การประเมินความเป็นพิษและฤทธิ์ต้านเบาหวานของสารสกัดน้ำจากเปลือกต้นลูกน้ำนม

นายฮาว แวน ด็อง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเวชศาสตร์
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ปีการศึกษา 2560

EVALUATION OF TOXICITY AND ANTIDIABETIC ACTIVITY OF THE AQUEOUS EXTRACT FROM *CHRYSOPHYLLUM CAINITO* L. STEM BARK

Hau Van Doan

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biomedical Sciences

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EVALUATION OF TOXICITY AND ANTIDIABETIC ACTIVITY OF THE AQUEOUS EXTRACT FROM *CHRYSOPHYLLUM CAINITO* L. STEM BARK

Suranaree University of Technology has approved this thesis submitted in fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ฮาว แวน ด็อง : การประเมินความเป็นพิษและฤทธิ์ต้านเบาหวานของสารสกัดน้ำจากเปลือกต้นลูกน้ำนม (EVALUATION OF TOXICITY AND ANTIDIABETIC ACTIVITY OF THE AQUEOUS EXTRACT FROM *CHRYSOPHYLLUM CAINITO* L. STEM BARK.). อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.นวลน้อย จูฑะพงษ์, 116 หน้า

ต้นลูกน้ำนม *(Chrysophyllum cainito L.)* ถูกนำมาใช้เป็นยาสมุนไพรเพื่อรักษาผู้ป่วยโรคเบาหวานในหลายประเทศ โดยเฉพาะอย่างยิ่งในประเทศเวียดนาม อย่างไรก็ตามยังขาดหลักฐานทางวิทยาศาสตร์ที่สนับสนุนการใช้พืชดังกล่าว การศึกษานี้มีวัตถุประสงค์เพื่อประเมินฤทธิ์ต้านเบาหวานของสารสกัดน้ำจากเปลือกต้น*ลูกน้ำนม*ควบคู่ไปกับกลไกการออกฤทธิ์ นอกจากนั้นยังทำการศึกษาพิษของสารสกัดอีกด้วย จากการวิเคราะห์ด้วยวิธีที่ใช้ DPPH ABTS และ FRAP พบว่าสารสกัดจากเปลือกต้นน้ำนมมีฤทธิ์แรงในการต้านอนุมูลอิสระเทียบเท่าได้กับกรดแอสคอบิคและบิวทิเลทไฮดรอกซีโทลูอีน จากนั้นจึงทำการศึกษาความเป็นพิษเฉียบพลันในหนูขาว พบว่าไม่มีหนูขาวแสดงอาการพิษหรือตายจากการป้อนสารสกัดเป็นเวลา 14 วันแม้ในขนาดสูงสุดที่ 4000 มก./กก. สารสกัดจากเปลือกต้นลูกน้ำนมในขนาด 250 และ 500 มก./กก. สามารถลดพื้นที่ใต้กราฟของระดับน้ำตาลในเลือดจากการทดสอบความทนทานต่อน้ำตาลในหนูปกติได้อย่างมีนัยสำคัญ (P<0.05) ในการศึกษาด้วยโมเดลที่ใช้สาร alloxan ในการชักนำให้หนูเป็นเบาหวานพบว่า การป้อนสารสกัดเพียงครั้งเดียวในขนาด 250 และ 500 มก./กก. สามารถลดน้ำตาลในเลือดของหนูหลังอดอาหารได้อย่างมีนัยสำคัญเช่นเดียวกับยา glibenclamide

จากนั้นได้ทำการศึกษาหากลไกการออกฤทธิ์ของสารสกัดในการลดน้ำตาลในเลือดแบบเฉียบพลัน พบว่า สารสกัดไม่สามารถยับยั้งการดูดซึมน้ำตาลกลูโคสผ่านลำไส้เล็กส่วนกลางที่แยกจากร่างกายของหนูถีบจักรได้ อย่างไรก็ตามสารสกัดในขนาด 50 มก./มล. เพิ่มการนำกลูโคสเข้าสู่กล้ามเนื้อหน้าท้องที่แยกจากหนูถีบจักรได้อย่างมีนัยสำคัญเมื่อให้ร่วมกับอินซูลิน (P<0.05) และพบว่าสารสกัดยังมีฤทธิ์ยับยั้งเอนไซม์ α-glucosidase ที่แรงกว่า acarbose เมื่อนำสารสกัดทดสอบเป็นระยะเวลานานขึ้นแบบ sub-chronic พบว่าทั้งสารสกัดและยา glibenclamide ไม่สามารถลดน้ำตาลในเลือดของหนูได้ ซึ่งคาดว่าระดับความรุนแรงของการเป็นเบาหวานของหนูในระยะหลังของการทดลองน่าจะสูงเกินกว่าประสิทธิภาพของยาที่จะให้ผลในการออกฤทธิ์

ในกรณีของการศึกษาพิษวิทยาในสัตว์ทดลอง ผู้วิจัยยังได้ทำการศึกษาผลของสารสกัดต่อการพัฒนาการในปลาม้าลาย ความเข้มข้นของสารสกัดที่ทำให้ตัวอ่อนตาย 50% คำนวณได้ที่ 25.75 ± 1.51 มคก./มล. เมื่อทำการศึกษาความเป็นพิษในหลอดทดลองพบว่าสารสกัดทำให้เกิดการยับยั้งการเจริญเติบโตของเซลล์ HepG2 ด้วยค่า IC_{50} = 121.75 ± 7.98 มคก./มล. (โดยวิธี MTT) และ

169.38 ± 10.40 มคก./มล. (โดยวิธี Trypan blue exclusive) สารสกัดทำให้เกิดการตายของเซลล์เนื่องจากไปกระตุ้นกระบวนการแก่ ผลการทดลองจากการวิเคราะห์วัฏจักรของเซลล์แสดงให้เห็นว่าสารสกัดไปเพิ่มจำนวนเซลล์ที่อยู่ในระยะ sub-G1 ให้มากขึ้น สุดท้ายไม่พบว่าสารสกัดมีฤทธิ์ปกป้องการถูกทำลายภายใต้สภาวะความเครียดออกซิเดชันต่อเซลล์ HepG2

