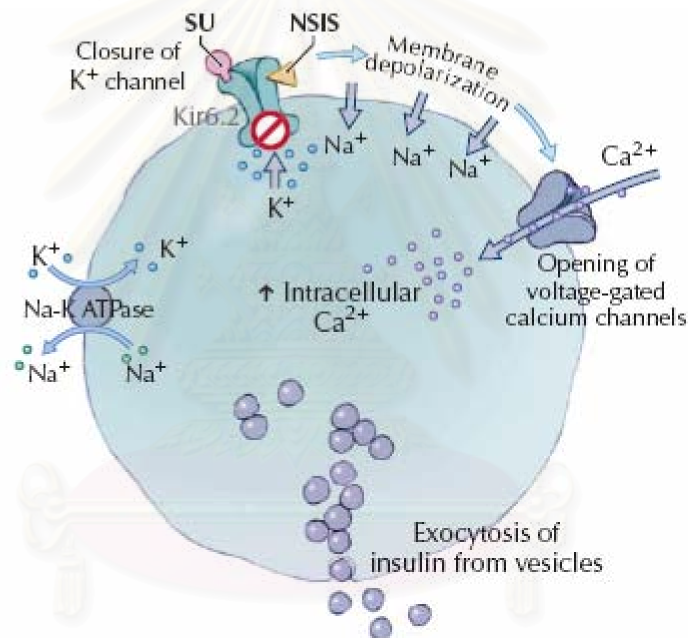

Figure 3 The site of action of sulfonylureas (SU) and non-SU insulin secretagogues (NSIS) [99]

Biguanides

The major mechanisms of action of biguanides are to improve peripheral tissue sensitivity to insulin and to suppress hepatic glucose production by reducing gluconeogenesis [100]. Metformin does not cause weight gain and may even facilitate weight loss (partly by its slight anorexic effect) [101]. The drug also reduces triglyceride level (particularly if elevated), has a small effect in reducing total and LDL cholesterol, and slightly increases HDL cholesterol [102]. In monotherapy, the

advantage of agent is for obese patients, in an attempt to avoid weight gain and increased insulin levels. Metformin should be avoided in patients with renal or hepatic insufficiency, in alcoholics, and in those with cardiopulmonary insufficiency or other known risk for lactic acidosis.

α-Glucosidase inhibitors

α-Glucosidase inhibitors such as acarbose, voglibose reduce intestinal absorption of starch, dextrin, and disaccharides by inhibiting the action of intestinal brush border α-glucosidase. Inhibition of this enzyme results in slowering the absorption of carbohydrate, then the postprandial rise in plasma glucose is blunted in both normal and diabetes subjects. Acarbose also competitively inhibits glucoamylase and sucrase but has weak effects on pancreatic α-amylase. It reduces postprandial plasma glucose levels on IDDM and NIDDM subjects. However, only small improvements in hemoglobin A1c values have been reported. The drug is poorly absorbed [7]. The major side effect of α-glucosidase inhibitors is gastrointestinal intolerance (flatulence, soft stool or diarrhea, mild abdominal pain) due to both osmotic effects and bacterial fermentation of undigested carbohydrates in the distal bowel. Acarbose may represent a good alternative in elderly people at higher risk of sulfonylureas- induced hypoglycemia and metformin-induced lactic acidosis [2].

Thiazolidinediones

Thiazolidinediones are a new class of compounds that work by enchancing insulin action and thus promoting glucose utilization in peripheral tissues, possibly by stimulating nonoxidative glucose metabolism in muscle and suppressing gluconeogenesis in the liver. This action is attributed to the stimulation of a new class of nuclear receptors (PPAR-γ) [2]. In addition, preliminary data show reduced plasma triglyceride levels and a slight increase in HDL cholesterol levels [103]. Since these drugs act by improving insulin action and do not affect pancreatic insulin secretion, they do not tend to cause hypoglycemia [104].

Insulin

Exogenous insulin may become necessary to compensate for the secretory failure of β-cell in the presence of marked insulin resistance in type 2 diabetes [2]. In order to successful with an intensive insulin regimen, the patient and the medical team must consider the following aspects of insulin therapy, such as the type of insulin, the time course of action of each type of insulin, the techniques, absorption characteristic, and devices available for insulin delivery, the potential complications of insulin therapy; and the stage of insulin therapy, from initial regimens to pattern control concepts to the principles of intensive insulin therapy.

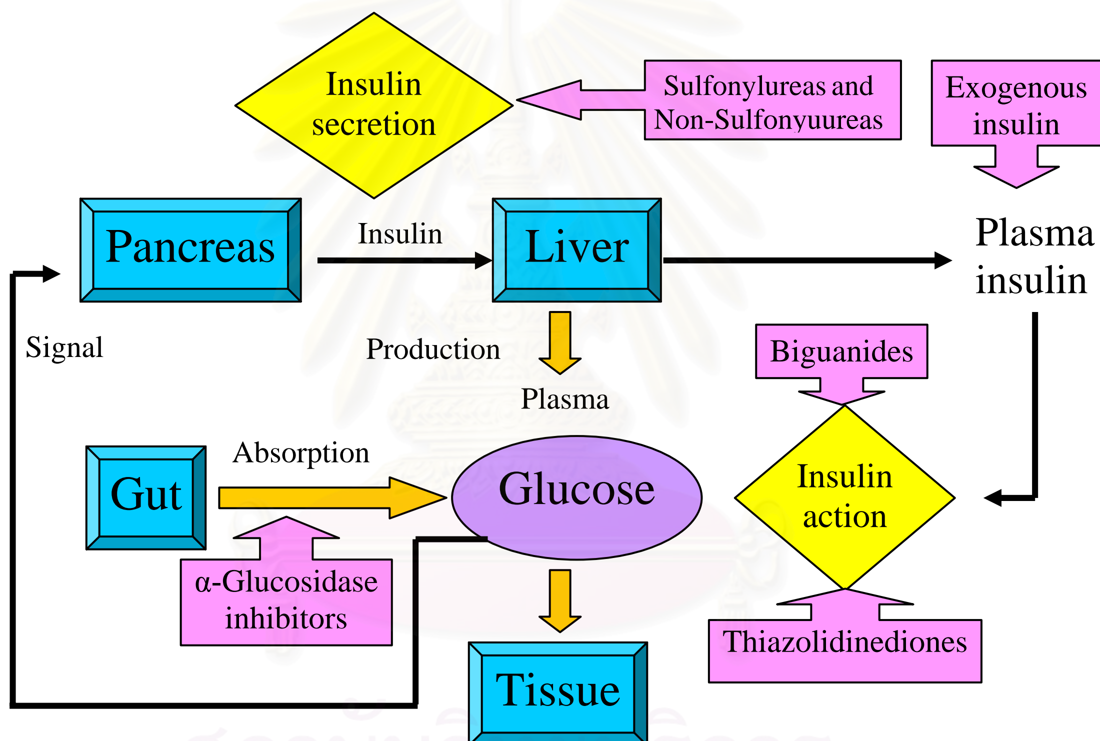

Figure 4 Current status of drug treatment of type 2 diabetes: sites of action of sulfonylureas, metformin, acarbose, thiazolidinediones and exogenous insulin [2]

3. INSULIN

Insulin is a polypeptide, consisting of two peptide chains (the A-chain and the B-chain) connected together with two disulphide bond. The prohormone precursor of insulin is a single peptide chain known as proinsulin, which is converted to insulin by proteolytic cleavage. This results in the removal of a peptide, known as C-peptide. In the prohormone, C-peptide connects the two peptide chains of insulin. Both insulin and the C-peptide are stored in granules in the β–cell of the pancreas [105].

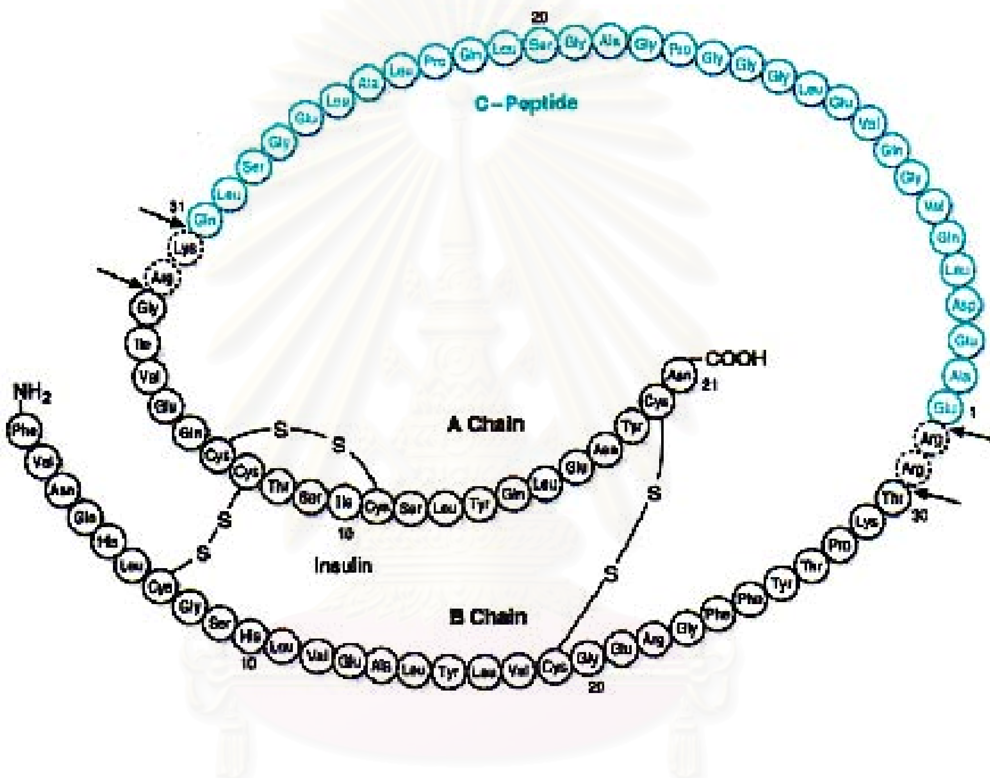

Figure 5 Structure of insulin [12]

The release of insulin into the blood is stimulated by high levels of glucose and amino acid. The hormone acts on most tissue of the body, but muscle, adipose tissue and liver are quantitatively the most important. However, some tissue, such as brain and erythrocytes, which are obligatory utilizes of glucose for fuel, are not sensitive to insulin.

Glucose enters the β cell via specific glucose transporter proteins known as GLUT 2. Glucose is phosphorylated through the action of glucokinase to from glucose-6-phosphate, which is metabolized through glycolysis, the TCA cycle and oxidative phosphorylation. These reactions result in an increase in ATP levels within

the β cell. As the β cell ATP/ADP ratio increase, the activity of a membrane-bound, ATP-dependent K^+ channel (K^+_{ATP}) is inhibited (i.e., the channel is closed). The closing of this channel leads to a membrane depolarization , which activates a voltage-gated Ca^{2+} channel that allows Ca^{2+} to enter the β cell such that intracellular Ca^{2+} levels increase significantly. The increase in intracellular Ca^{2+} stimulates the fusion of insulin containing exocytotic vesicles with the plasma membrane, resulting in insulin secretion. Thus, an increase in glucose levels within the β cell initiates insulin release [12].

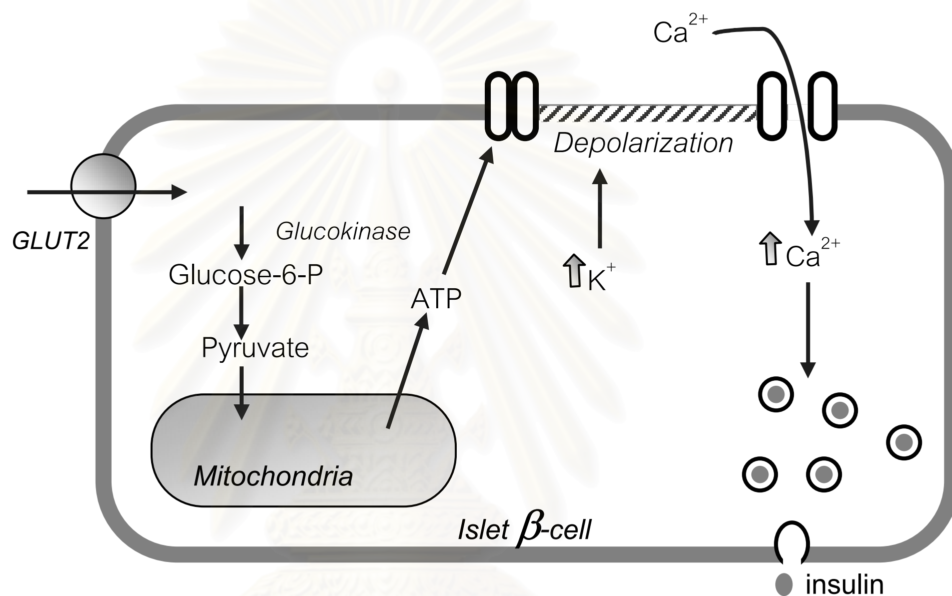

Figure 6 Mechanism of glucose stimulated insulin secretion from pancreatic β cell [98]

As insulin is secreted, the synthesis of new insulin molecules is stimulated, so that secretion is maintain until blood glucose levels fall. Insulin is rapidly removed from the circulation and degraded by the liver (and, to a less extent, by kidney and skeletal muscle), so that blood insulin levels decrease rapidly once the rate on secretion slows.

There are many factors other than the blood glucose concentration can modulate insulin release. The pancreatic islets are innervated by the autonomic nervous system, including a branch of the vagus nerve. These neural signals help to coordinate insulin release with the secretory signals initiated by the ingestion of fuels. However, signals from the central nervous system are not required for insulin secretion. Certain amino acids also can stimulate insulin secretion, although the

amount of insulin release during a high-protein meal is very much lower than that released by a high-carbohydrate meal. Gastric inhibitory polypeptide (GIP, a gut hormone released after the ingestion of food) also aids in the onset of insulin release. Epinephrine, secreted in response to fasting, stress, trauma and vigorous exercise, decreases the release of insulin. Epinephrine release signals energy utilization, which indicates that less insulin needs to be secreted, as insulin stimulates energy storage [12].

4. α-GLUCOSIDASE ENZYME

Digestion of Carbohydrate

In the digestive tract, dietary polysaccharides and disaccharides are converted to monosaccharide by glycosidases, enzymes that hydrolyze the glycosidic bonds between the sugars. All of these enzymes exhibit some specificity for the sugar, the glycosidic bonds (α or β), and the number of saccharide units in the chain. The monosaccharides formed by glycosidases are transported across the intestinal mucosal cells into the interstitial fluid and subsequently enter the bloodstream.

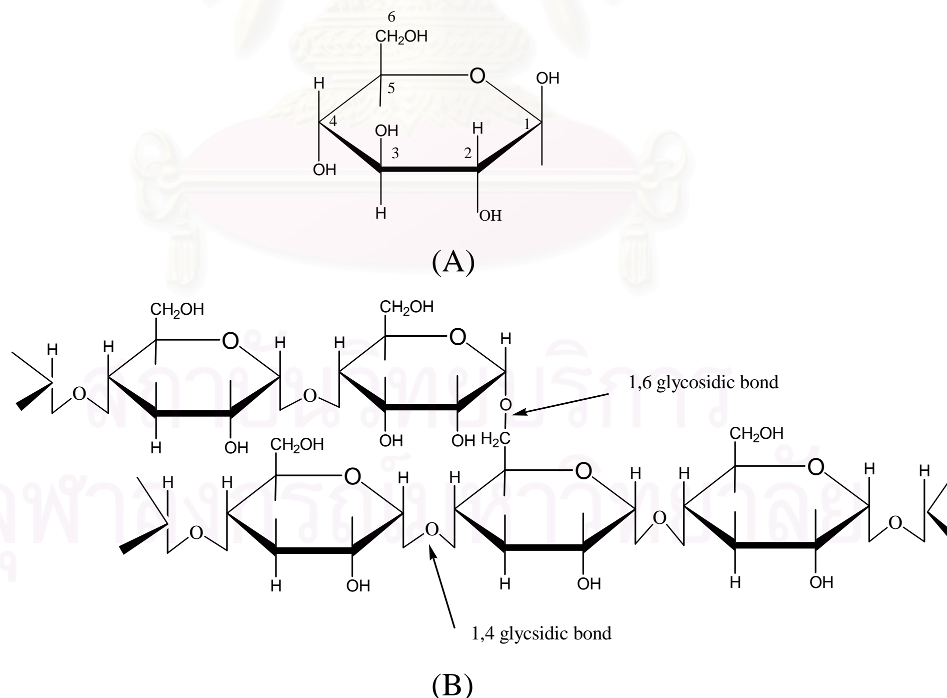

Figure 7 **(A) Structure of glucose showing the conventional numbering system for the carbon atoms**

(B) Portion of amylopectin molecule showing α-1,4 and α-1,6 glycosidic linkages [105]

Disaccharidases of the intestinal brush-border membrane

The dietary disaccharides lactose and sucrose, as well as the products of starch digestion, are converted to monosaccharides by glucosidases attached to the membrane in the brush-border of absorptive cell. Glucosidase hydrolyzes internal α-1,4 bonds between glycosyl residues in the disaccharide chains. The different glucosidase activities are found in four glycoproteins: glucoamylase, the sucrase-maltase complex, the smaller glycoprotein trehalase, and lactase-glucosylceramidase. These glucosidases are collectively called the small intestine disaccharidases, although glucoamylase is really an oligosaccharidase [12].

1. **Glucoamylase:** Split α-1,4 glycosidic bonds between glucosyl units, being sequentially with the residue at the tail end (nonreducing end) of the chain. This is an exoglucosidase. Substrates include amylase, amylopectin, glycogen and maltose.
2. **Sucrase-isomaltase complex:** It has two catalytic sites such as sucrase-maltase catalytic site and isomaltase-maltase catalytic site.