การศึกษานี้ เป็นการแสดงหลักฐานทางตรงต่อฤทธิ์ต้านเบาหวานของสารสกัดน้ำจากเปลือกลำต้นลูกน้ำนมเป็นครั้งแรก พร้อมกับเสนอกลไกการออกฤทธิ์ที่เป็นไปได้ของสารสกัดคือการกระตุ้นการนำน้ำตาลกลูโคสเข้าเซลล์และการยับยั้งเอนไซม์ α-glucosidase ข้อมูลที่ได้จากการศึกษานี้สนับสนุนการใช้พืชชนิดนี้พร้อมกับให้ข้อมูลด้านความปลอดภัยและข้อควรระวังในการใช้รักษาผู้ป่วยโรคเบาหวาน สำหรับสารออกฤทธิ์ในสารสกัดควรจะได้มีการศึกษาต่อไป

สาขาวิชาปรีคลินิก ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2560 ลายมือชื่ออาจารย์ที่ปรึกษา_____

HAU VAN DOAN : EVALUATION OF TOXICITY AND ANTIDIABETIC ACTIVITY OF THE AQUEOUS EXTRACT FROM *CHRYSOPHYLLUM CAINITO* L. STEM BARK. THESIS ADVISOR : ASSOC. PROF. NUANNOI CHUDAPONGSE, Ph.D. 116 PP.

CHRYSOPHYLLUM CAINITO/ TOXICITY/ DIABETES/ ALLOXAN/ APOPTOSIS/ GLUCOSE UPTAKE/ α-GLUCOSIDASE INHIBITION.

Chrysophyllum cainito L. has been used as an alternative medicine for the treatment of diabetic patients in many countries, especially in Vietnam. However, there is very limited scientific rationale for this medical use. The present study aimed to evaluate the antidiabetic activity of the extract from *C. cainito* stem bark (CE) and the possible mechanisms underlying this action. Furthermore, the toxicity of CE was also investigated. CE showed strong antioxidant activity comparable to the ascorbic acid and butylated hydroxytoluene in DPPH, ABTS, and FRAP assay. The acute toxicity study in rat showed no sign of toxicity or mortality after a single administration of CE up to 4000 mg/kg in 14 days of observation. The chosen doses of CE at 250 and 500 mg/kg significantly reduced the area under curve of blood glucose level in oral glucose tolerance test in normal mice ($P<0.05$). In alloxan-induced diabetic mice model, similar to glibenclamide, a single dose of both 250 and 500 mg/kg of CE significantly decreased fasting blood glucose level.

The mechanisms underlying the acute effect on blood glucose were evaluated. From the isolated rat jejunum experiment, the CE at any doses used did not inhibit glucose absorption. However, the CE at 50 μg/ml significantly increased the amount of

glucose uptake by isolated abdominal muscles from mice in the presence of insulin ($P<0.05$). It was found that the CE produced stronger inhibition of α-glucosidase activity than acarbose. The sub-chronic treatment of CE was examined and found that neither CE nor glibenclamide reduce blood glucose level after 21 days, possibly due to the high severity of the animal diabetic state.

In term of toxicology, *in vivo* study on development was also conducted in zebrafish models. It was demonstrated that the concentration that cause a half of embryos death was 25.75 ± 1.51 µg/ml. It was found from the *in vitro* cytotoxicity that CE significantly inhibited HepG2 cell growth with IC_{50} = 121.75 ± 7.98 µg/ml (MTT assay) and IC_{50} = 169.38 ± 10.40 µg/ml (Trypan blue exclusive assay). The action of CE that induced cells death was by triggering cell apoptotic program. Cell cycle analysis demonstrated that CE markedly increased the percentage of cells in sub-G1 phase. CE at non-toxic concentrations had no protective activity on oxidative stress-damaged HepG2 cells.

Direct evidence of antidiabetic activity of CE with possible modes of action, glucose uptake stimulation and α-glucosidase inhibitory effect was reported for the first time herein. These data support the potential use of this plant for the treatment of diabetic patients with safety and caution information.

School of Preclinic Student's Signature _____

Academic Year 2017 Advisor's Signature _____

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Hau Van Doan

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LIST OF ABBREVIATIONS

AA	Ascorbic acid
ABTS	2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid
Akt	Protein kinase B
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
AST	Aspartate aminotransferase
ATCC	The American Type Culture Collection
ATP	Adenosin triphosphate
AUC	Area under the curve
BG	Blood glucose level
BHT	Butylated hydroxytoluene
BUN	Blood urea nitrogen
CE	*Chrysophyllum cainito* stem bark extract
DDP-4	Dipeptidylpeptidase-4
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
FBS	Fetal bovine serum
FRAP	Ferric reducing antioxidant power

LIST OF ABBREVIATIONS (Continued)

GAE	Gallic acid equivalents
GLP-1	Glucagon-like peptide-1
Glib	Glibenclamide
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
HbA1c	Glycated haemoglobin
HDL	High-density lipoprotein cholesterol
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC_{50}	Concentration of CE that inhibits the activity of chemical or biological funtions by a half.
KRB	Kreb's Ringer bicarbonate buffer
LC_{50}	Dose of CE that cause death to 50% of animals
LDL	Low-density lipoprotein cholesterol
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
OECD	The Organisation for Economic Co-operation and Development
OGTT	Oral glucose tolerance test
PBS	Phosphate buffered saline
PDK1	Pyruvate dehydrogenase kinase 1
PGO	Peroxidase-glucose oxidase
PI3K	Phosphatidylinositide 3-kinases

LIST OF ABBREVIATIONS (Continued)

PNPG	p-nitrophenyl-α-D-glucopyranoside
PPARγ	Peroxisome proliferator-activated receptor gamma
RO	Reverse osmosis
ROS	Reactive oxygen species
SEM	Standard error of the mean
SGLT2	Sodium-glucose co-transporter 2
SNK	Student-Newman-Keuls
STZ	Streptozotocin
SUR	Sulfonylureas receptor
TBHP	tert-butyl hydroperoxide
TDZ	Thiazolidinedione
ZHE1	Zebrafish hatching enzyme 1

CHAPTER I

INTRODUCTION

In recent years, people around the world have confronted many severe health problems. As stated by World Health Organization, the most of death in 2015 caused by ischemic heart disease followed by stroke, chronic obstructive pulmonary disease, lung cancer, diabetes and lower respiratory infections (World Health Organization Fact sheets, available at www.who.int/mediacentre/factsheets/fs310/en/ accessed on March 10, 2018).

Diabetes mellitus, one of the most common metabolic disorders, has been reported to affect approximately 415 million people worldwide in 2015 and the number of cases has been estimated to increase to 642 millions in 2040 (Ogurtsova et al., 2017). According to the International Diabetes Federation, approximately 5.0 million people died from diabetes in 2015 equivalent to one death every six seconds globally (International Diabetes Federation, 2015). In Thailand, there were approximately 4% of total mortality due to diabetes and the incidences of diabetes were found in 9.6% of the population. These amounts in Vietnam were 3% and 4.9% respectively (World Health Organization, 2016). The expenditure on diabetes was estimated to $376 billion in 2010 and $490 billion in 2030 worldwide. Each person could spend $1,330 on diabetes in 2010. The amount of money depends on sex differences, living area, age, as well as country income (Zhang et al., 2010).

Microvascular and macrovascular complications play a major role in diabetic

morbidity and mortality in both type 1 and type 2. Prolonged hyperglycemia in blood stream increases the risk of cardiovascular diseases which primarily kill the patients. Microvasular serious complications can be counted for retinopathy, nephropathy, and neuropathy (Fowler, 2008).

Diabetic medications have exhibited many adverse effects which can bring the patient to other health problems from nausea, headache, weight gain to urinary tract infections (SGLT2 Inhibitors) (Geerlings, Fonseca, Castro-Diaz, List, and Parikh, 2014; Liu et al., 2017) or even heart failure (Thiazolidinediones) (Hernandez, Usmani, Rajamanickam, and Moheet, 2011; Nesto et al., 2003). Therefore, antidiabetic medicinal plants are of interest due to natural products are sources of novel drug leads (Ezuruike and Prieto, 2014; Malviya, Jain, and Malviya, 2010).

Chrysophyllum cainito is a tropical fruit tree of which biological activities have been demonstrated so far. The benefits of *C. cainito* are included hypoglycemic, antioxidant, wound healing, antihypertensive, anti-inflammatory and antibacterial activities. To date, there is no scientific evidence on the effect of *C. cainito* stem bark provided, especially on diabetic remedy although traditional use of stem bark in diabetes was recorded in Côte-d'Ivoire, a country located in the West of Africa (Koffi, Konan Édouard, and Kouassi, 2009). In the Southwest of Vietnam, diabetic patients also used the stem bark as a folk medicine. Therefore, this study is designed to evaluate the antidiabetic activity of the stem bark in animal model and its underlying mechanism of action. Moreover, the toxicity of new agent has been considered lately in parallel with their benefits (Moreira, Teixeira, Monteiro, De-Oliveira, and Paumgartten, 2014; Nasri and Shirzad, 2013). Hence, the toxicity of *C. cainito* extract will also be examined in this study. The findings of the present study will provide pharmacological evidence

for the use of *C. cainito* as well as its toxicity profile.

CHAPTER II

LITERATURE REVIEW

2.1 Diabetes mellitus

Diabetes mellitus (or diabetes, DM) is a chronic disease characterized by long-term hyperglycemia status. The characteristic is derived from insufficient insulin or the utilize of insulin improperly. In the body, insulin is secreted by pancreatic β-cells located in islets of Langerhans. The role of insulin in glucose homeostasis is summarized in Figure 2.1.

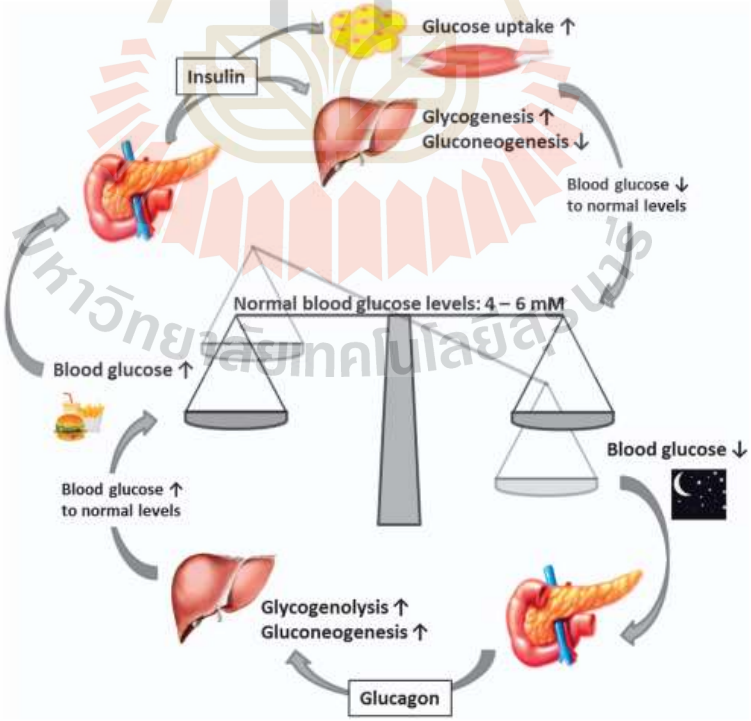


Figure 2.1 The control of blood glucose in the body (Röder et al., 2016).

Insulin helps to lower blood glucose by increasing glucose uptake at glucose-stored cells. In the other hand, when glucose level is lower than normal, glucagon is secreted to generate glucose from degrading liver glycogen (Röder, Wu, Liu, and Han, 2016).

There are two major types of diabetes mellitus, type 1 and type 2, though other types of diabetes can occur during women pregnant or from side effects of a medication.

2.1.1 Type 1 diabetes

Type 1 diabetes (juvenile diabetes) mostly occurs in youth although the symptom has also found in adulthood. Type 1 diabetes is caused by autoimmune system leading to the destruction of the β-cells. Thus, the deficiency in insulin production causes the rise of glucose concentration in the blood stream. Insulin injection is essential in the therapy of type 1 diabetes along with some physical activity and healthy diet strategy (National institute of diabetes and digestive and kidney diseases, 2013).

2.1.2 Type 2 diabetes

Type 2 diabetes (adult-onset diabetes, type 2 DM) is diagnosed more often in elderly than young, and more popular in overweight or obese people (National institute of diabetes and digestive and kidney diseases, 2013). Pathogenesis and therapy of type 2 diabetes were described by Stumvoll and colleagues in Figure 2.2 (Stumvoll, Goldstein, and van Haeften, 2005). Insulin is a key hormone regulate glucose homeostasis. Both insulin resistance (IR) and insulin deficiency are the patterns of type 2 diabetes. Insulin becomes resistance when cells do not utilize insulin properly which prominently show in the liver and skeletal muscle. Under this condition, the body usually increases cell function to secrete more insulin. The overwork may cause damage to cells over time because of the toxicity from high glucose or by itself leading to cell

dysfunction. Insulin resistance is closely associated with obesity and poor physical exercise. Approximately 90% of diabetes cases are suffered from type 2. However, human being can prevent type 2 diabetes with the changing of healthy lifestyles such as healthy diet, keeping healthy body weight, taking physical activity often, and avoiding smoking and alcoholic consumption (Zheng, Ley, and Hu, 2017).