   - **Sucrase-maltase;** Splits sucrose, maltose and maltotriose

   - **Isomaltase-maltase;** Splits α-1,6 bonds in a number of limit dextrins, as well as the α-1,4 bonds in maltose and maltotriose.
3. **Trehalase:** Split bond in trehalose, which is 2 glucosyl units linked α-1,1 through their anomeric carbons.
4. **β-Glycosidase complex (lactase-glucosylceramidase):** It has two catalytic sites such as glucosyl-ceramidase catalytic site and lactase catalytic site.

   - Glucosyl-ceramidase; Split β-glycosidic bonds between glucose or galactose and hydrophobic residues, such as the glycolipid glucosylceramide and galactosylceramide.

   - **Lactase;** Split the α-1,4 bonds between glucose and galactose. To a lesser extent also splits the α-1,4 bonds between some cellulose disaccharides.

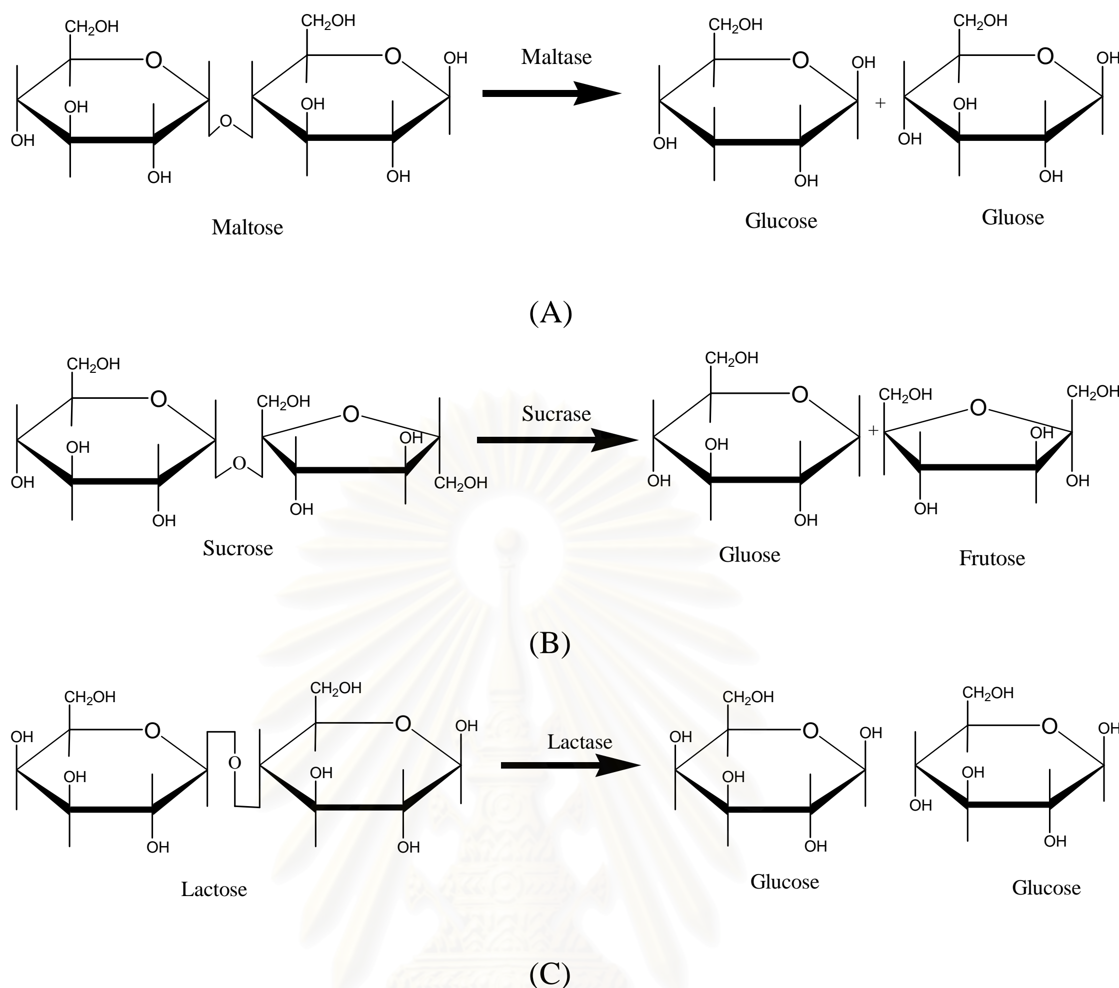

Figure 8 Degradation of disaccharides by brush border disaccharidase [105]

(A) Maltose (B) sucrose (C) lactose

Mechanism of enzyme action

The earliest model of enzyme function is the "lock and key" model. This model assumes that enzyme has rigid, three-dimension shapes with an active site on the surface (the lock) that will accept only substrates of a specific shape (the key). However, the active site in most enzymes is not rigid, and will change conformation to accommodate an incoming substrate. The "induced fit" model reflects this view of enzyme active site, indicating the specificity of enzyme action by proposing that the enzyme changes the conformation when binding to the substrate.

The induced fit model of the enzyme action can be used to explain the variety of enzyme activity that is reduced by inhibitors. In competitive inhibition, there is a competition between the substrate and an inhibitor for the active site of the enzyme.

Competitive inhibitors often have molecular structure similar to the substrate of the enzyme, and once the inhibitor combines with the enzyme the active site is block, preventing further catalytic action [106].

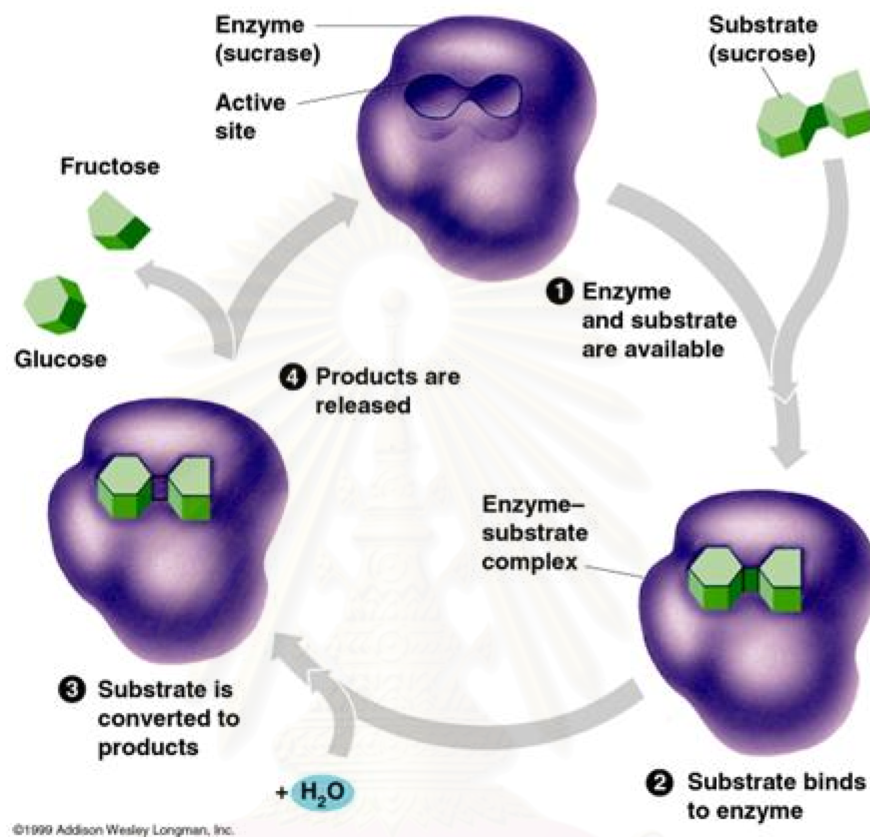

Figure 9 Lock and key enzyme model [107]

Once the carbohydrates have been split into monosaccharides, the sugars are transported across the intestinal epithelial cells and into the blood for distribution to all tissues. The pathways for transporting monosaccharides (glucose, fructose and galactose) across the intestinal epithelial cells are discussed as the followings [12];

1. Na^+-dependent transporters

Na^+-dependent glucose transporters (SGLT1), which were located on the luminal side of the absorptive cells, lead these cells to concentrate glucose from the intestinal lumen. A low intracellular Na^+ concentration is maintained by a Na^+, K^+-ATPase on the serosal (blood) side of the cell that uses the energy from ATP cleavage to pump Na^+ out of the cell into the blood. Thus, the transport of glucose from a low

concentration in the lumen to a high concentration in the cell is promoted by the cotransportion in the cell. It is promoted by the cotransport of Na^+ from a high concentration in the lumen to a low concentration in the cell (secondary active transport).

2. Facilitative glucose transporters

Facilitative glucose transporters, which do not bind Na^+, are located on the serosal side of the cells. Glucose move via the facilitative transporters from the high concentration inside the cell to the lower concentration in the blood without the expenditure of energy. In addition to the Na^+-dependent glucose transporters, facilitative transporters for glucose also exist on the lumen side of the absorptive cells.

3. Galactose and fructose absorption through glucose transporters

Galactose is absorbed through the same mechanisms as glucose. It enters the absorptive cells on the luminal side via Na^+-dependent glucose transporters and facilitative glucose transporters and is transported through the serosal side on the facilitative glucose transporters.

Fructose both enters and leaves absorptive epithelial cells by facilitative diffusion, apparently via transporter proteins that are part of the GLUT family. The transporter on the luminal side has been identified as GLUT5. Although this transporter can transport glucose, it has much higher activity with fructose.

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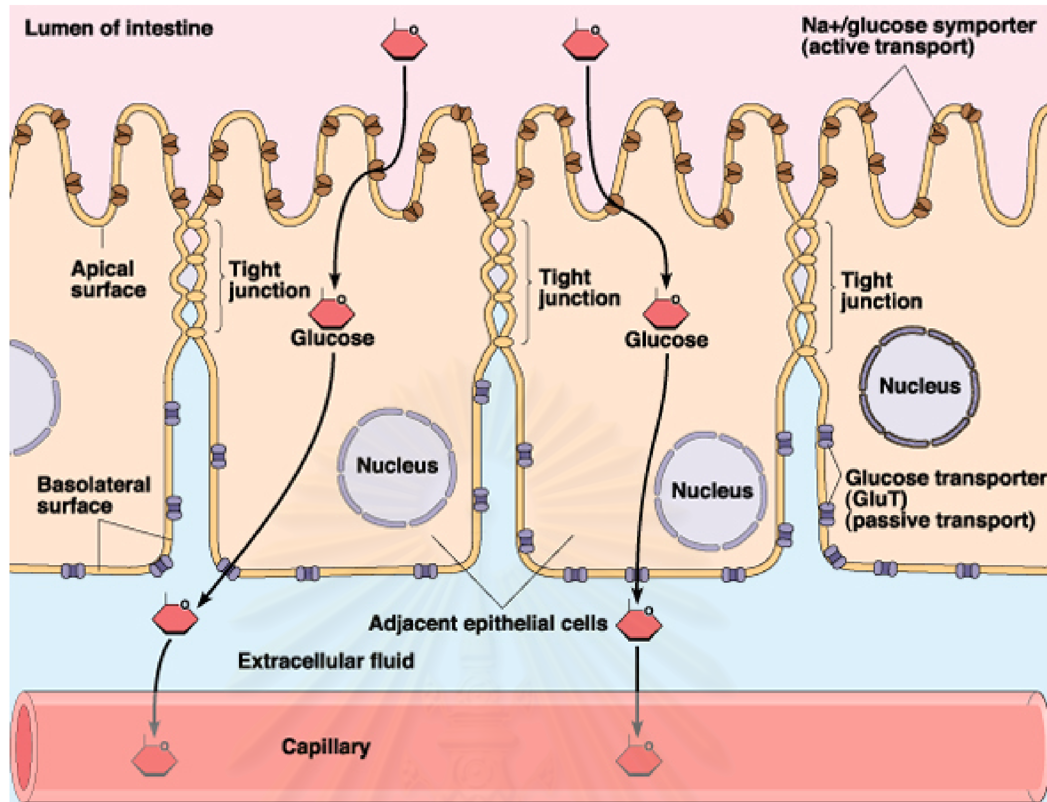


Figure 10 The transcellular transporter of glucose across the intestinal epitilium [108]

5. STREPTOZOTOCIN

Diabetes can be investigated either in animals induced diabetic by chemical compounds or diabetic strains. There may be a specific damage of insulin producing β-cells, a temporary inhibition of insulin release and/or production or a decreased efficacy of insulin in target tissue. From all compounds mainly streptozotocin and alloxan are used to induce diabetes.

The mechanism of streptozotocin action

Streptozotocin (STZ, 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) is synthesized by *Streptomycetes achromogenes* and is used to induce both IDDM and NIDDM.

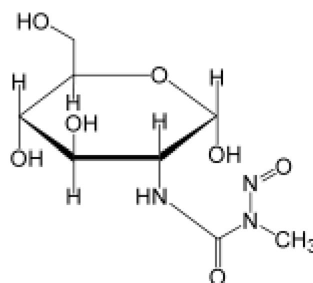


Figure 11 The chemical structure of streptozotocin (STZ) [109]

The range of the STZ dose, which is frequently used as a single intravenous injection in adult rats to induce IDDM, is between 40 and 60 mg/kg body weight [110], but higher doses are also used. STZ is also efficacious after intraperitoneal administration of a similar or higher dose, but single dose below 40 mg/kg body weight may be ineffective [111].

NIDDM can be induced in rats by intravenous or intraperitoneal treatment with 100 mg/kg body weight STZ on the day of birth. At 8-10 weeks of age and thereafter, rats neonatally treated with STZ showed mild basal hyperglycemia, an impaired response to the glucose tolerance test [112] and a loss of β cell sensitivity to glucose [113].

Streptozotocin action in β cells is associated with characteristic changes in blood insulin and glucose concentrations. Two hours after injection, the hyperglycemia is observed together with a drop in blood insulin. About six hours later, hypoglycemia occurs with high levels of blood insulin. Finally, hyperglycemia develops and blood insulin levels decrease [114]. These changes in blood glucose and insulin concentrations reflect abnormalities in β cell function. STZ impairs glucose oxidation [115] and decreases insulin biosynthesis and secretion [116-117]. It was observed that STZ at first abolished the β cell response to glucose. Then, temporary return of responsiveness appears which is followed by its permanent loss and cells are damaged [114].

STZ is taken up by pancreatic β cells *via* glucose transporter GLUT2. A reduced expression of GLUT2 has been found to prevent the diabetogenic action of STZ [118-119].

Intracellular action of STZ results in changes of DNA in pancreatic β cells leading to DNA fragmentation [120-121]. STZ damaged β cell by alkylation of DNA

[122-123]. The alkylating activity of STZ is related to its nitrosourea moiety, especially at the O^6 position of guanine.

Because STZ is a nitric oxide (NO) donor and NO was found to bring about the destruction of pancreatic islet cells, which is another pathway of STZ-induced DNA damage [124, 119]. In addition, STZ was found to generate reactive oxygen species, which make to DNA fragmentation and induce other deleterious changes in the cells [125, 115]. The action of STZ on mitochondria and increased activity of xanthine oxidase result in superoxide anion production. It was demonstrated that STZ inhibits the Krebs cycle [126] and substantially decreases oxygen consumption by mitochondria [117]. These effects strongly limit mitochondrial ATP production and cause depletion of this nucleotide in β cells [117,127]. NO is partially mediated restriction of mitochondria ATP generation. This molecule was found to bind to the iron-containing aconitase inhibiting enzyme activity [128].

Augmented ATP dephosphorylation increases the supply of substrate for xanthine oxidase (β cells possess high activity of this enzyme) and enhances the production of uric acid, which is the final product of ATP degradation [129]. Then, xanthine oxidase catalyses reaction in which the superoxide anion is formed [130], resulting in a generation of superoxide anion, hydrogen peroxide and hydroxyl radicals [129, 125]. The inhibition of xanthine oxidase by allopurinol restricts the cytotoxic effect of STZ *in vitro.* Pretreatment of β cells with this inhibitor prevented the STZ-induced decrease of insulin secretion [129].

It can be concluded that potent alkylating properties of STZ are the main reason of its toxicity. However, the synergistic action of both NO and reactive oxygen species may also contribute to DNA fragmentation and other damages caused by STZ. NO and reactive oxygen species can act separately or form the highly toxic peroxynitrate (ONOO; Figure 12). Therefore, intracellular antioxidants or NO scavengers substantially attenuate STZ toxicity.

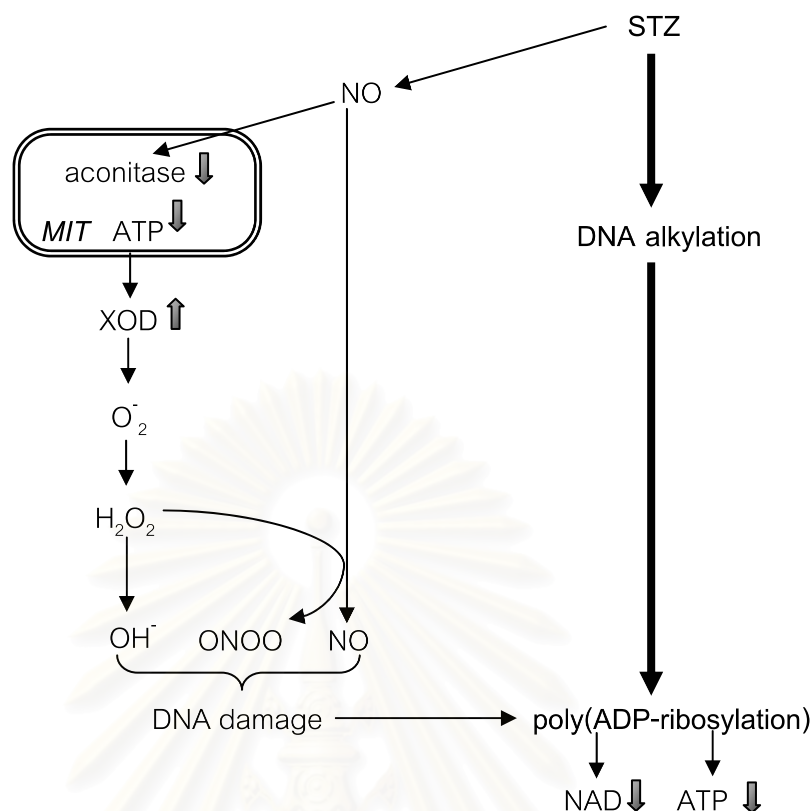


Figure 12 The mechanism of streptozotocin (STZ)-induced toxic events in β cells of rat pancreas. MIT –mitochondria; XOD – xanthine oxidase [131]

STZ-induced DNA damage activates poly ADP ribosylation [132]. This process leads to depletion of cellular NAD+, promote reduction of the ATP content [133] and subsequent inhibition of insulin synthesis and secretion [117].

In summary, streptozotocin enters the β cell *via* a glucose transporter (GLUT2) and causes alkylation of DNA. DNA damage induces activation of poly ADP-ribosylation, a process that is more important for the diabetogenicity of streptozotocin than DNA damage itself. Poly ADP-ribosylation leads to depletion of cellular NAD+ and ATP. Enhanced ATP dephosphorylation after streptozotocin treatment supplies a substrate for xanthine oxidase resulting in the formation of superoxide radicals. Consequently, hydrogen peroxide and hydroxyl radicals are also generated. Furthermore, the toxic amounts of nitric oxide that are liberated from STZ inhibit aconitase activity and participate in DNA damage. As a result of the streptozotocin action, β cells undergo the destruction by necrosis [131].

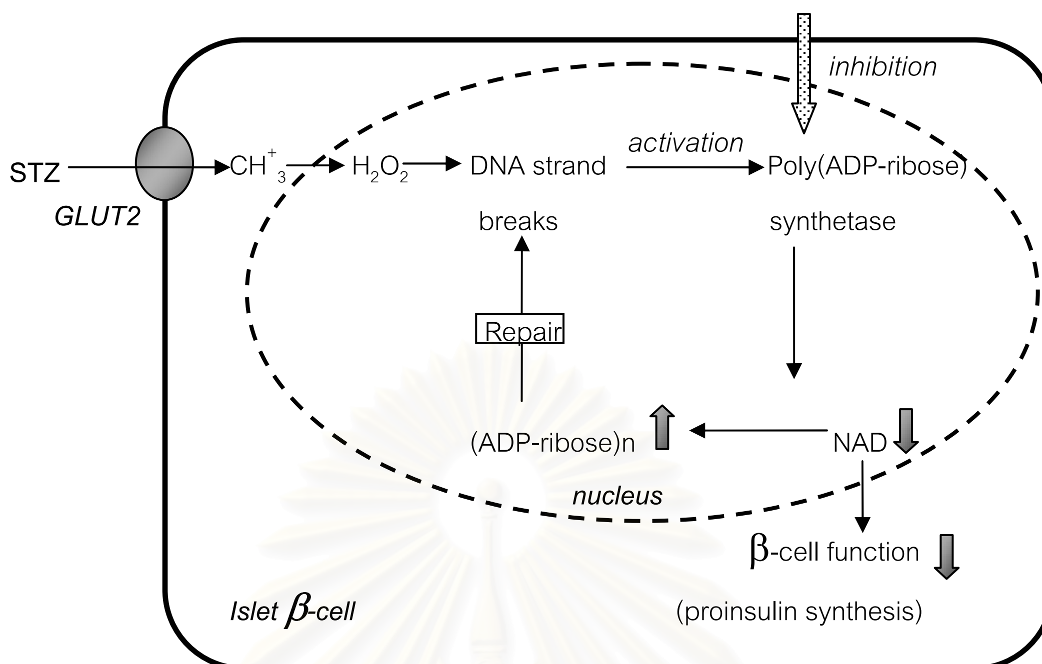
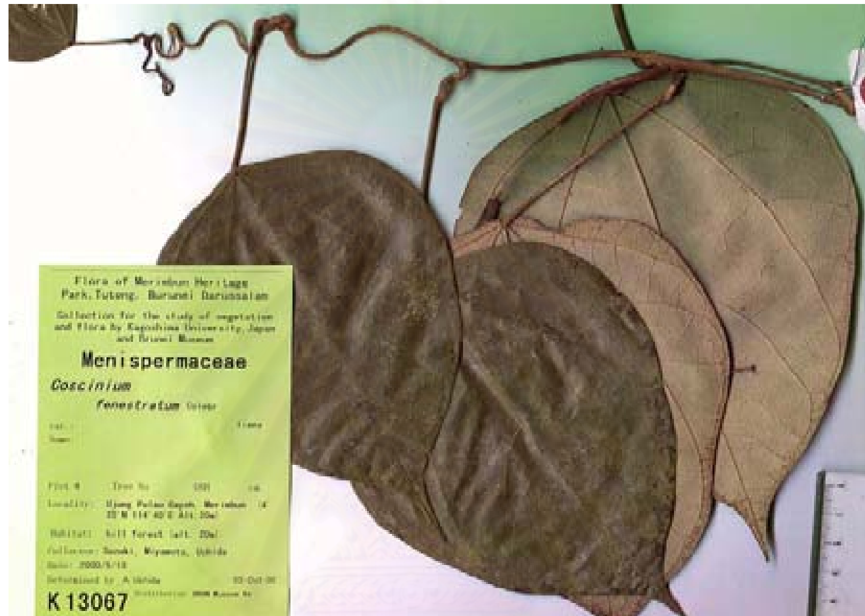


Figure 13 The mechanism action of streptozotocin (STZ) [134]

6. *COSCINIUM FENESTRATUM*

Coscinium fenestratum (Gaertn.) Colebr. or "Hamm" is in family Menispermaceae. The pharmacognostic characteristics of "Hamm" are similar to "Khamin-krua", for example, *leaves*: usually broadly ovate or ovate, rarely subpanduriform with basal, lateral lobes, 11-33 by 8-23 cm, base broadly rounded, truncate or shallowly cordate, rarely broadly obtuse, apex acuminate, upper surface glabrescent, usually drying smooth, midrib and other main nerves sunken; lower surface often whitish tomentellous, palmately 5-7 nerves at base and also usually two pairs of distal lateral nerves thinly coriaceous; petiole 3-16 cm, inserted up to 0.8 (-2.7) cm from basal margin of lumina; *Inflorescences*: flowers in several-flowered, globose heads 6-7 mm diameter, on peduncles 10-30 mm, arranged in a raceme 5-11 cm, inflorescences arising singly or a few together, bracts subulate, 4-5 mm long; *Male flowers*: sessile or with pedicels, up to 1 mm; *Sepals* broadly elliptic to obovate, the inner 3-6 spreading, yellow, 1.5-2 mm long, the outermost, smaller, 1-1.5 mm long, inserted lower; *Stamens*: 6, 1 mm long; *Female flowers*: *Sepals* as in male flowers; *Staminodes*: 6, claviform, 1 mm long; *Carples*: 3, curved-ellipsoidal, 2 mm long,

densely pilose; style filiform, recurved; *Infructescenes*: with carpophore globose, tomentellous, 7-8 mm diameter, beraring 1-3 drupes; *Drupes*: subglobose, tomentellous, brown to orange or yellowish, 2.8-3 cm diameter, pericarp drying woody, ca 1 mm thick, endocarp bony, 2.2-2.5 cm diameter, wall 3 mm thick covered with anastomosing fibrous ridges; condyle deeply intrusive, thickly clavate; *Seeds*: whitish, subglobose, enveloping the condyle [135].



(A)

(B)

Figure 14 ***Coscinium fenestratum***

(A) ***C. fenestratum*** **plants** **(B) Dried pieces stem of** ***C. fenestratum***

Present compound of *C. fenestratum* stem are comprise of isoquinoline alkaloid in form of protoberberines, for example, Berberine: 0.08%, Berberine, oxo: 0.0185%, Canadine, 8-oxo: (-): 0.023%, Corypalmine, Iso: 8-oxo: (-): 0.009%, Palmatine, oxy: 0.009%, Thaicanine, 8-oxo: (-): 0.005%, Thalfendine, Tetrahydro: 8-oxo: (-): 0.0175% and steroids such as Sitosterol,beta: 0.25% [136]. Kaewpradub A. [137] has reported that 4 kinds of alkaloid are found from the stem of *C. fenestratum*. Three of them are protoberberine alkaloids such as berberine, jatrorrhizine and tetrahydropalmatine. The other one is aporpine alkaloids, crebanine. Furthermore, some minor alkaloids from *C. fenestratum* are also found, for example, oxyberberine, tetrahydroberberine (canadine), sitosterol and tigmasterol [138].

The biological activities of *C. fenestratum* have been investigated. Palasuntheram C *et al*. [139] has been found that the water extract of *C. fenestratum* (6.25-18.75 mg/ml) showed antibacterial activities against *Clostridium botulinum* , *Clostridium novyi* , *Clostridium perfringens* , *Clostridium sporogenes* , *Stahhylococcus aureus* and *Clostridium tetani* in agar plate.

Namba T *et al*. [140] has been found that MeOH extract of *C. fenestratum* 5.0 µg/ml showed a mitogenic activity against Lymphocytes in cell culture and MeOH and MeOH-H_2O (1:1) extract showed an antibacterial activity against *Streptococcus mutans* at IC_{50} of 140 µg/ml and 240 µg/ml, respectively [141].

Singh GB *et al.* [142] has been found that a 50% ethanol extract of *C. fenestratum* stem material (AECF) has possessed hypotensive action in anaesthetised dogs, rats and guinea pigs in a dose-related pattern. However, the fall in blood pressure was not modified by alpha and beta adrenergic blockers, cholinergic and histaminergic antagonists or by ganglion blocking agents. The effect was more pronounced in spinal-transected animals. AECF non-specifically inhibited the pressor responses to epinephrine, norepinephrine, dimethylphenylpiperazenium iodide (DMPP) and depressor responses to acetylcholine and histamine. AECF failed to exhibit any hypotension when administered via cannula into the lateral cerebral ventricle. Given orally to mice, AECF did not exhibit grossly observable central nervous effects up to doses of 800 mg/kg. The oral LD_{50} was estimated to be 1200 mg/kg in mice.

Hattori M *et al*. [143] has been found that MeOH and MeOH-H_2O (1:1) extract showed an antiviral activity against virus-herpes simplex 1 at dose 100 µg/ml in agar plate.

Ueda JY *et al*. [144] has been found that MeOH and MeOH-H2O (1:1) extract of *C. fenestratum* has antiproliferative activities against human HT-1080 fibrosarcoma cells. *C. fenestratum* showed selective activity against lung carcinoma and/or lung metastatic cell lines, A549, LLC and B16-BL6.

Venukumar MR. and Latha MS. [145] has been found that an antioxidant effect of methanol extract of *C. fenestratum* stem powder. The methanol extract was treated in carbon tetrachloride-intoxicated rat liver for 90 days (daily, orally at the dose of 60 mg/kg body weight). The decreased activities of antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase in carbon tetrachloride-intoxicated rats, and its retrieval towards near-normalcy in the methanol extract co-administered animals, revealed the effectiveness of *C. fenestratum* in combating oxidative stress due to hepatic damage.

Namking M, *et al*. [146] has been found that *C. fenestratum* was a nontoxic substance and presented no significant risk of acute toxicity on structures and functions of liver, kidneys, heart, lungs, testis and ovaries.

Paopadetkarn A. [147] has been found that crude water extract of *C. fenestratum* (0.1-1 mg/kg) show the hypoglycemic effect in normal rats. In repeated dose of CE at 1 g/kg body weight/day in normal rats, for 14 days, CE show no different in blood glucose concentration. Blood chemistry and hematological value were not different between the groups of CE or distilled water. Histopathological findings revealed non-remarkable lesions of liver, exocrine pancreas and pancreatic islet in both groups. CE fed daily in diabetic rats for 14 days suppressed serum cholesterol and triglyceride. The LD_{50} of CE was greater than 20 g/kg body weight.

Tran QL *et al.* [148] has been found that the methanol extract of *C. fenestratum* had the strongest antiplasmodial activity by inhibiting the growth of the chloroquine-resistant *Plasmodium falciparum* strain FCR-3 with EC_{50} value of 0.5 µg/ml. Activity-guided fractionation led to identification of berberine as the major active constituent.

Venukumar MR. and Latha MS. [149] investigated anti-hepatotoxic activity of methanol extract of *C. fenestratum* stem (MEC) against carbon tetrachloride-induced hepatopathy in rats. Hepatotoxic rats were treated with MEC for a period of 90 days (60 mg/kg body weight, daily, orally by intubation). Anti-hepatotoxic effect was studied by assaying the activities of serum marker enzymes like aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma glutamyl

transpeptidase, lactate dehydrogenase etc. and glucose (6) phosphate dehydrogenase in liver, the concentrations of total proteins, total lipids, triglycerides, phospholipids and cholesterol in serum, liver and kidney. The activities of all the marker enzymes registered a significant elevation in carbon tetrachloride-treated rats, which were significantly recovered towards an almost normal level in animals co-administered with MEC. Other biochemical changes induced by carbon tetrachloride showed reliable signs of retrieving towards the normalcy. In addition, histopathological analysis confirmed the biochemical investigations.

Yin *et al.* [150] studied the effects of berberine on glucose metabolism *in vitro*. The action of berberine was compared with metformin and troglitazone (TZD) by using HepG2 cell line, phenotypically similar to human hepatocytes. It was used for glucose consumption (GC) studies. In this study, they found that the glucose-lowering effect of berberine decreased as the glucose consumption increased. The maximal potency was reached in the presence of 5.5 mmol/L glucose, and it’s effect was abolished when the glucose consumption increased to 22.2 mmol/L. This effect was not dependent on insulin concentration, which was similar to that of metformin and was different from that of TZD, whose glucose-lowering effect is insulin dependent. These observations suggest that berberine is able to exert a glucose-lowering effect in hepatocytes, which is insulin independent and similar to metformin, but has no effect on insulin secretion.

Furthermore, other alkaloids such as berberrubine and the ester derivatives of berberruine had a strong anti-tumor activity [151], minor alkaloids as sitosterol produced hypo cholesterolemic activity [152] and hypoglycemic activity [153].

C. fenestratum has been widely used as a traditional medicine. The request to use this plant in diabetes is increasing. However, the informations biological activities and pharmacological effects of *C. fenestratum* are limited, therefore, the study of pharmacologic effects of *C. fenestratum* has been necessary and it may possible be developed to be a new drug for treating diabetes in the future.

CHAPTER III

MATERIALS & METHODS

1. Materials

1.1 Animals

Male Sprague-Dawley Rats, weighing 100-150 g and 200-250 g, were purchased from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. All animals were maintained in the animal room of Faculty of Veterinary Science, Chulalongkorn University, Bangkok at least one week before use. Rats were fed *ad libitum* with commercial pellet diet (CP 082 mice feed) and water.

1.2 Chemicals

- Acarbose (Glucobay®, Bayer, Thailand)
- Calcium chloride (Merck, Germany)
- Citrate buffer (Sigma, U.S.A.)
- Dextran (Sigma, U.S.A.)
- Diethyl ether (Lab-scan, Thailand)
- Glibenclamide (Danonil®, Hoechst Marion Roussel, Germany)
- Glucose (Merck, Germany)
- Heparin (Leo Pharmaceutical, Denmark)
- Hepes (Sigma, U.S.A.)
- Insulin radioimmunoassay kit (DPC®, U.S.A.)
- Intestinal acetone powders from rat (Sigma, U.S.A.)
- Magnesium sulphate heptahydrate (Merck, Germany)
- Maltose (Acros Organics, U.S.A.)
- O-dianisidine (Sigma, U.S.A.)
- PGO enzyme (Sigma, U.S.A.)
- Pentobarbital sodium (Nembutal®, Sanofi, France)
- Potassium chloride (Merck, Germany)
- Potassium phosphate (Merck, Germany)
- Rat insulin

- Sodium chloride (Merck, Germany)
- Sodium hydrogen carbonate (Merck, Germany)
- Streptozotocin (Sigma, U.S.A.)
- Sucrose (Ajax Finechem, Australia)

1.3 Equipments

- Autopipets (Gilson, France)
- Blood glucose monitor (Advantage®,Roche Diagnostics Co,Thailand)
- Centrifuge
- Feeding tube
- Gamma counter (The nucleus, Model 600B)
- Hot plate (Sangi Model 14405)
- Microplate
- Microplate reader (Tecan A-5082)
- Microtube
- Peristalsis pump (Mimipulse II, Gilson, France)
- pH meter
- Rotary evaporator
- Vortex mixer
- Water bath shaker (Grant, England)
- Water pump (Gilson, France)
- Weighting machine

2. Methods

2.1 Preperation, extraction and characterization feature of the extract from *C. fenestratum*

The ethanol extraction of *C. fenestratum* was performed by Dr. Damrong Sommit, Department of Chemistry, Faculty of Science, Mahanakorn University of Technology, Bangkok. Since Singhana B. [154] has purified and reported that berberine is the major active compound of *C. fenestratum*, we confirmed the presence of berberine in this extract by using thin layer chromatography (TLC) and nuclear magnetic resonance (NMR) techniques (Appendix A).