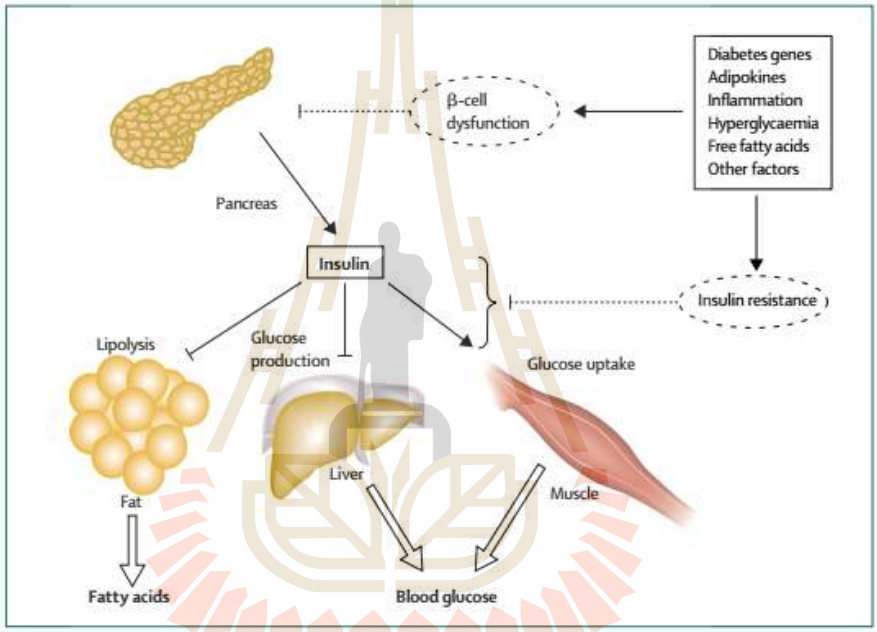


Figure 2.2 Pathophysiology of hyperglycemia and increased circulating fatty acid in type 2 diabetes (Stumvoll et al., 2015).

2.1.2.1 Insulin deficiency in type 2 DM

As mentioned above, insulin deficiency is one of main pathophysiological characters of type 2 DM. The insufficiency of insulin is due to the impair in cellular secretory individually or to the loss of β-cell mass. The abnormality of glucose-stimulated insulin secretion may contribute to the reduction of insulin level.

The defective glucose-stimulated insulin secretion is also a result of impairing of glucose sensing (Cantley and Ashcroft, 2015). Mechanism of glucose-stimulated insulin secreton is described in Figure 2.3.

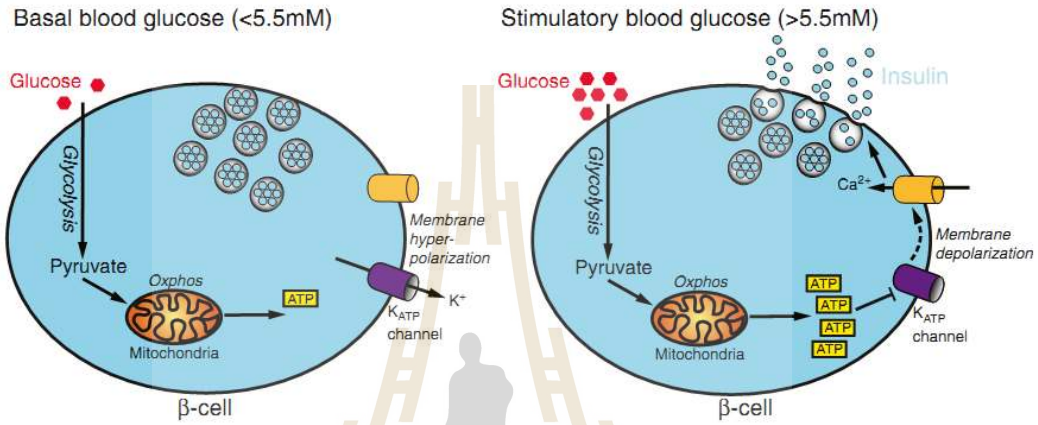


Figure 2.3 Mechanism of glucose-stimulated insulin secretion in β-cells. The rise of extracellular glucose increases ATP production (right panel) resulting in the closure of K_{ATP} channels, depolarization in plasma membrane, calcium influx and insulin vesicle exocytosis (Cantley and Ashcroft, 2015).

Oxidative stress also leads to the impairment of insulin secretion in type 2 DM (Robertson, 2006). The progressive destruction of pancreatic β-cells increases over time independently with risk factors of type 2 DM (Ozougwu, 2013). The significant reduction of β-cells mass is found in type 2 DM patients. Relative β-cell volume deficit, increasing of apoptosis and low replication of β-cells have been shown in obesity and lean type 2 DM patients compared to non-diabetes (Guillausseau et al., 2008).

2.1.2.2 Insulin resistance in type 2 DM

Insulin resistance is an important indicator for diagnosis of DM and a crucial target in DM management. The association of genetic expression of lipoprotein lipase to peroxisome proliferator–activated receptor (PPARγ) expression and mitochondrial function are widely known for contributions to the muscle insulin resistance in type 2 DM. Insulin resistance in muscle is risk factor for the progression of type 2 DM (Taylor, 2012).