2.2 Preperation of streptozotocin-induced diabetic rats

Male Sprague-Dawley rats weighing 100-150 g were fasted overnight. Diabetes was induced by intravenous injection of streptozotocin (STZ) in a single dose of 50 mg/kg body weight via tail vein. STZ was dissolved in cold citrate buffer solution (pH 4.5) at a concentration of 50 mg/ml, immediately before use. Fasting blood glucose (FBG) level in the animals was measured 3 days after STZ injection. The rats with FBG level higher than 250 mg/dl were included in the study.

2.3 Plasma glucose determination

Blood samples were collected from tail vein using heparinized 10 μl/tube and then centrifuged to separate plasma. The plasma was collected for determination of glucose concentration by glucose oxidase/peroxidase reaction (Sigma, U.S.A.).

2.4 Insulin determination

Effluent from perfused rat pancreas was collected for determination of insulin concentration by insulin radioimmunoassay kits (DPC®, U.S.A.).

3. Experimental protocol

3.1 The hypoglycemic effects of crude ethanol extract of *C. fenestratum* on serum glucose levels in oral several sugar-loaded normal rats

The hypoglycemic effects of *Coscinium fenestratum* extract (CFE) were investigated using oral glucose tolerance test (OGTT) as a tool. Three different kinds of sugar, including glucose, maltose and sucrose were fed to rats as substrates. The rats in each group were divided into 5 subgroups which contained 8 animals each. Then, they were treated with different doses of CFE and glibenclamide or acarbose as followings:

Glucose

- Group 1, control group; distilled water 0.5 ml/rats
- Group 2, treatment group; CFE 250 mg/kg body weight
- Group 3, treatment group; CFE 500 mg/kg body weight
- Group 4, treatment group; CFE 1000 mg/kg body weight
- Group 5, positive control group; glibenclamide 5 mg/kg body weight

Maltose

- Group 1, control group; distilled water 0.5 ml/rats
- Group 2, treatment group; CFE 250 mg/kg body weight
- Group 3, treatment group; CFE 500 mg/kg body weight
- Group 4, treatment group; CFE 1000 mg/kg body weight
- Group 5, positive control group; acarbose 3 mg/kg body weight

Sucrose

- Group 1, control group; distilled water 0.5 ml/rats
- Group 2, treatment group; CFE 250 mg/kg body weight
- Group 3, treatment group; CFE 500 mg/kg body weight
- Group 4, treatment group; CFE 1000 mg/kg body weight
- Group 5, positive control group; acarbose 3 mg/kg body weight

Glibenclamide 5 mg was dissolved with 80% tween 100 µl, and then dissolved with distilled water 900 µl. The other treatments were dissolved in distilled water. After 5 minutes of administration, the rats were fed with 3 g/kg body weight of glucose, maltose or sucrose solution. Blood samples were collected from tail vein by tail milking at 0, 30, 60 and 120 min. Blood glucose concentration was determined by glucose oxidase test [155].

3.2 The hypoglycemic effects of crude ethanol extract of *C. fenestratum* on serum glucose levels in oral several sugar-loaded streptozotocin-induced diabetic rats

The investigation of hypoglycemic effect of *Coscinium fenestratum* extract (CFE) in diabetic rats were performed in oral glucose tolerance test (OGTT) as same as in normal rats. The treatment regimens were as followings:

Glucose

- Group 1, control group; distilled water 0.5 ml/rats
- Group 2, treatment group; CFE 500 mg/kg body weight
- Group 3, positive control group; glibenclamide 5 mg/kg body weight

Maltose

- Group 1, control group; distilled water 0.5 ml/rats
- Group 2, treatment group; CFE 500 mg/kg body weight
- Group 3, positive control group; acarbose 3 mg/kg body weight

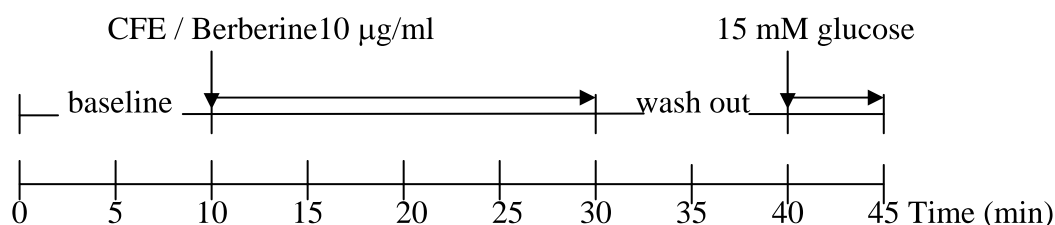
Sucrose

- Group 1, control group; distilled water 0.5 ml/rats
- Group 2, treatment group; CFE 500 mg/kg body weight
- Group 3, positive control group; acarbose 3 mg/kg body weight

Glibenclamide 5 mg was dissolved with 80% tween 100 µl, and then dissolved with distilled water 900 µl. The other treatments were dissolved in distilled water. After 5 minutes of administration, the rats were fed with 3 g/kg body weight of glucose, maltose or sucrose solution. Blood samples were collected from tail vein by tail milking at 0, 30, 60 and 120 min. Blood glucose concentration was determined by glucose oxidase test [155].

3.3 The stimulatory effects on insulin secretion from perfuse rat pancreas

The stimulatory effect on insulin secretion of C. fenestratum was performed by *in situ* pancreatic perfusion. Male Sprague-Dawley rats weighing 250-350 g were used in this experiment. Rats were fasted overnight. The operation was performed as previously described [156]. Briefly, the rats were anesthetized with pentobarbital sodium 60 mg/kg body weight via intraperitoneal and were maintained at 37ºC on a hot plate during the experiment. The celiac arteries were cannulated with a polyvinyl tubing (0.625 mm ID), then the pancreata were immediately perfused with the Krebs-Ringer bicarbonate buffer (KRB) supplemented with 10 mM HEPES, 5.5 mM glucose, 1 % dextran and 0.2 % BSA as a basal medium. The KRB was continuously aerated with 95% O_2 + 5% CO_2 at pH 7.4. The flow rate was 1 ml/min, and the effluent fluid from the portal vein, which was cannulated with a vinyl tubing (1.12 mm ID), was ~1 ml/min. The rats were euthanized immediately after the placement of cannulas and the beginning of the flow. After an equilibration period of 20 min, the effluent fluid was collected every minute. After the baseline period of 10 min, the perfusate containing 10 µg/ml of CFE or berberine was administered for 20 min, followed by a washout period with the basal medium for 10 min. The perfusate containing glucose (15 mM) was administered as a positive control for 6 min at the end of the experiments. The insulin concentration was determined by insulin radioimmunoassay kits (DPC®, U.S.A.).



3.4 The inhibitory effect of *C. fenestratum* on rat intestinal α-glucosidase (AGH)

3.4.1 Preparation of AGH solution from rat intestinal acetone powder

A crude enzyme solution was prepared from rat intestinal acetone powder (Sigma, U.S.A.) with slightly modification [157]. Rat intestinal acetone powder 30 mg was dissolved in 1 ml of cold 0.9 % NaCl solution and mixed with vortex mixer. After centrifugation at 5000g for 30 min, 4 ºC, the supernatant was directly subjected to inhibitory assay. Crude enzyme solution 1 unit contained 0.5 mg of protein.

3.4.2 Assay for AGH inhibitory activity

The AGH inhibitory activity assay was done as previously described [157] with minor modification to make a smaller assay scale. The substrate (maltose 37 mM or sucrose 37 mM) and the test compounds (*C. fenestratum* or acarbose) were dissolved in 0.1 M phosphate buffer solution (pH 7.0). Crude enzyme solution 20 μl and the test compounds 40 μl were pre-incubated simultaneously for 10 min. After pre-incubation period, the substrate 140 μl was added and incubate at 37 ºC for 30 and 60 min for maltose and sucrose, respectively. The assay tubes were immediately immersed in boiling water for 10 min to stop reaction. Glucose concentration was determined by glucose oxidase test [158]. The IC_{50} value was calculated using Sigma Plot 8.0 program.

4. Statistical analysis

All data were expressed as mean ± standard error of mean (S.E.M.). Statistical comparisons were made using one-way analysis of variance (ANOVA), with post-hoc range tests followed by the least significant difference test (LSD) at p-value less than 0.05 ($p<0.05$).

CHAPTER IV

RESULTS

1. The hypoglycemic effects of the crude ethanol extract of *Coscinium fenestratum* on serum glucose levels in oral several sugar-loaded normal rats

The effects of *C. fenestratum* extract (CFE) on plasma glucose levels were studied by performing OGTT and three different kinds of sugar (glucose, maltose and sucrose) were used as substrates. The rats in each group of sugar were divided into 5 subgroups. The control group was fed with distilled water 0.5 ml/rat. Other three groups of rat were fed orally with three different doses of CFE (250, 500 and 1000 mg/kg). For the last group of rat, glibenclamide at a concentration of 5 mg/kg or acarbose 3 mg/kg was used as a positive control in rats receiving glucose or maltose and sucrose. All treatments were administered to rats for 5 min before sugar feeding.

Glucose-loaded group

The hypoglycemic effects of CFE in glucose loaded normal rats were shown in figure 15 and table 6. The percentage decrease of plasma glucose levels, compared with the control group, was shown in table 7. Before treatment, the fasting plasma glucose levels of all groups were not significantly different. The CFE (250-1000 mg/kg) significantly decreased ($p<0.05$) plasma glucose levels in a dose-dependent manner. The CFE at a concentration of 250 mg/kg decreased plasma glucose level by 30.55 % and 20.01 % at 30 and 60 min after glucose feeding, respectively. The plasma glucose levels of CFE 250 mg/kg group, at 30, 60 and 120 min were 141.55 ± 16.52, 148.32 ± 7.99 and 120.84 ± 5.99 mg/dl, respectively and those of the control group were 203.82 ± 14.60, 185.41 ± 15.16 and 139.66 ± 9.85 mg/dl, respectively. The CFE (500 mg/kg) decreased plasma glucose level by 29.51 and 20.84 % at 30 and 60 min after glucose feeding, respectively, and their plasma glucose concentrations at 30, 60 and 120 min, were 143.67 ± 10.25, 146.44 ± 12.82 and 122.55 ± 14.89 mg/dl, respectively. The CFE (1000 mg/kg) and glibenclamide (5 mg/kg) showed significantly decrease ($p<0.05$) in plasma glucose levels when compared with control group during 30-120 min after glucose feeding. The plasma glucose levels of CFE 1000 mg/kg group, at 30, 60 and 120 min were 100.28 ± 5.58, 103.93 ± 7.46 and

93.24 ± 6.63 mg/dl, respectively and the percentage decreases were 50.80, 43.95 and 33.23 %, respectively. In addition, the plasma glucose levels of glibenclamide group were 169.15 ± 8.76, 148.54 ± 11.24 and 107.12 ± 8.38 mg/dl and the percentage decrease were 17.01, 19.89 and 23.30 %, respectively.

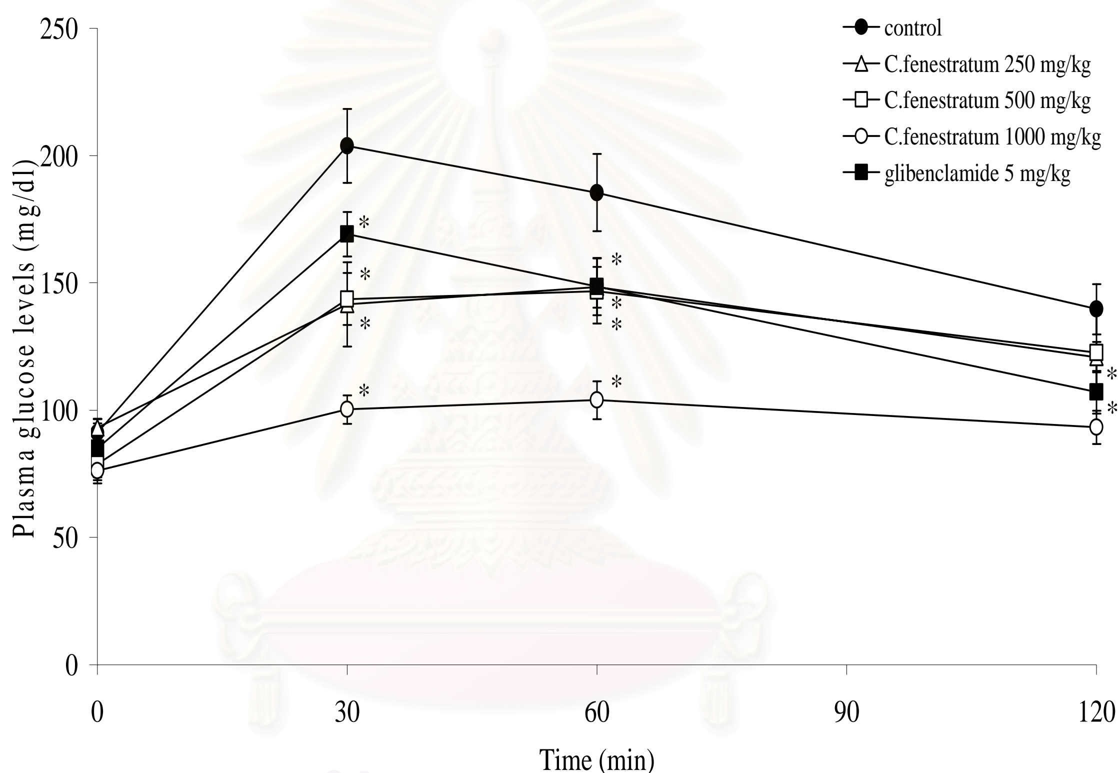


Figure 15 Effects of *C. fenestratum* on oral glucose tolerance test (OGTT) in normal rats using glucose as a substrate (n=8).

All data presents in Mean ± S.E.M.

* Significant at $p<0.05$, compared with control group at the same time

Table 6 **Effects of *C. fenestratum* on oral glucose tolerance test (OGTT) in normal rats using glucose as a substrate (n=8).**

Groups	Plasma glucose levels (mg/dl)			
	Time (min) after treatment			
	0	30	60	120
Control	91.16±3.62	203.82±14.60	185.41±15.16	139.66±9.85
C. fenestratum 250 mg/kg	93.06±3.67	141.55±16.52*	148.32±7.99*	120.84±5.99
C. fenestratum 500 mg/kg	78.72±6.11	143.67±10.25*	146.77±12.82*	122.55±14.89
C. fenestratum 1000 mg/kg	76.27±5.05	100.28±5.58*	103.93±7.46*	93.24±6.63*
Glibenclamide 5 mg/kg	85.09±11.47	169.15±8.76*	148.54±11.24*	107.12±8.38*

All data presents in Mean ± S.E.M.

* Significant at $p<0.05$, compared with control group at the same time

Table 7 **The percentages decrease of plasma glucose levels of *C. fenestratum* in glucose loaded group compared with control group in normal rats.**

Groups	% decrease of plasma glucose levels			
	Time (min) after treatment			
	0	30	60	120
C. fenestratum 250 mg/kg	-2.09	30.55*	20.01*	13.47
C. fenestratum 500 mg/kg	13.65	29.51*	20.84*	12.25
C. fenestratum 1000 mg/kg	16.33	50.80*	43.95*	33.23*
Glibenclamide 5 mg/kg	6.66	17.01*	19.89*	23.30*

* Significant at $p<0.05$, compared with control group at the same time

Maltose-loaded group

The hypoglycemic effects of CFE in maltose loaded normal rats were shown in figure 16 and table 8. The percentage decrease of plasma glucose levels, compared with control group, was shown in table 9. Before treatment, the fasting plasma glucose levels of all groups were not significantly different. The CFE (250 and 500 mg/kg) significantly decreased plasma glucose level in a dose-dependent manner, but not 1000 mg/kg of the CFE. The CFE (250 mg/kg) reduced plasma glucose concentrations by 16.28 and 36.19 % at 60 and 120 min after maltose feeding, respectively. The CFE (500 mg/kg) significantly decreased ($p<0.05$) plasma glucose levels by 34.04, 24.43 and 18.42 % at 30, 60 and 120 min after maltose feeding, respectively. The plasma glucose levels of CFE 250 mg/kg group, at 30, 60 and 120 min, were 154.22 ± 5.31, 146.39 ± 5.18 and 97.51 ± 2.97 mg/dl and those of the control group were 183.89 ± 19.74, 174.87 ± 14.25 and 152.81 ± 5.57 mg/dl, respectively. The plasma glucose levels of CFE 500 mg/kg group, at 30, 60 and 120 min, were 121.30 ± 9.49, 132.15 ± 7.34 and 124.66 ± 5.79 mg/dl. For the positive control, acarbose 3 mg/kg, significantly decreased ($p<0.05$) plasma glucose levels by 25.84 and 29.14 % at 30 and 120 min after maltose feeding, respectively and the plasma glucose levels at 30, 60 and 120 min were 136.38 ± 4.21, 156.62 ± 3.60 and 108.28 ± 5.23 mg/dl, respectively.

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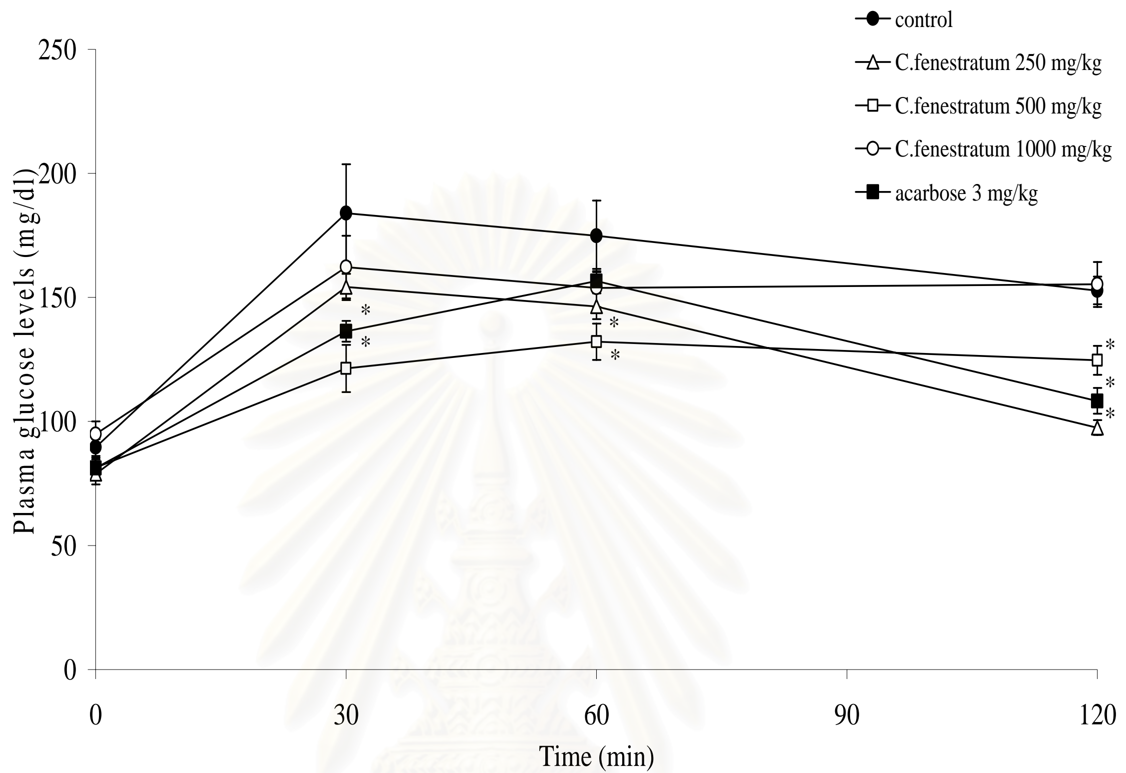

Figure 16 **Effects of *C. fenestratum* on oral glucose tolerance test (OGTT) in normal rats using maltose as a substrate (n=8).**

All data presents in Mean ± S.E.M.

* Significant at $p<0.05$, compared with control group at the same time

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Table 8 **Effects of *C. fenestratum* on oral glucose tolerance test (OGTT) in normal rats using maltose as a substrate (n=8).**

Groups	Plasma glucose levels (mg/dl)			
	Time (min) after treatment			
	0	30	60	120
Control	89.53±3.40	183.89±19.74	174.87±14.25	152.81±5.57
C. fenestratum 250 mg/kg	78.76±4.08	154.22±5.31	146.39±5.18*	97.51±2.97*
C. fenestratum 500 mg/kg	81.42±3.34	121.30±9.49*	132.15±7.34*	124.66±5.79*
C. fenestratum 1000 mg/kg	94.81±5.10	162.27±12.59	153.84±7.73	155.26±9.03
Acarbose 3 mg/kg	81.24±4.21	136.38±4.21*	156.62±3.60	108.28±5.23*

All data presents in Mean ± S.E.M.

* Significant at $p<0.05$, compared with control group at the same time

Table 9 **The percentages decrease of plasma glucose levels of *C. fenestratum* in maltose loaded group compared with control group in normal rats.**

Groups	% decrease of plasma glucose levels			
	Time (min) after treatment			
	0	30	60	120
C. fenestratum 250 mg/kg	12.02	16.13	16.28*	36.19*
C. fenestratum 500 mg/kg	9.05	34.04*	24.43*	18.42*
C. fenestratum 1000 mg/kg	-5.90	11.76	12.03	-1.60
Acarbose 3 mg/kg	9.26	25.84*	10.44	29.14*

* Significant at $p<0.05$, compared with control group at the same time

Sucrose-loaded group

The hypoglycemic effects of CFE in sucrose loaded normal rats were shown in figure 10 and table 10. The percentage decrease of plasma glucose levels, compared with control group, was shown in table 11. Before treatment, the fasting plasma glucose levels of all groups were not significantly different. The CFE (250-1000 mg/kg) significantly decreased ($p<0.05$) plasma glucose level in a dose-dependent manner. The CFE (250 mg/kg) decreased plasma glucose levels by 17.81 % at 30 min after sucrose feeding and their plasma glucose concentrations at 30, 60 and 120 min, were 126.02 ± 9.99, 143.65 ± 8.66 and 131.91 ± 8.79 mg/dl, respectively. The CFE (500 mg/kg) reduced plasma glucose levels by 32.96 and 29.92 % at 30 and 60 min after sucrose feeding, respectively. The plasma glucose levels of CFE 500 mg/kg group, at 30, 60 and 120 min, were 102.79 ± 6.15, 104.73 ± 6.21 and 111.59 ± 4.91 mg/dl, respectively. In addition, the CFE (1000 mg/kg) decreased plasma glucose levels by 39.24 and 35.99 % at 30 and 60 min after sucrose feeding, respectively. The plasma glucose levels of CFE 1000 mg/kg group, at 30, 60 and 120 min, were 93.16 ± 8.36, 95.66 ± 7.92 and 112.13 ± 9.29 mg/dl, respectively. For the positive control group, acarbose 3 mg/kg, significantly decreased ($p<0.05$) plasma glucose levels by 31.54 and 25.73 % at 30 and 60 min after sucrose feeding, respectively and their plasma glucose concentrations at 30, 60 and 120 min were 104.97 ± 6.19, 111.00 ± 8.87 and 102.20 ± 4.11 mg/dl, respectively.

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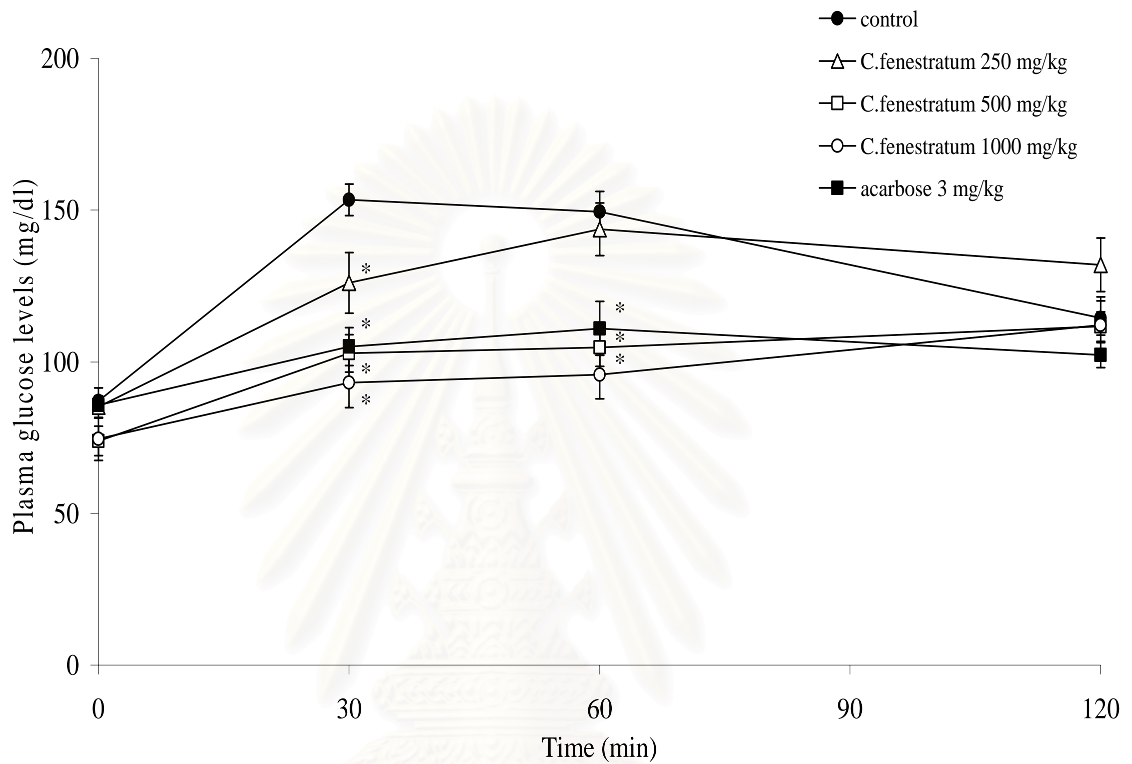

Figure 17 **Effects of *C. fenestratum* on oral glucose tolerance test (OGTT) in normal rats using sucrose as a substrate (n=8).**

All data presents in Mean ± S.E.M.

* Significant at $p<0.05$, compared with control group at the same time

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Table 10 Effects of *C.fenestratum* on oral glucose tolerance test (OGTT) in normal rats using sucrose as a substrate (n=8).

Groups	Plasma glucose levels (mg/dl)			
	Time (min) after treatment			
	0	30	60	120
Control	87.02±4.32	153.32±5.19	149.44±6.65	114.44±5.68
C. fenestratum 250 mg/kg	85.10±3.66	126.02±9.99*	143.65±8.66	131.91±8.79
C. fenestratum 500 mg/kg	73.91±4.80	102.79±6.15*	104.73±6.21*	111.59±4.91
C. fenestratum 1000 mg/kg	74.58±7.06	93.16±8.36*	95.66±7.92*	112.13±9.29
Acarbose 3 mg/kg	85.73±2.84	104.97±6.19*	111.00±8.87*	102.20±4.11

All data presents in Mean ± S.E.M.

* Significant at $p<0.05$, compared with control group at the same time

Table 11 The percentages decrease of plasma glucose levels of *C. fenestratum* in sucrose loaded group compared with control group in normal rats.

Groups	% decrease of plasma glucose levels			
	Time (min) after treatment			
	0	30	60	120
C. fenestratum 250 mg/kg	2.21	17.81*	3.88	-15.27
C. fenestratum 500 mg/kg	15.07	32.96*	29.92*	2.49
C. fenestratum 1000 mg/kg	14.30	39.24*	35.99*	2.01
Acarbose 3 mg/kg	1.49	31.54*	25.73*	10.70

* Significant at $p<0.05$, compared with control group at the same time

2. The hypoglycemic effects of crude ethanol extract of *Coscinium fenestratum* on serum glucose levels in oral several sugar-loaded streptozotocin-induced diabetic rats

The investigation of hypoglycemic effects of *C. fenestratum* extract in diabetic rats were performed by oral glucose tolerance test (OGTT). Since the CFE 500 mg/kg has shown the hypoglycemic effect in all three kinds of sugar feeding in normal rats, we decided to use this single dose in diabetic rats. The streptozotocin-induced diabetic rats with FBG level higher than 250 mg/dl were included in this study.

Glucose-loaded group

The effects of CFE in glucose loaded diabetic rat were shown in figure 18 and table 12. The percentage decrease of plasma glucose levels, compared with control group, was shown in table 13. Before treatment, the fasting plasma glucose levels of all groups were not significantly different. The CFE (500 mg/kg) and glibenclamide (5 mg/kg) significantly decreased ($p<0.05$) plasma glucose levels by 19.53 and 15.41 % at 30 min after glucose feeding, respectively. The plasma glucose levels of CFE group at 30, 60 and 120 min were 487.00 ± 26.63, 506.34 ± 22.35 and 476.74 ± 19.07 mg/dl, respectively and those of the control group were 605.23 ± 26.13, 580.88 ± 22.23 and 530.70 ± 24.16 mg/dl, respectively. For the glibenclamide positive control group, the plasma glucose concentrations at 30, 60 and 120 min were 511.95 ± 35.85, 554.50 ± 40.76 and 512.12 ± 39.09 mg/dl, respectively.

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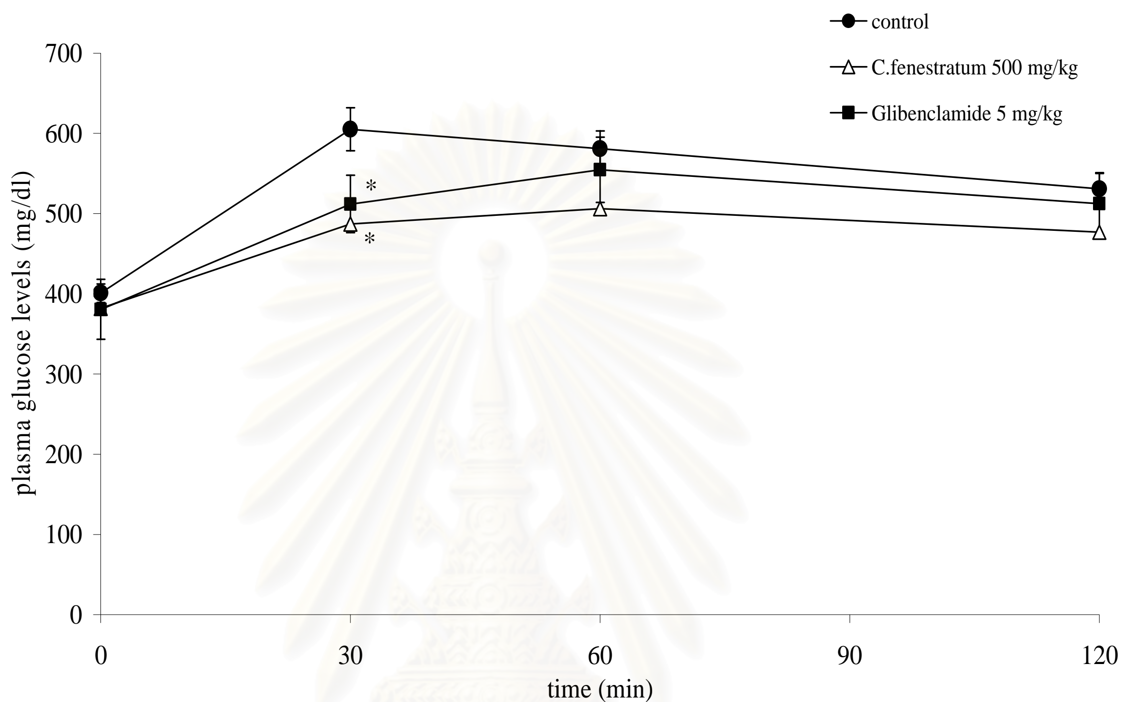

Figure 18 **Effects of *C. fenestratum* on oral glucose tolerance test (OGTT) in diabetic rats using glucose as a substrate (n=8).**

All data presents in Mean ± S.E.M.

* Significant at $p<0.05$, compared with control group at the same time

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Table 12 Effects of *C. fenestratum* on oral glucose tolerance test (OGTT) in diabetic rats using glucose as a substrate (n=8).

Groups	Plasma glucose levels (mg/dl)			
	Time (min) after treatment			
	0	30	60	120
Control	400.84±27.16	605.23±26.13	580.88±22.23	530.70±24.16
C.fenestratum 500 mg/kg	381.74±11.64	487.00±26.63*	506.34±22.35	476.74±19.07
Glibenclamide 5 mg/kg	380.62±37.19	511.95±35.85*	554.50±40.76	512.12±39.09

All data presents in Mean ± S.E.M.

* Significant at $p<0.05$, compared with control group at the same time

Table 13 The percentages decrease of plasma glucose levels of *C. fenestratum* in glucose loaded group compared with control group in diabetic rats.

Groups	% decrease of plasma glucose levels			
	Time (min) after treatment			
	0	30	60	120
C. fenestratum 500 mg/kg	4.77	19.53*	12.83	10.17
Glibenclamide 5 mg/kg	5.04	15.41*	4.54	3.50

* Significant at $p<0.05$, compared with control group at the same time

Maltose-loaded group

The hypoglycemic effects of CFE in maltose loaded diabetic rat were shown in figure 19 and table 14. The percentage decrease of plasma glucose levels, compared with control group, was shown in table 15. Before treatment, the fasting plasma glucose levels of all groups were not significantly different. The CFE (500 mg/kg) significantly decreased ($p<0.05$) plasma glucose levels by 11.88 % at 60 min after maltose feeding, however, acarbose (3 mg/kg) failed to decreased plasma glucose levels in diabetic rats. The plasma glucose concentrations of CFE group at 30, 60 and 120 min were 522.36 ± 11.73, 512.58 ± 12.22 and 496.91 ± 21.54 mg/dl, respectively and those of the control group were 531.07 ± 22.86, 581.66 ± 25.40 and 507.38 ± 32.14 mg/dl, respectively. In addition, the plasma glucose levels of acarbose group were 496.48 ± 13.36, 522.33 ± 21.50 and 473.85 ± 21.29 mg/dl, respectively.