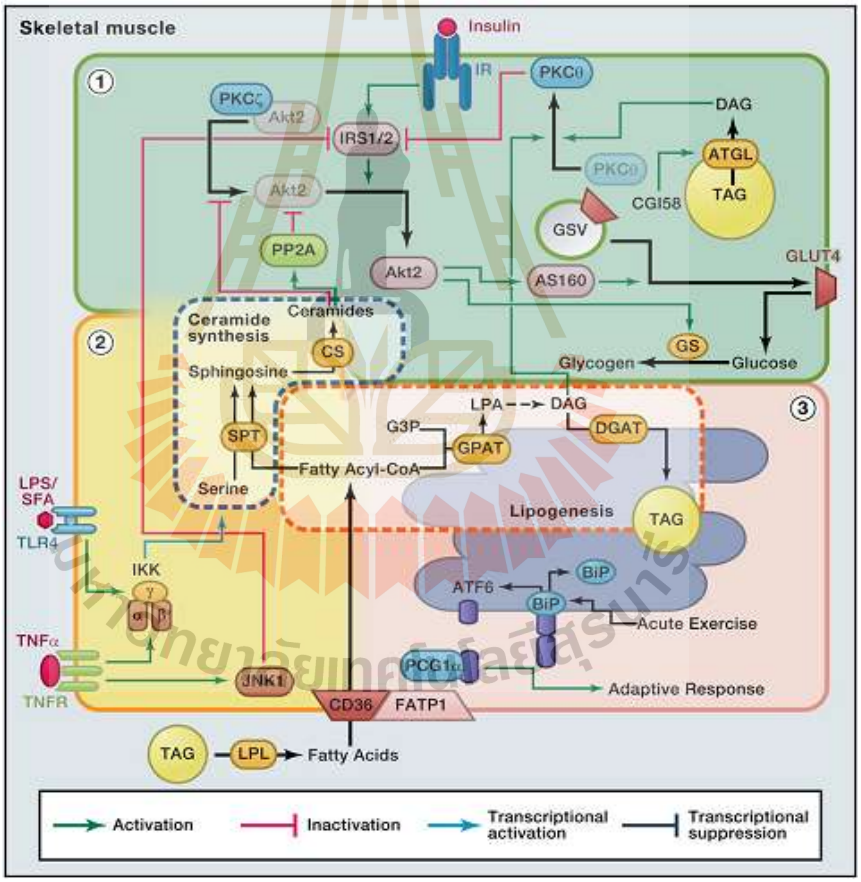


Figure 2.4 Insulin signaling pathway in muscle insulin resistance (Samuel and Shulman, 2012).

In skeletal muscle, lipid induces insulin resistance by contributing to the impaired insulin signaling, decreased insulin-stimulated glucose uptake and impaired glucose transporter 4 translocation. The aggregation of etopic lipid in liver leads to the resistance of hepatocytes to insulin. The impaired insulin signaling pathway has been found to mediate through the activation of protein kinase C (PKCs). Figure 2.4 depicts the signaling pathway where insulin resistance occures in muscle (Samuel and Shulman, 2012). At molecular level of insulin resistance, insulin receptor substrate-2, protein kinase B and forkhead transcription factor FOXO1a are of distinct interest. Phosphoinositide-dependent kinase 1-independent phosphorylation of protein kinase Cε has been shown to inhibit the expression of insulin receptor. Additionally, insulin resitance are affected by mitochondrial dysfunction and adipokines (e.g TNF-α, leptin, adiponectin) (Saini, 2010).

2.3 Diabetes complications

Complications of diabetes are found in both type 1 and type 2 diabetes. Diabetes complications are divided into microvascular and macrovascular complications. Diabetes complications can affect to eye visions, kidney, amputations, heart diseases or even death. Diabetic nephropathy is the major factor of renal failure end stage. Kidney disease contributes to the development of hypertension and macrovascular complication. Diabetic condition is worse following diabetic kidney disease since kidney disease reduces renal gluconeogenesis and insulin clearance. Kidney disease also decreases glucose filtration and excretion (Pecoits-Filho et al., 2016). Blindness is a final result of diabetic retinopathy over years whereas nervous degeneration during high sugar condition relates to pain, neurological disability, sensory loss, and wound

healing. Cardiovascular complications in diabetes are initiated with the accumulation of atherosclerosis, developing to myocardial infarction, ischemia, stroke and diastolic dysfunction (Forbes and Cooper, 2013).

2.4 Diabetes diagnosis and antidiabetic drugs

2.4.1 Diagnostic criteria

The identification of diabetes can be done rapidly by checking fasting blood glucose or random blood glucose. According to WHO, a new diabetic case is diagnosed with fasting blood glucose level ≥126 mg/dl (≥7.0 mmol/l) and 2-hour plasma glucose level ≥ 200 mg/dl after glucose loading or a random glucose level ≥ 200 mg/dl. The diagnostic criteria are summarized in Table 2.1 (Word Health Organization, 2016).

Recently, glycated haemoglobin (HbA1c) test has been applied to indicate diabetes worldwide. A level of HbA1c ≥ 6.5% indicates diabetic status whereas the value below 6.5% requires more test to identify (World Health Organization, 2011). Florkowski has reviewed that the HbA1c measurement is a convenient test and strong indicator for diabetes diagnosis because the integrated glycemia is a consequence of entire lifespan of the red blood cell. It can determine chronic hyperglycemia and the developing complications. However, this test also exhibits the disadvantages. Due to the haemolysis and other conditions, HbA1c level can be reduced thereby the result will be influenced (Florkowski, 2013).

Table 2.1 Diabetes diagnosis and glucose tolerance diagnostic criteria (World Health Organization, 2006).

Diabetes	
Fasting plasma glucose 2–h plasma glucose*	≥7.0mmol/l (126mg/dl) **or** ≥11.1mmol/l (200mg/dl)
Impaired Glucose Tolerance (IGT)	
Fasting plasma glucose 2–h plasma glucose*	<7.0mmol/l (126mg/dl) **and** ≥7.8 and <11.1mmol/l (140mg/dl and 200mg/dl)
Impaired Fasting Glucose (IFG)	
Fasting plasma glucose 2–h plasma glucose*	6.1 to 6.9mmol/l (110mg/dl to 125mg/dl) **and (if measured)** <7.8mmol/l (140mg/dl)

* Venous plasma glucose 2–h after ingestion of 75g oral glucose load

* If 2–h plasma glucose is not measured, status is uncertain as diabetes or IGT cannot be excluded

2.4.2 Antidiabetic drugs

In order to provide a complete care for an individual with diabetes, it is required to understand the risk factors, the status of complications, and the previous treatment applications of the patients. Life style intervention along with pharmacological intervention and daily mornitoring of blood glucose are the keys to optimize the diabetic care for patients (Chaudhury et al., 2017). Accoding to Chaudhury and co-workers, life style changes clearly affect the prevention or delay the onset of diabetes in clinical trial. Life style modifications are strongly recommended to the patients with prediabetic diagnosised, impair glucose tolerance, and impair fasting glucose. Besides diet monitoring and exercise, moderate weight lost (7%), control alcohol and sodium

consumption are also considered. Pharmacological management of diabetics involve the use of medicines which are classified into insulin therapy and oral hyperglycemic drugs.

Insulin therapy

Insulin plays a crucial role in the regulation of glucose. In the pancreas, β-cells secrete insulin to maintain a normal range of sugar in the circulation. The β-cells are destroyed and clearance in type 1 diabetes, therefore, exogenous insulin is an essential treatment. Insulin injection can be applied to type 2 diabetes when the symptom is severe. Different generations of insulin have been developed to increase the efficiency of insulin and their duration of action (Table 2.2) (Tahrani, Bailey, Del Prato, and Barnett, 2011). There are available of rapid-acting insulin, short-acting insulin, intermediate-acting insulin, long-acting insulin or pre-mixed (Gomez-Perez and Rull, 2005).

Oral hypoglycemic drugs

As mentioned above, insulin insensitivity and low level of insulin production are aspects of type 2 diabetes. It is a complex of metabolic disorders with hypertension, heart disease, inflammation, hyperlipidemia, etc. Therefore, the purpose of treatment includes protecting the patient from long-term complications. Antidiabetic drugs are classified based on its mechanism of action. They are increasing insulin secretion, inhibiting glucose synthesis from liver, increasing insulin sensitivity, and interfering sugar absorption in gastrointestinal tract (Tahrani et al., 2011). The initial prescription is usually with a single agent, however, the combination of drugs will give a better effect on controlling blood sugar (Jovanovic et al., 2004; Kawamori et al., 2014; Raskin et al., 2004). The major classes of antidiabetic agents are as follow.

2.4.2.1 Sulfonylureas

Sulfonylureas are the first group of oral hypoglycemic agents that increase insulin secretion from pancreatic β-cells. Figure 2.5 is an illustration of the mechanism action of gliclazide on pancreatic β-cells (Proks, Reimann, Green, Gribble, and Ashcroft, 2002). By biding to the sulfonylurea receptor (SUR) subunit 1 of the ATP-sensitive potassium chanel (K_{ATP}) located in plasma membrane (Proks et al., 2002), they promote the chanel closure. K_{ATP} locked by glucose or sulfonylureas causes depolarization of pancreas β-cell membrane leading to the opening of voltage-gated calcium ion channels. Thus, the Ca^{2+} influx increases. The rising of Ca^{2+} in β-cells stimulates the release of insulin-containing secretory granules.

K_{ATP} channels are also located in cardiac, skeletal and smooth muscles, however, in these tissues the channels are contributed by different SURs which also have specific sensitivity to the drugs. This is an explanation of the adverse effect of this group on cardiovascular system.

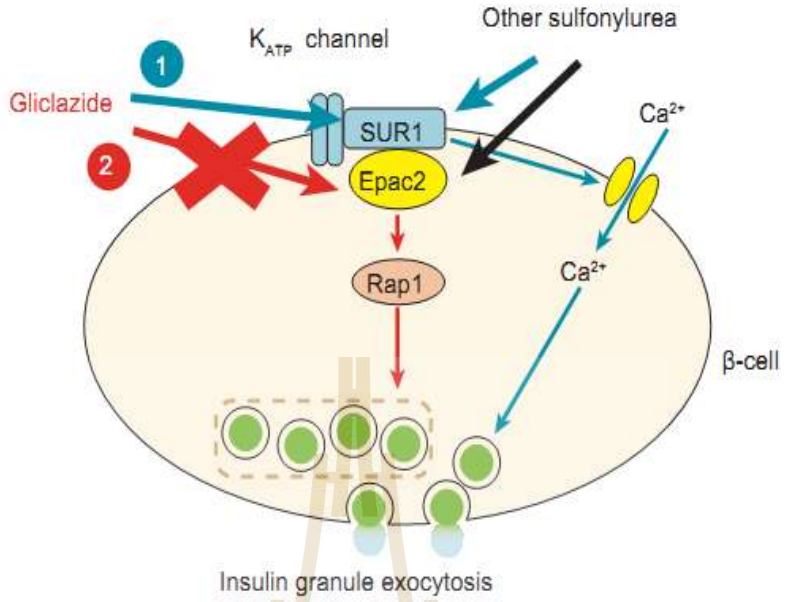


Figure 2.5 The mechanism of action of the insulin secretagoague gliclazide on pancreatic β-cells (Proks et al., 2002).

2.4.2.2 Meglitinides

Meglitinides provide a rapid action on insulin secretion, therefore, it offers a better control of postprandial glucose control. Meglitinides have short-term effect, thus, the risk of late hypoglycemia is decrease. Nateglinide and repaglinide are two derivative meglitinides using in the treatment of type 2 DM. They possess the effect on reducing postprandial glucose elevation and reducing the level of HbA1c. Repaglinide has been found to have more effects on reducing fasting plasma glucose and glucagon secretion than nateglinide. Repaglinide has mechanism of action similarly to sulfonylureas which is mediated by K_{ATP} channel. Nateglinide also binds to SURs and inhibits K_{ATP} channels and effects on stimulating insulin secretion (Guardado-Mendoza, Prioletta, Jiménez-Ceja, Sosale, and Folli, 2013).

2.4.2.3 Biguanides

Biguanides (mainly Metformin) are well-known as an inhibitor of hepatic glucose production in the treatment of type 2 DM. The efficiency of metformin depends on the dose and the duration of treatment. Metformin has been found to act via both MAPK-dependent and independent mechanisms on hepatic gluconeogenesis. Metformin also exerts its inhibition effect on the Complex I of mitochondrial respiratory chain in the liver, thus suppressing ATP production. Metformin plays a role of MAPK activator to inhibit the glucose production from hepatocyte. The action is due to the inhibition of fructose-6-bisphosphatase by AMP and downregulation of the gluconeogenic enzymes PEPCK and glucose-6-phosphatase. It also ameliorates insulin sensitivity and lowers cAMP, thus diminishing the expression of gluconeogenic enzymes (Rena, Hardie, and Pearson, 2017).

2.4.2.4 Thiazolidinediones

Thiazolidinediones (TDZs) are used in controlling of type 2 DM via acting on the sensitivity of insulin receptor. Binding of TDZs to peroxisome proliferator-activated receptor gamma (PPARγ) leads to the formation of heterodimers with retinoid-X receptors (RXRs) which targets to specific DNA sequences found on the promoters of PPARγ target genes. As a result, this binding stimulates the transcription of the genes involving in the metabolism of glucose and lipids. The antihyperglycemic effect of TDZs are due to their insulin-sensitizing activity, therefore, TDZs therapy is not associated with the risk of hypoglycemia (Cariou, Charbonnel, and Staels, 2012). The mechanism of TDZs action is summarized in Figure 2.6.