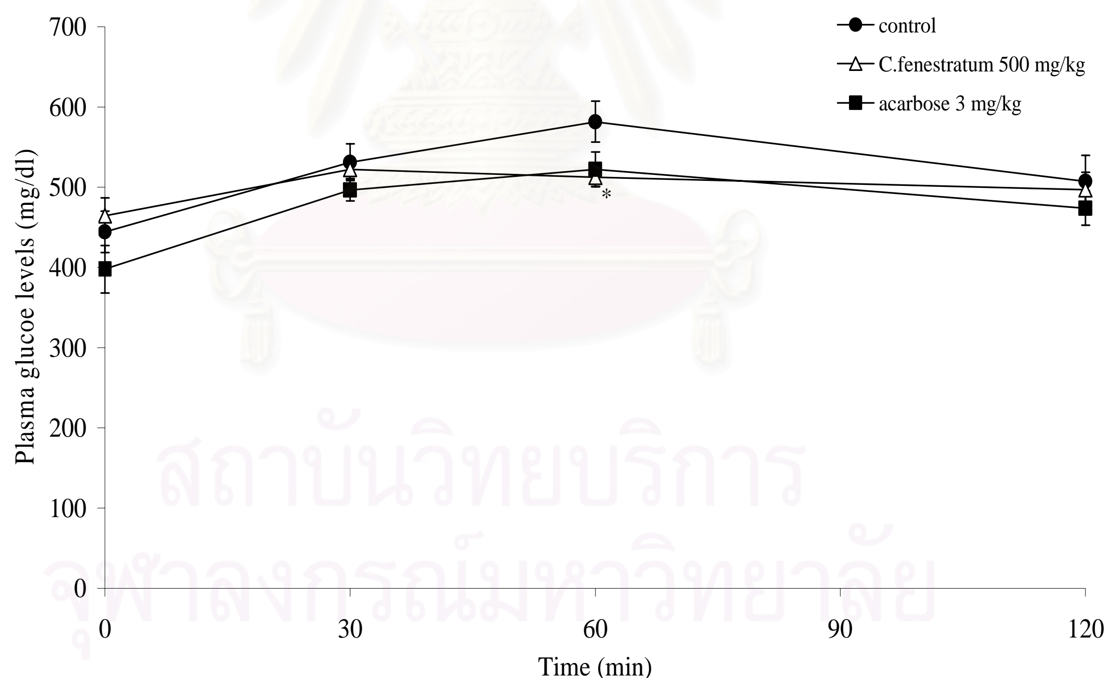

Figure 19 Effects of *C. fenestratum* on oral glucose tolerance test (OGTT) in diabetic rats using maltose as a substrate (n=8).

All data presents in Mean ± S.E.M.

* Significant at $p<0.05$, compared with control group at the same time

Table 14 Effects of *C. fenestratum* on oral glucose tolerance test (OGTT) in diabetic rats using maltose as a substrate (n=8).

Groups	Plasma glucose levels (mg/dl)			
	Time (min) after treatment			
	0	30	60	120
Control	444.32±25.59	531.07±22.86	581.66±25.40	507.38±32.14
C.fenestratum 500 mg/kg	464.60±22.17	522.36±11.73	512.58±12.22*	496.91±21.54
Acarbose 3 mg/kg	397.68±29.62	496.48±13.36	522.33±21.50	473.85±21.29

All data presents in Mean ± S.E.M.

* Significant at $p<0.05$, compared with control group at the same time

Table 15 The percentages decrease of plasma glucose levels of *C. fenestratum* in maltose loaded group compared with control group in diabetic rats.

Groups	% decrease of plasma glucose levels			
	Time (min) after treatment			
	0	30	60	120
C. fenestratum 500 mg/kg	-4.56	1.64	11.88*	2.06
Acarbose 3 mg/kg	10.50	6.51	10.20	6.61

* Significant at $p<0.05$, compared with control group at the same time

Sucrose-loaded group

The hypoglycemic effects of CFE in sucrose loaded diabetic rats were shown in figure 20 and table 16. The percentage decrease of plasma glucose levels, compared with control group, was shown in table 17. Before treatment, the fasting plasma glucose levels of all groups were not significantly different. Although both the CFE (500mg/kg) and acarbose (3 mg/kg) had a tendency to decrease plasma glucose levels, but their effects did not show any significant differences when compared with the control group. The plasma glucose concentrations of CFE group at 30, 60 and 120 min were 500.60 ± 50.59, 534.47 ± 52.95 and 486.05 ± 50.39 mg/dl, respectively and those of control group were 565.27 ± 14.67, 567.95 ± 14.86 and 525.29 ± 17.38 mg/dl, respectively. For the acarbose positive control group, the plasma glucose levels at 30, 60 and 120 min were 487.34 ± 27.36, 481.93 ± 16.45 and 495.11 ± 21.18 mg/dl, respectively.

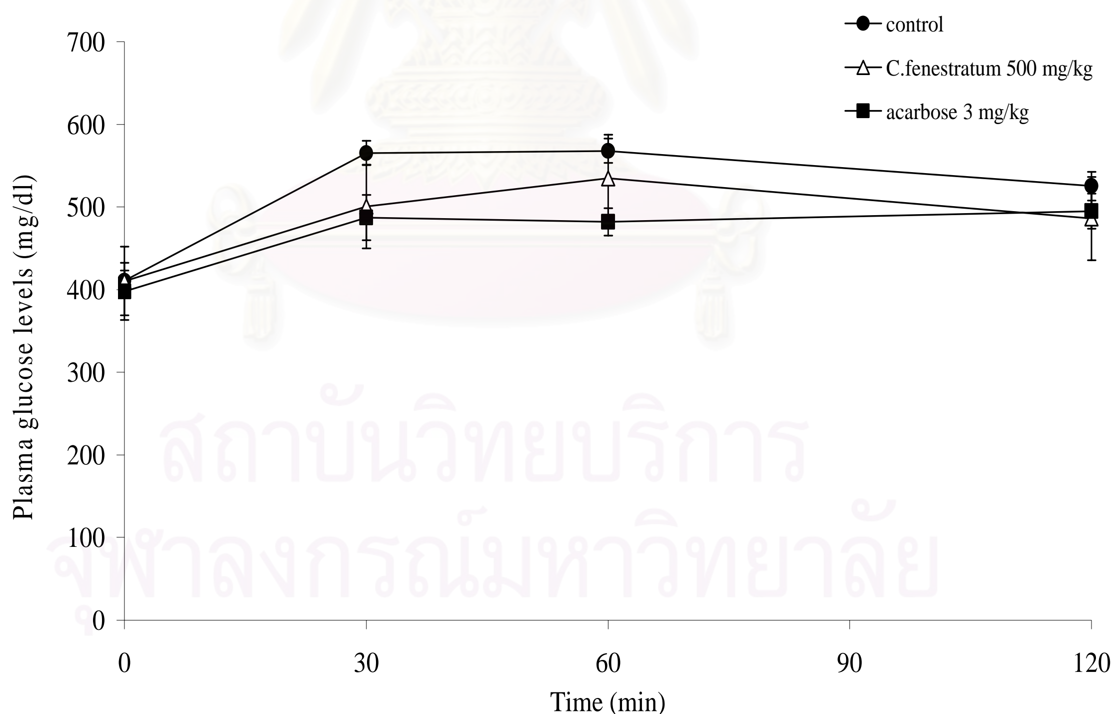

Figure 20 **Effects of *C. fenestratum* on oral glucose tolerance test (OGTT) in diabetic rats using sucrose as a substrate (n=8).**

All data presents in Mean ± S.E.M.

* Significant at $p<0.05$, compared with control group at the same time

Table 16 Effects of *C. fenestratum* on oral glucose tolerance test (OGTT) in diabetic rats using sucrose as a substrate (n=8).

Groups	Plasma glucose levels (mg/dl)			
	Time (min) after treatment			
	0	30	60	120
Control	410.60±12.36	565.27±14.67	567.95±14.86	525.29±17.38
C. fenestratum 500 mg/kg	410.38±41.69	500.60±50.59	534.47±52.95	486.05±50.39
Acarbose 3 mg/kg	397.79±34.86	487.34±27.36	481.93±16.45	495.11±21.18

All data presents in Mean ± S.E.M.

* Significant at $p<0.05$, compared with control group at the same time

Table 17 The percentages decrease of plasma glucose levels of *C. fenestratum* in sucrose loaded group compared with control group in diabetic rats.

Groups	% decrease of plasma glucose levels			
	Time (min) after treatment			
	0	30	60	120
C. fenestratum 500 mg/kg	0.05	11.44	5.89	7.47
Acarbose 3 mg/kg	3.12	13.79	15.15	5.74

* Significant at $p<0.05$, compared with control group at the same time

3. The stimulatory effects of *C. fenestratum* on insulin secretion from perfused rat pancreas

The effects of crude extract *C. fenestratum* and berberine on insulin secretion were performed by *in situ* pancreatic perfusion. The CFE and berberine 10 µg/ml were administered for 20 min. The profile of insulin release was shown in figure 21 together with basal control, which was obtained by perfusion with KRB alone for 40 min. The CFE increased insulin secretion in a biphasic pattern: a peak followed by a sustained phase. Within 20 min of administration, it stimulated three biphasic pattern profiles, in which the maximum insulin secretions were 2.98-, 3.85- and 3.55-fold, respectively, over the basal control group. However, the berberine at the same dose as the CFE slightly and gradually increased insulin secretion from the rat pancreas with the maximum of 1.33-fold over the basal control group. The effluent insulin concentration returned to the baseline during 10 min washing period and increased to 4 to 13.02 fold of the baseline value on the administration of 15 mM glucose (positive control).

The areas under the curve were calculated for 20 min of administration (Figure 22). The CFE significantly stimulated insulin secretion, but not berberine, compared with the basal control group.

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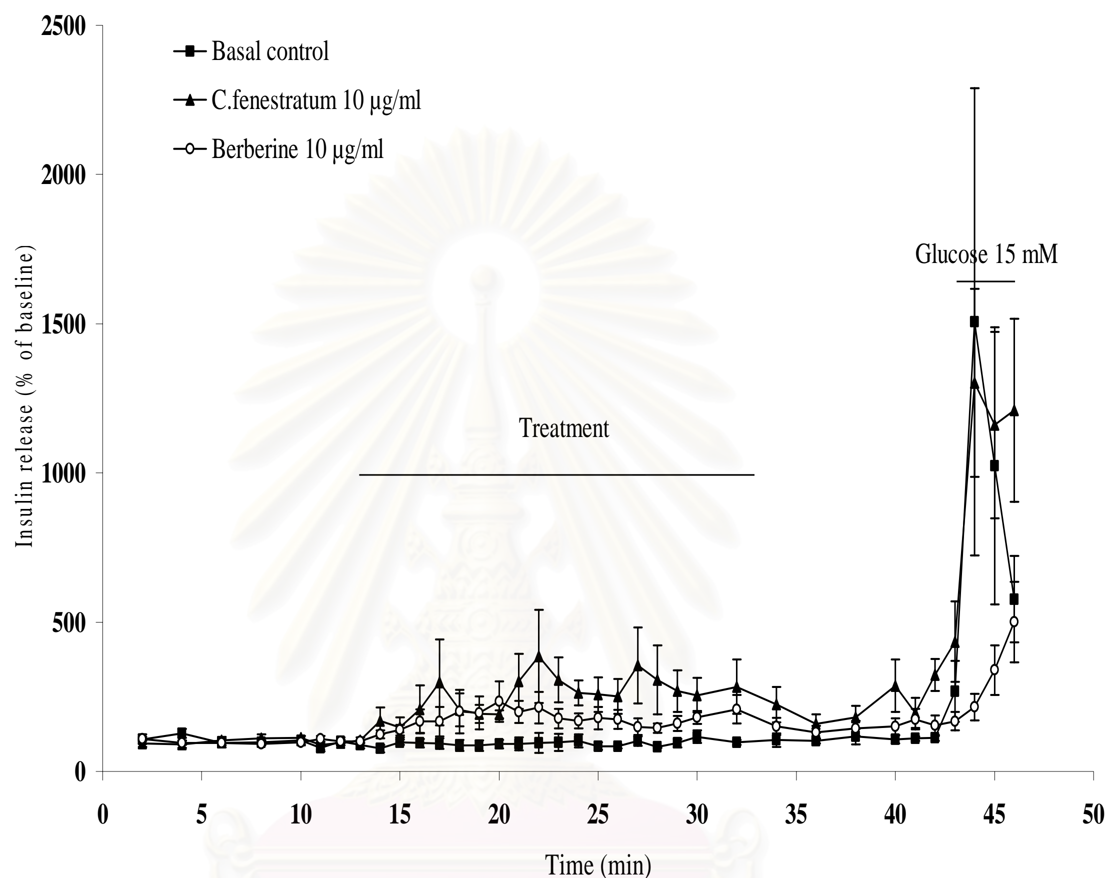

Figure 21 **Effect of *C. fenestratum* and berberine 10 µg/ml on insulin release from perfused rat pancreas.**

Treatments were performed for 20 min (heavy line).

Values are Mean ± S.E.M., n = 3-5.

Range of baseline insulin concentrations of effluents in each group:

Basal control : 16.01 ± 12.53 ng/ml

C. fenestratum : 6.52 ± 4.04 ng/ml

Berberine : 1.73 ± 0.32 ng/ml

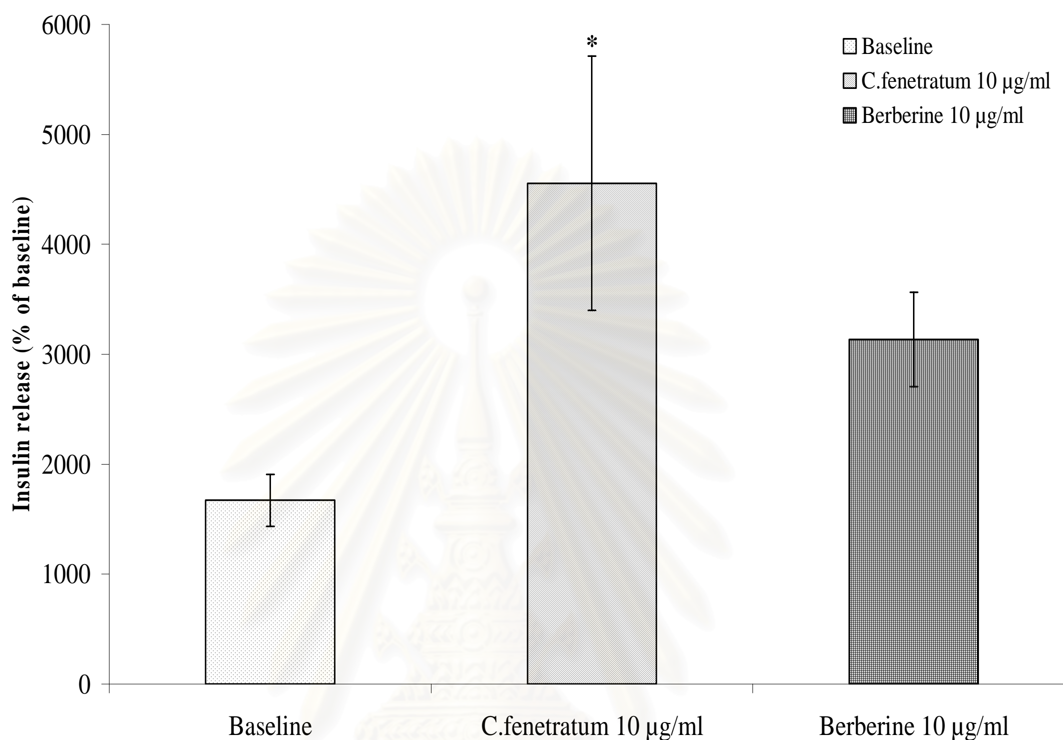

Figure 22 **Area under the curve of *C. fenestratum* and berberine 10 µg/ml on insulin release from perfused rat pancreas.**

AUC was calculated from 20 min insulin secretion curve.

Values are Mean ± S.E.M., n = 3-5.

* Significant at $p<0.05$, compared with the control group

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4. The inhibitory effect of *C. fenestratum* on rat intestinal α-glucosidase (AGH)

The inhibitory effects of CFE and acarbose on α-glucosidase (AGH) activity (maltase and sucrase) from rat intestinal acetone powder were shown in figure 23 and figure 24. The IC_{50} of CFE and acarbose were calculated and shown in table 18. The result showed that the inhibitory activities of CFE on maltase were higher than sucrase with the IC_{50} of 3.89 mg/ml and 11.22 mg/ml, respectively (Figure 4-15 and table 4-14). Similar to the CFE, acarbose was more effective to inhibit the activities of maltase than sucrase with the IC_{50} of 0.66 µg/ml and 6.76 µg/ml, respectively (Figure 4-16 and table 4-14). In addition, the inhibitory activities of acarbose on both enzymes were higher than those of the CFE.

Table 18 The inhibitory effect of *C. fenestratum* and acarbose against α-glucosidase from rat intestinal acetone powder.

Enzyme (Substrate concentration)	**IC_{50} (Concentration)**	
	CFE (mg/ml)	Acarbose (µg/ml)
Maltase (37 mM)	3.89	0.66
Sucrase (37 mM)	11.22	6.76

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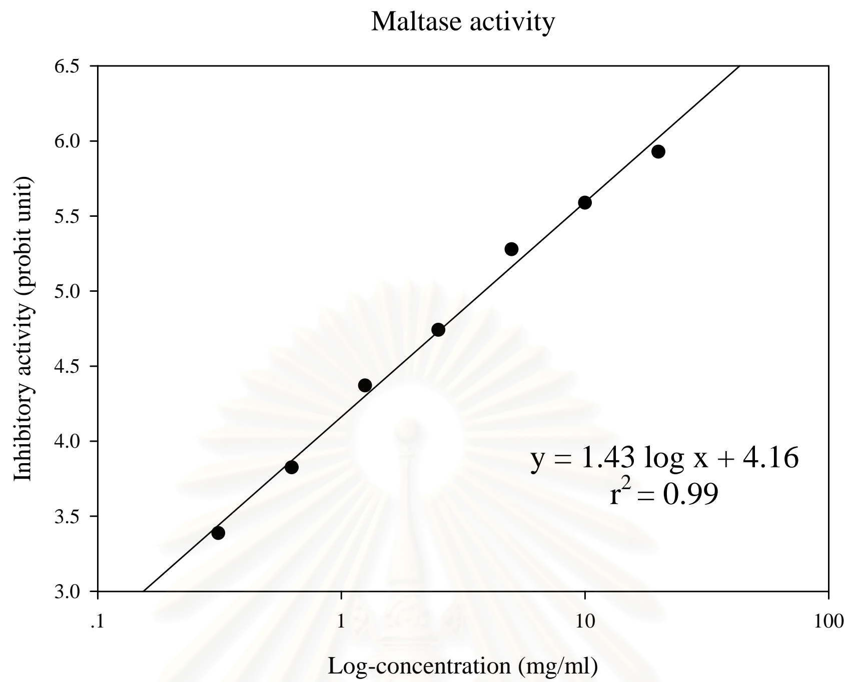
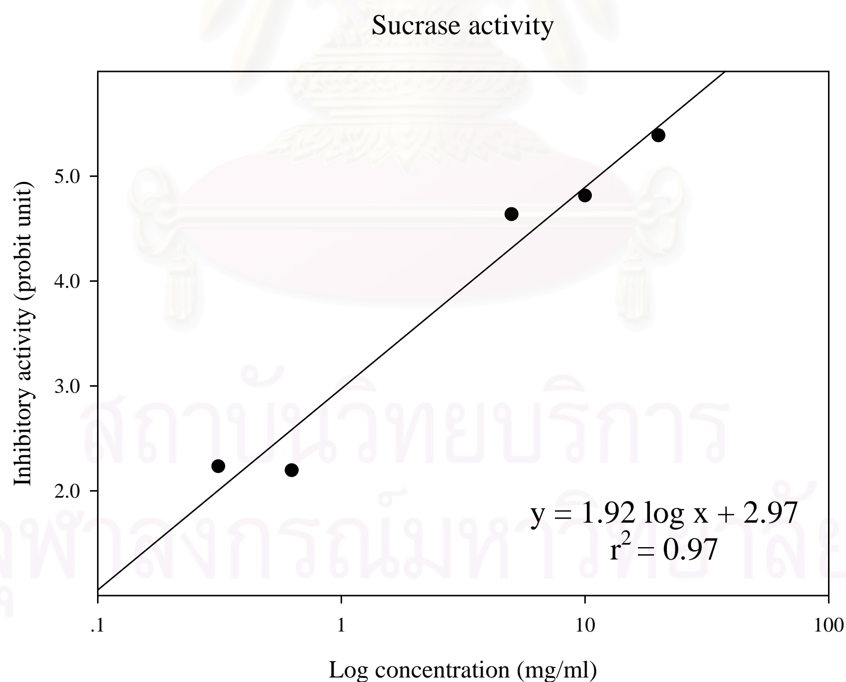


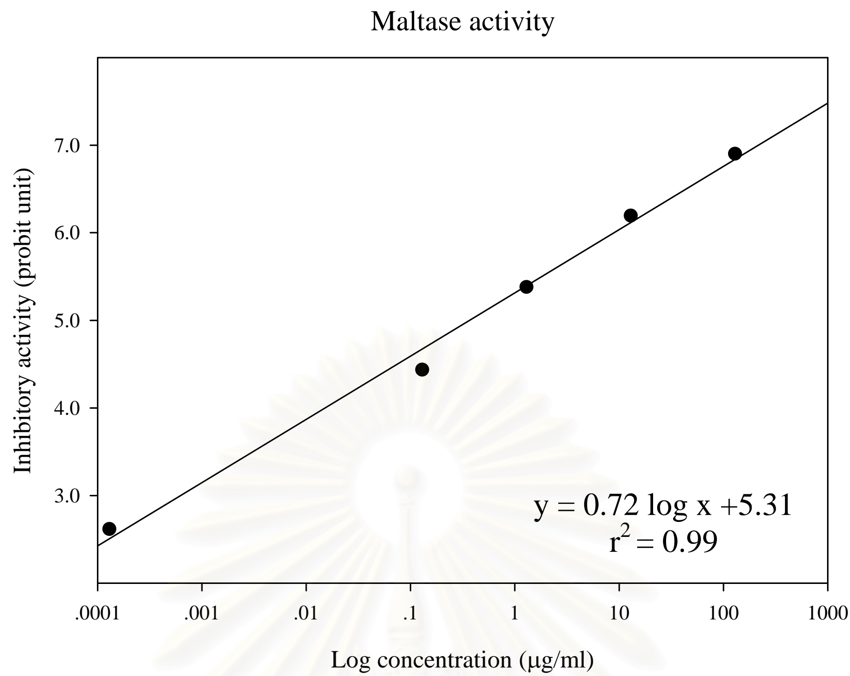
(A)



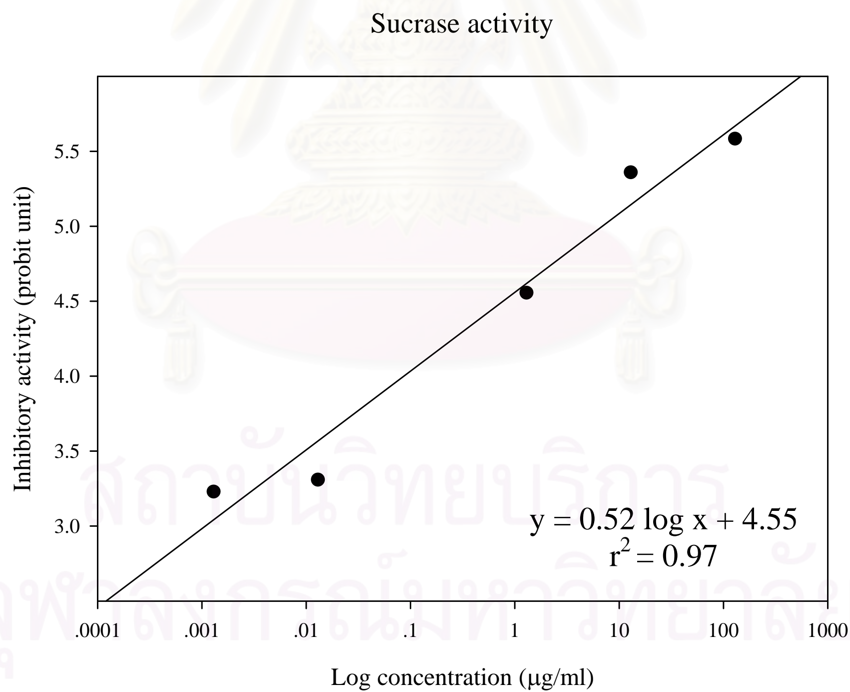
(B)

Figure 23 **Linear-regression of inhibitory activity (Probit unit) of *C. fenestratum*, 0.3125 – 20 mg/ml, against maltase and sucrase activity (n=6) from rat intestinal**

(A) Maltase activity (B) Sucrase activity



(A)



(B)

Figure 24 **Linear-regression of inhibitory activity (Probit unit) of acarbose, $1.2912x10^{-4}$ – $1.2912x10^{2}$ µg/ml, against maltase and sucrase activity (n=6) from rat intestinal**

(A) Maltase activity (B) Sucrase activity

CHAPTER V

DISCUSSION AND CONCLUSION

This research has been performed to investigate the hypoglycemic effects of the crude ethanol extract of *C. fenestratum* in normal and diabetic rats using oral glucose tolerance test as a tool for the study. In addition, the mechanisms underlying its action, direct stimulation of insulin secretion from perfused rat pancreas and inhibition of rat intestinal AGH, were validated.

1. Characteristic features of *C. fenestratum*

Berberine has been reported as a plant alkaloid that is widely distributed in the *Coscinium* plants and it is the major compound in *C. fenestratum* [136-137,154]. It was also found to have a similar effect to metformin on improving insulin sensitivity in high-fat-fed-rats [159]. In addition, it exerted a glucose-lowering effect in hepatocytes, which are insulin-independent, but had no effect on insulin secretion [150]. Therefore, in this study, we speculated that berberine should be one of the major compounds that exerted the antidiabetic activity in the crude ethanol extract of *C. fenestratum*.

Two lots of the crude ethanol extract of *C. fenestratum* used in this study were compared with berberine by thin layer chromatography (TLC). Both lanes of each extract exhibited a spot on TLC at the same position of berberine with R_f value of 0.54, which showed the presence of berberine in each lot of the crude extract. Furthermore, the NMR technique was used to support the above evidence. The ^{1}H and ^{13}C NMR (DMSO-d_6) spectrum displayed the chemical shifts of both protons and carbons very close to those of berberine, which confirmed the presence of berberine and the consistency of the characteristics of both lots of the crude ethanol extracts used in this study.

2. The hypoglycemic effects of the crude ethanol extract of *C. fenestratum* on serum glucose levels in oral several sugar-loaded normal rats

In normal rats, the effects of CFE at concentrations of 250-1000 mg/kg on lowering plasma glucose levels were investigated in the rats loaded with several kinds of sugar, glucose, maltose and sucrose. The results show that the extract at all doses used in this study inhibited the increase of serum glucose levels in all three kinds of sugar-loaded rats.

In glucose-loaded rats, the CFE (250-500 mg/kg) showed significant decrease in plasma glucose levels during 30-60 min after glucose administration, and the dose of CFE at 1000 mg/kg displayed a longer and more potent antihyperglycemic effect than the others, indicating a dose-dependent profile of this extract. These anti-hyperglycemic effects of the crude ethanol extract were more prolonged than those in crude water extract in which 0.1-1 g/kg of the crude water extract suppressed plasma glucose levels for only 30 min after glucose feeding [147]. This implies that both crude ethanol and water extracts may contain different chemical constituents that exerted anti-hyperglycemic effects.

Furthermore, we compared the hypoglycemic effects of this extract with an oral hypoglycemic agent, glibenclamide (5 mg/kg). Interestingly, we found that the anti-hyperglycemic effects of CFE (250-1000 mg/kg) were better than that of glibenclamide, especially at 1000 mg/kg dosage. Glibenclamide is a well-known sulfonylurea oral hypoglycemic drug in which it actions through stimulation of insulin release from pancreatic β-cell [98,160]. From these results, it may also postulated that at least more than one hypoglycemic mechanisms may be involved in this extract, one effective via insulin secretagouge similar to the action of glibenclamide and the other acting probably via extra-pancreatic action and/or insulin-like effect.

In maltose-loaded rats, the CFE (250-500 mg/kg) significantly decreased plasma glucose levels in a dose-dependent manner, however, 1000 mg/kg of CFE failed to do so, indicating too high of this dosage. We also compared the hypoglycemic effects of this extract with acarbose, an α-glucosidase inhibitor drug. The result showed that the anti-hyperglycemic activity of CFE was similar to acarbose. In sucrose-loaded rats, the CFE (250-1000 mg/kg) significantly decreased plasma glucose levels in a dose-dependent manner and their anti-hyperglycemic activities were also similar to acarbose. These *in vivo* results suggested that the CFE

probably inhibit α-glucosidase, the small intestinal enzymes that breakdown non-absorbable complex carbohydrate into absorbable monosaccharides, especially maltase and sucrase. Such an action leads to delay and reduce rise in postprandial blood glucose level [2,155].

3. The hypoglycemic effects of the crude ethanol extract of *C. fenestratum* on serum glucose levels in oral several sugar-loaded streptozotocin-induced diabetic rats

Streptozotocin (STZ) -induced diabetic rats was used as a model to investigate the activity of hypoglycemic agents in several experiments [161-162]. STZ destroyed pancreatic β-cell, resulting in insufficiency of insulin release [131]. In order to induce type 2 diabetic rats, we decided to inject STZ (50 mg/kg) intravenously to the rats. Since Bunnitigon [163] has demonstrated that STZ at the dose of 45 mg/kg IV could induce diabetic state with plasma glucose levels about 353 mg/dl and Arison et al. [164] have reported that there was some evidence of β-cell regeneration in animal given a low dose of STZ (50 mg/kg), but not in higher doses (65-100 mg/kg). The effects of CFE on suppressing plasma glucose levels in diabetic rats have also been investigated by loading several kinds of sugar, glucose, maltose and sucrose, as in normal rats. However, we used only single dose of CFE at 500 mg/kg since it has demonstrated the statistical hypoglycemic effects in all three kinds of sugar feeding in normal rats. Glibenclamide or acarbose were also used as positive controls in glucose- or maltose- and sucrose-loaded experiments, respectively.

In glucose-loaded diabetic rats, both CFE and glibenclamide (5 mg/kg) significantly decreased plasma glucose levels with similar effects at 30 min after glucose administration. In maltose-loaded rats, only CFE, but not acarbose, could significantly suppress the plasma glucose levels at 60 min after maltose loaded. In addition, neither CFE nor acarbose demonstrated the statistical significant in hypoglycemic effect in sucrose-loaded diabetic rats. These results implied that CFE seemed to exert more effective hypoglycemic activity in normal rats than in STZ-induced diabetic rats. However, both acarbose and glibenclamide, which are commercial oral hypoglycemic drugs, also showed similar results as in CFE. It is possible that too wide range of plasma glucose level (253.78 - 585.38 mg/dl) has been used in this experiment. In addition, the experimental design may not be appropriate. We should investigate the hypoglycemic effects of the extract on fasting or non-

fasting plasma glucose levels, not those after several sugars feeding, in diabetic rats. Although the hypoglycemic effect of CFE in diabetic rats may not be impressive compared to those in normal rats, the ethanol extract still be able to show higher effect than the crude water extract of *C. fenestratum*. Paopadetkarn, has demonstrated that only high concentration of crude water extract (1 g/kg) could suppress non fasting plasma glucose level at 2 and 3 hr after oral administration in diabetic rats [147]. However, the crude ethanol extracts at lower concentration (500 mg/kg) significantly decreased plasma glucose levels after loading with 3 g/kg glucose solution in this study.

4. The stimulatory effects of *C. fenestratum* on insulin secretion from perfused rat pancreas

To confirm our speculation on the insulin secretagouge mechanism, we further investigated the direct effect of CFE (10 μg/kg) on insulin secretion from perfused rat pancreas. We found that the CFE significantly increased insulin secretion in a biphasic pattern: a peak followed by a sustained plateau. As we mentioned before that berberine has been found to be the active compound in *C. fenestratum*, we, therefore, examined its effect on insulin secretion. Berberine (10 μg/ml) slightly and gradually stimulated insulin secretion in a monophasic pattern with the maximum of 1.33 fold over baseline. However, this increment was not statistically significant when comparing the area under the curve of insulin secretion with the basal control group. These results confirmed that the *in vivo* hypoglycemic activity of *C. fenestratum* was due to stimulating insulin secretion from pancreatic β-cells. However, the CFE was more effective in stimulating insulin secretion than berberine, which implied the possibility of other compounds in the crude extract that can stimulate insulin secretion. Basnet et al. [153], reported that the other compound was found in the CFE, such as sitosterol, produced hypoglycemic activity. It is possible that the sitosterol in the crude extract may stimulate insulin secretion. In order to warrant this statement, further work on investigation of sitosterol-induced insulin secretion need to be performed. The result from the perfusion study was consistent with the previous reported by Yin et al. [150] in which berberine had no effect on insulin secretion in βTC3 cells, a β-cell line derived from transgenic mice expressing a hybrid insulin gene-oncogene. [165] In addition, one interesting point on the period of positive

control (15 mM glucose) at the end of perfusion study, we found that high concentration of glucose in berberine-perfused rat pancreas stimulated insulin secretion much lower than those of basal control- and the CFE-perfused rat pancreas. This may due to the cytotoxic effect of berberine that has been reported in human hepatocytes, HepG2 cells [150]. However, the crude extract did not show any cytotoxic effect. This indicates that some other compounds in the crude extract may help to counteract or protect the functional β-cells from deterioration by berberine so that they remain active and produce insulin.

5. The inhibitory effect of *C. fenestratum* on rat intestinal α-glucosidase (AGH)

As we mentioned before that the mechanisms underlying anti-hyperglycemic effect of this extract may be involved more than one pathway. One of them was insulin secretagouge and the others may act probably via extra-pancreatic action and/or insulin-like effect. Since berberine has been reported to inhibit the activity of disaccharidase, maltase and sucrase, in differentiated intestinal epithelial cells (Caco-2) [166], we, therefore, investigated these effects of the crude ethanol extracts on rat intestinal α-glucosidase.

The inhibitory activity of CFE on sucrase and maltase were investigated *in vitro*. The results showed that CFE could inhibit the activities of maltase better than sucrase, which was similar to acarbose. The IC_{50} of CFE on maltase and sucrase were 3.89 mg/ml and 11.22 mg/ml, respectively and the IC_{50} of acarbose on maltase and sucrase were 0.66 µg/ml and 6.76 µg/ml, respectively. Our result was opposite to the other reported by Pan et. al, in which berberine could inhibit sucrase activity better than maltase in Caco-2 cells [166]. It is possible that berberine may react differently in different kinds of cells or again some other components in the crude extract may also play role on this acting.

Compared to berberine, known α-glucosidase inhibitor and sulfonylurea oral hypoglycemic agent, this crude ethanol extract may have some advantages in the treatment of patients with diabetes mellitus. Berberine has shown some cytotoxic activity in human hepatocytes, HepG2 cells [150]. α-Glucosidase inhibitor is associated with high incidence of gastrointestinal side effects such as diarrhea,

abdominal distension and flatulence, which may be due to a fermentation of unabsorbed carbohydrate causing bacterial overgrowth in colon [167-169]. Sulfonylurea drugs may also cause hypoglycemic adverse reactions, including coma [170-171]. Hence, this CFE may be worth to be developed as a new drug for treatment of diabetes mellitus in the future. However, further biochemical and pharmacological studies in purification and isolation of the other active compounds in this extract, as well as elucidation of their mechanisms of action are needed.

From all of the above-mentioned discussion, we concluded that the crude ethanol extract of *C. fenestratum* had anti-hyperglycemic action in both normal and STZ-induced diabetic rats. The CFE was more potent in reducing plasma glucose levels than glibenclamide in glucose-loaded rats, and exerted similar action as acarbose in decreasing plasma glucose levels in maltose- and sucrose-loaded rats. From perfused rat pancreas, the CFE was more potent in stimulating insulin secretion than berberine. In addition, the CFE was found to effectively suppress the activity of rat intestinal α-glucosidase; maltase and sucrase. Taken together, these results suggested that the mechanisms underlying hypoglycemic activity of CFE were at least partly due to stimulation of insulin secretion and inhibition of intestinal α-glucosidase, maltase and sucrase.