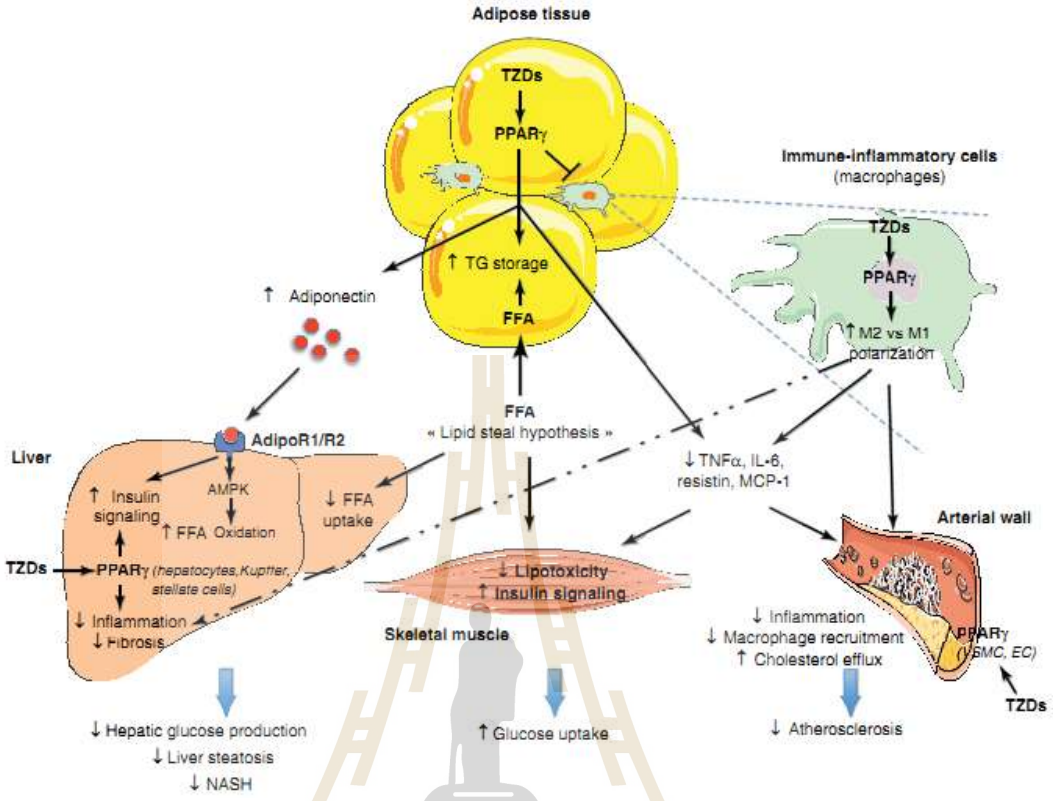


Figure 2.6 Mechanism action of Thiazolidinediones (TDZs) (Cariou et al., 2012)

2.4.2.5 Glucagon-like peptide-1 and dipeptidylpeptidase-4 inhibitors

Glucagon-like peptide-1 (GLP-1) receptor agonists and dipeptidylpeptidase-4 (DPP4) inhibitors are two new classes of antidiabetic drugs targeting on the insulin secretion by incretin hormone (GLP-1). GLP-1 increases insulin production while inhibits the secretion of glucagon when glucose level is high. In addition, GLP-1 consumption can delay the emptying of gastric and food intake is also decreased by its anorexic action. GLP-1 helds the glucose-lowering effect and weight-loss effect which are benefit for diabetic patient with overweight/obesity. Therefore, GLP-1 is a crutial goal in diabetic research. However, the half-life of GLP-1 is short

due to the deactivative effect of DPP-4. Therefore, the use of DPP-4 inhibitor can prolong the activity of endogenous incretin hormones (Garber, 2011).

2.4.2.6 α-Glucosidase inhibitors

α-Glucosidase inhibitors are the antidiabetic drugs for type 2 most effectively in postprandial glucose monitor. The inhibition of α-glucosidase enzymes produced in the brush border of enterocytes minimizes the absorbable monosaccharides into blood stream. It is because the carbohydrate consumed is in the form of non-absorbable oligosaccharides and polysaccharides which are required to be hydrolyzed into absorbable monosaccharides. Acarbose is the most popular drug of this family. Acarbose also provides reversible effect on impaired glucose tolerance to normal glucose tolerance (Derosa and Maffioli, 2012).

2.4.2.7 Amylin

Amylin is a peptide that co-secreted with insulin by the islet of Langerhangs. It delays gastric emptying, blocks glucagon secretion and acts as a satiety enhancing agent even though human amylin may play a part in the contributing to β-cell destruction in type 2 diabetes (Schmitz, Brock, and Rungby, 2004). The co-administration of amylin or amylin analogues with insulin produces the synergictic effect of insulin on the reducing glucose level (Adeghate and Kalász, 2011).

The drug classes and mechanisms are summarized in Table 2.2.

Table 2.2 Antidiabetic drugs (Tahrani et al., 2011).

Agents	Examples	Mechanism(s) of action	Advantages	Disadvantages
Sulphonyureas	Gliclazide Glipizide Glimepiride Glibenclamide	Increase insulin secretion by binding to sulfonylurea receptor 1, resulting in depolarisation and calcium influx that initiates insulin secretion	Long-term safety Low cost	Hypoglycaemia, weight gain, possible need for self-monitoring blood glucose, careful dose titration
Biguanide	Metformin	Suppresses hepatic glucose output Increases insulin sensitivity in muscle Interferes with glucose and lactate metabolism in the gut. Might increase concentrations of endogenous glucagon-like peptide 1	Long-term safety Weight neutral Low risk of hy-poglycaemia Low cost	Gastrointestinal side effects, possible link to lactic acidosis, avoid in deteriorating renal function or hypoxaemic states
Meglitinides	Nateglinide Repaglinide	Bind to sulfonylurea receptor 1 on the β cell, but at a different site to sulfonylure-as, resulting in a more rapid and shorter insulin response	Rapid, short acting Suitable for prandial use	Few data for long-term safety, weight gain, hypoglycaemia. Self-monitoring of blood glucose (but less than with sulfonyl-ureas)

Table 2.2 Antidiabetic drugs (Continued).