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APPENDICES

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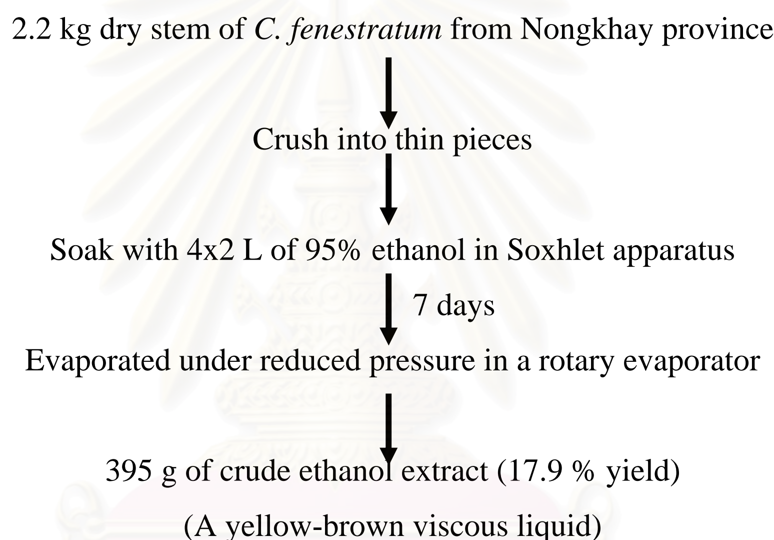
APPENDIX A

Extraction and characterization feature of *C. fenestratum*

1. Extraction of *C. fenestratum*

The ethanol extraction of *C. fenestratum* was performed by Dr. Damrong Sommit, Department of Chemistry, Faculty of Science, Mahanakorn University of Technology, Bangkok. The method for ethanolic extraction of *C. fenestratum* was shown as following in the below diagram.



2. Characteristic feature of *C. fenestratum*

It has been reported that berberine is the major active constituent in *C. fenestratum* [136-137,154]. In this study, we used two different lots of the crude extract from *C. fenestratum*. Therefore, in order to confirm the consistent characteristics of our extracts, the active compound in each lot was compared with berberine using thin layer chromatography (TLC) and nuclear magnetic resonance (NMR) techniques. Both lanes of each extract exhibited a spot on TLC at the same position of berberine with R_f value of 0.54 in 20% methanol in dichloromethane as a solvent system, which indicated the presence of berberine in both lots of the extract (Figure 25). The structure of berberine was shown in Figure 26.

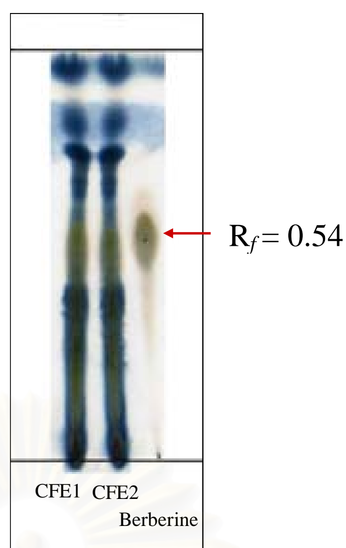


Figure 25 TLC of crude extract of *C. fenestratum* compared with berberine (R_f = 0.54)

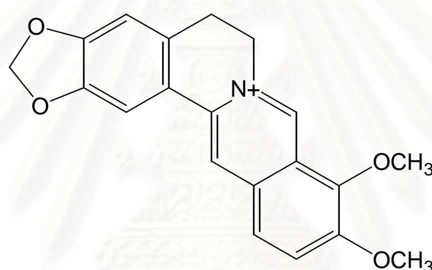


Figure 26 Structure of berberine [154]

Figure 28 to Figure 30 show ^{1}H and ^{13}C NMR (DMSO-d_6) spectrum which displayed the characteristic features of the crude extract. The ^{1}H and ^{13}C NMR chemical shifts of the extract were compared with berberine reported in literature as shown in table 19 [137,172]. The spectrums reveal the chemical shifts of both protons and carbons very close to those of berberine.

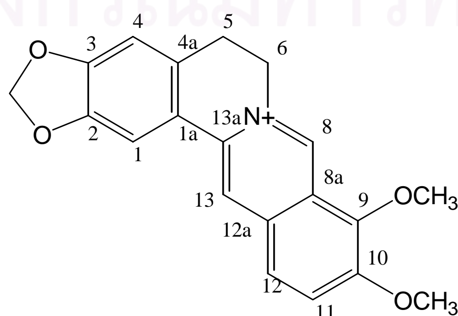


Figure 27 The chemical shift position of berberine [154]

Table 19 The comparison of the ^{1}H and ^{13}C NMR chemical shifts of crude ethanol extract of *C. fenestratum* with berberine [137,172].

Position	^{1}H NMR		^{13}C NMR	
	Berberine	*C. fenestratum*	Berberine	*C. fenestratum*
1	7.81	7.770	105.5	105.905
1a	-	-	120.5	120.883
2	-	-	147.7	148.147
3	-	-	149.8	150.290
4	7.10	7.078	108.5	108.902
4a	-	-	130.7	131.125
5	3.22	3.208	26.4	26.4
6	4.95	4.928	55.2	55.2
8	9.91	9.871	145.5	145.890
8a	-	-	121.4	121.867
9	-	-	143.7	144.090
9-OCH_3	4.11	4.099	62.0	62.380
10	-	-	150.4	150.4
10-OCH_3	4.08	4.069	57.1	57.492
11	8.22	8.197	126.7	127.137
12	8.01	8.016	123.6	124.055
12a	-	-	132.9	133.421
13	8.96	8.892	120.3	120.646
13a	-	-	137.5	137.920
OCH_2O	6.19	6.168	102.3	102.3

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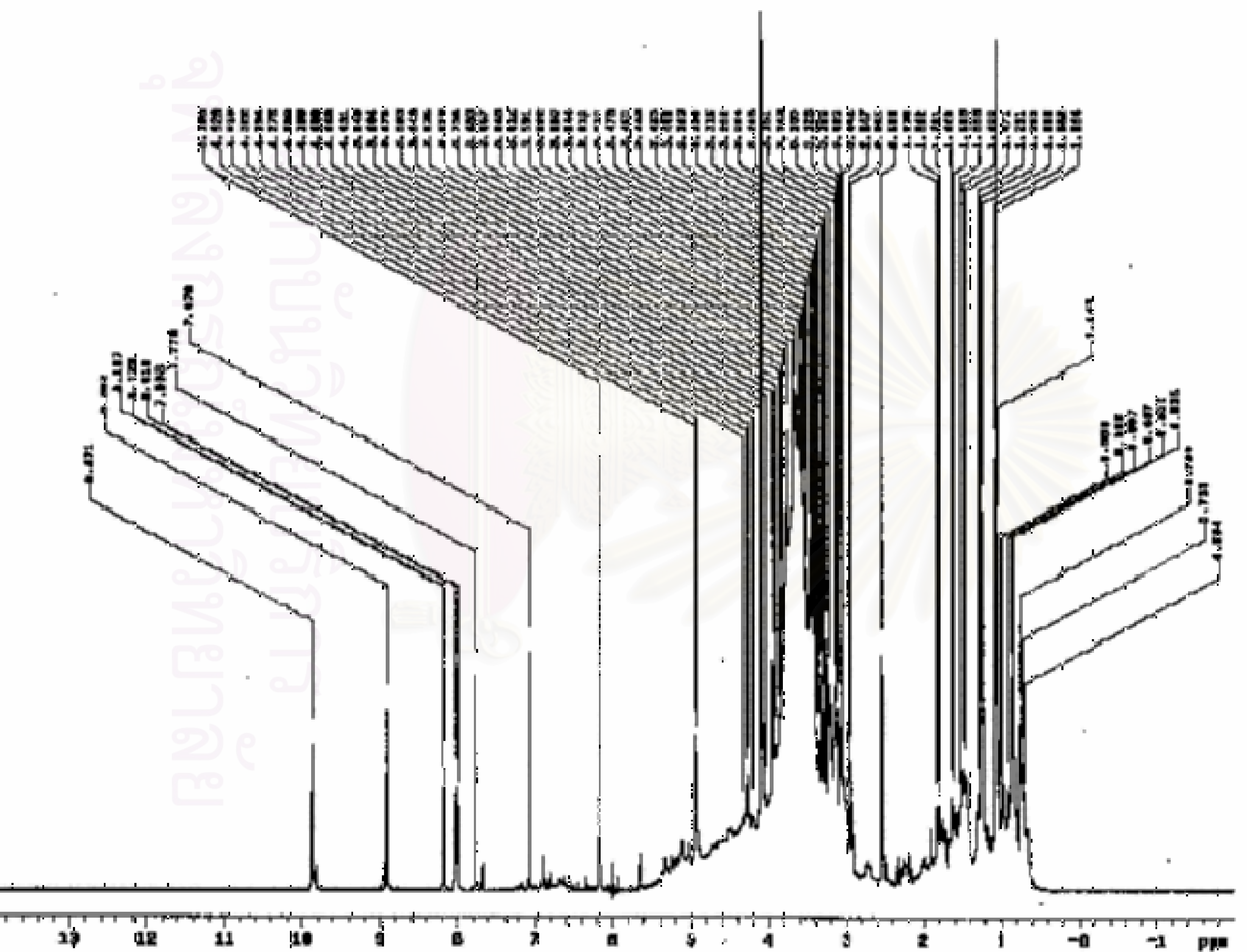

Figure 28 The ^1H NMR (DMSO-d_6) spectrum of *C. fenestratum*

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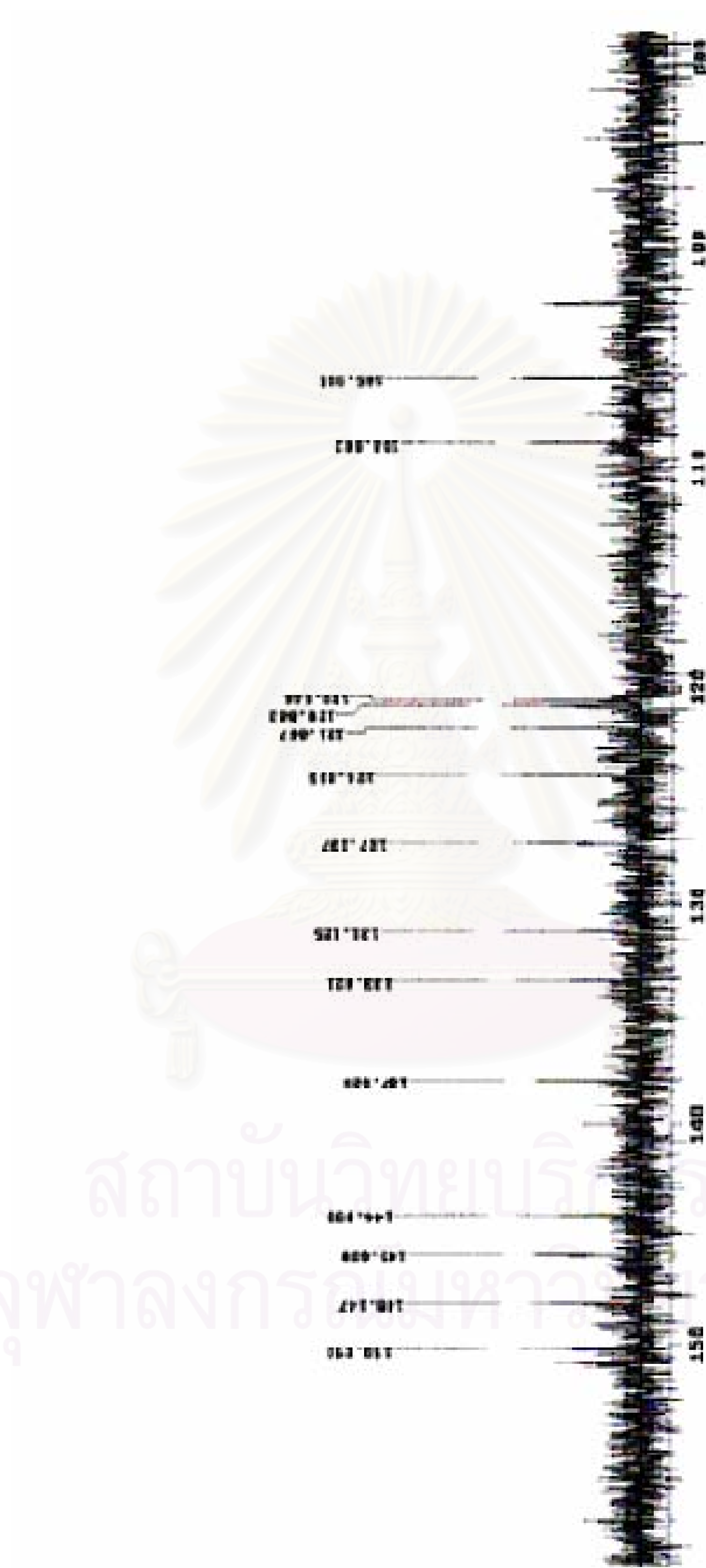

Figure 29 The ^{13}C NMR (DMSO-d_6) spectrum of *C. fenestratum*

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Figure 30 The ^{13}C NMR (DMSO-d_6) spectrum of *C. fenestratum* (continue)

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APPENDIX B

Glucose determination

Glucose Oxidase/Peroxidase Test*

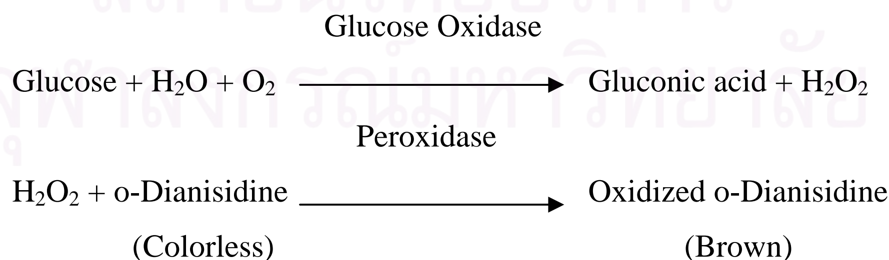
Reagents:

1. The PGO enzyme solution was prepared by adding the contents of 1 capsule of PGO enzymes to 100 ml of water.
2. The o-dianisidine solution was prepared by dissolving 50 mg of o-dianisidine dihydrochloride in 20 ml of water.
3. The PGO enzyme reaction solution was prepared by mixing 100 ml of the PGO enzyme solution and 1.6 ml of the o-dianisidine solution.
4. Glucose standard solution was prepared in serial dilution at 0, 18.75, 37.5, 75, 150 and 300 mg/dl.

Assay procedure:

1. Add 10 µl of sample to 1 ml of PGO enzyme reaction solution in the tube and mix well.
2. Incubate the tube at 37ºC for 30 min.
3. Read absorbance at 450 nm by spectrophotometer (Tecan A-5082).
4. Create glucose standard curve.
5. Calculate glucose concentration from standard curve.

Note: This procedure was base upon the following coupled enzymatic reactions.



The intensity of the brown color produced is a qualitative measurement of the original glucose concentration.

*** Available from Sigma product information**

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APPENDIX C

Insulin determination

Radioimmunoassay*

Reagents:

1. The ^{125}I insulin solution was prepared by adding 50 ml of distilled water to ^{125}I insulin (DPC®) 1 bottle.
2. Rat insulin standard solution was prepared at 5, 12.5, 50, 75, 100 and 1000 ng/ml.

Assay procedure:

1. Add 100 µl of sample to insulin Ab-coated tubes (DPC®, U.S.A.).
2. Add 0.5 ml of ^{125}I insulin solution to every tube and mix well.
3. Incubate for 18-24 min at 15-28ºC.
4. Decant thoroughly.
5. Count for 2 min in a gramma counter (The nucleus, Model 600B).
6. Create rat insulin standard curve.
7. Calculate insulin concentration from standard curve.

*** Available from COAT-A-COUNT Insulin (DPC®, U.S.A.)**

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APPENDIX D

Krebs-Ringer bicarbonate buffer (KRB)

Stock solution

Stock solution 1:

- NaCl 27.7 g/l

Stock solution 2:

- $CaCl_2$ 1.494 g/l

Stock solution 3:

- KH_2PO_4 0.648 g/l

Stock solution 4:

- KCL 1.413 g/l
- $NaHCO_3$ 8.401 g/l
- $MgSO_4.7H_2O$ 1.173 g/l

Working solution

1. Use each stock solution 50 ml made to 200 ml of KRB.
2. Add 5.5 mM glucose, 1% Dextran, 10 mM HEPES and 0.2% BSA as a supplementary.
3. Incubate for 20 min at 37℃ with carbogen.
4. Adjust to pH 7.4

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APPENDIX E

Phosphate buffer solution*

Stock solution

Stock solution A

- KH_2PO_4 9.073 g/l

Stock solution B

- $Na_2HPO_4 \cdot 7\ H_2O$ 17.817 g/l

Working solution

1. Add stock A 41.3 ml and stock B 58.7 ml.
2. Adjust to pH 7.0

*** Available from Merck Tables**

BIOGRAPHY

Ms. Wanlaya Jittaprasatsin was born on April 7, 1976 in Bangkok, Thailand. She graduated Bachelor's degree of Nursing Science in 1998 from Mahidol University. She had worked as a nurse at Siriraj Hospital from in 1998 to 2002.

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