Agents	Examples	Mechanism(s) of action	Advantages	Disadvantages
Thiazolidined-iones	Pioglitazone Rosiglitazone	PPARγ agonists act primarily in the adipose tissue to increase subcutaneous adipogenesis and reduce release of free fatty acids Increase insulin sensitivity in muscle and liver	Low risk of hypoglycaemia might reduce blood pressure	Long-term safety not established: risk of weight gain, oedema, heart failure, and fractures
Glucagon-like peptide-1 mimetics	Exenatide Liraglutide	Binds to glucagon-like-peptide-1 receptor, causing increased glucose-dependent insulin secretion and glucagon suppression, delayed gastric emptying, and appetite suppression	Weight loss Low risk of hypo-glycaemia Possible effect on β-cell survival	Long-term safety not known Unconfirmed association with pancreastitis and medullary cell carcinoma GI side-effects, avoid in renal failure
Dipeptidylpe-ptidase-4 inhibitors	Sitagliptin Vildagliptin Saxaglitpin	Increase endogenous incretin concentrations	Weight neutral Low risk of hypo-glycaemia Possible effect on β-cell survival	Long-term safety not known. Unconfirmed association with pancreatitis

Table 2.2 Antidiabetic drugs (Continued).

Agents	Examples	Mechanism(s) of action	Advantages	Disadvantages
α-glucosidase inhibitors	Acarbose Miglitol	Inhibit carbohydrate degradation in gut	Weight neutral Low cost	Gastrointestinal side-effects
Amylin analogue	Pramlintide	A synthetic soluble analogue of human amylin, lowers postprandial glucose by centrally mediated satiety, suppressing postprandial glucagon secretion, and delaying gastric emptying	Weight loss	Unknown long-term safety Increases the risk of insulin-induced hypoglycaemia Only used with insulin
Insulin	Aspart, Lispro, Atrapid, Humulin S, Insuman rapid Insulatard, Glargine, Biphasic premixed	Directly activate the insulin receptor, decrease hepatic glucose output, increase peripheral use, and reduce lipolysis	More sustained glycaemic improvements compared with other drugs	Weight gain Hypoglycaemia Need for self-monitoring of blood glucose Fluid retention

2.5 Role of antioxidant in diabetes and diabetes management

Diabetes condition and oxidative stress expose a reciprocal impact. Under chronic hyperglycemia state, glucose toxicity will increase the formation of reactive oxygen species (ROS) in β-cell. The releasing of ROS leads to β-cell deterioration and the progression of pancreatic β-cell dysfunction (Figure 2.7) (Kaneto, Katakami, Matsuhisa, and Matsuoka, 2010).

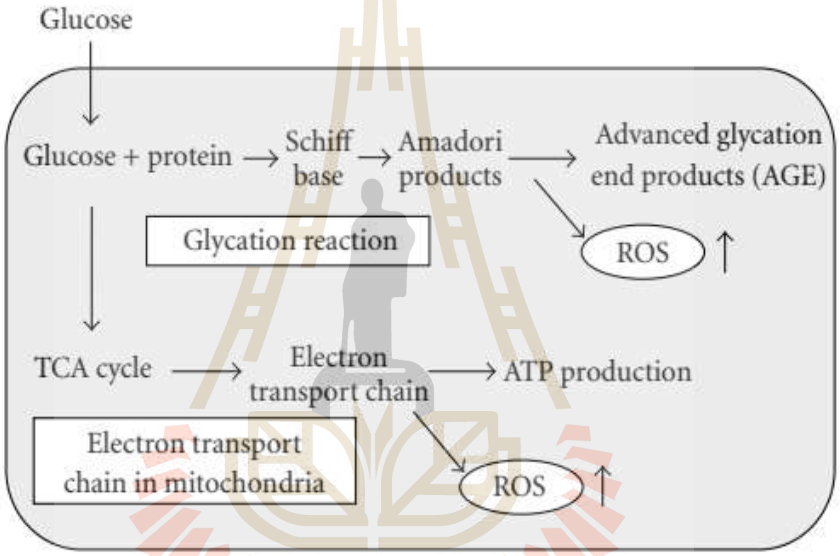


Figure 2.7 ROS generated under high glucose conditions (Kaneto et al., 2010).

ROS affects to pancreatic β-cells production of insulin and insulin sensitivity. As a result of low expression of antioxidant enzymes in the cell under long-time high glucose condition, the acceleration of mitochondria ROS suppresses the first phase of glucose–induced insulin secretion by interrupting glyceraldehyde 3-phosphate dehydrogenase activity (Sakai et al., 2003). Overproduction of oxidants from mitochondrial skeletal muscle increases the activation of p38 MAPK and other stress-

activated kinases. Those kinases associate with the inhibitory insulin-stimulated insulin signaling and diminish glucose transport activity (Henriksen, Diamond-Stanic, and Marchionne, 2011).

Free radicals control is an important approach in diabetes management. Many types of antioxidant agents such as vitamin E, C, β-carotene have been studied in diabetic treatment with interesting outcomes (Afkhami-Ardekani and Shojaoddiny-Ardekani, 2007; Bursell et al., 1999; Iino et al., 2005). As shown in Figure 2.8, Zucker Diabetic Fatty rats treated with the anti-oxidant n-acetylcysteine (NAC) prevented type 2 diabetic progression, such as abnormal changes in glycemia, compared to vehicle-treated rats. Unfortunately, no trials have been reported in humans to test this antioxidative effect on diabetic remedy (Robertson, 2006). However, antidiabetic medicinal plants mostly possess strong antioxidant activity which provide evidences to concede the role of antioxidants in diabetic treatment (Dey et al., 2015; Dias et al., 2012; Moodley, Joseph, Naidoo, Islam, and Mackraj, 2015).

Antioxidant activity of an agent can be measured *in vitro* by several methods through free radical producing compounds. DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, ABTS (2,2′-azino-bis (3-ethylbenzthiazoline -6-sulfonic acid) radical cation decolorization assay, FRAP (ferric reducing antioxidant power) assay or hydroxyl radical-scavenging assay are the most popular. Phenolics, flavonoids and anthocyanin have been identified as major compounds responsible for free radical scavenging activity of plant (Abdennacer et al., 2015; Alañón, García-Ruíz, Díaz-Maroto, Pérez-Coello, and Moreno-Arribas, 2015; Skotti, Anastasaki, Kanellou, Polissiou, and Tarantilis, 2014).

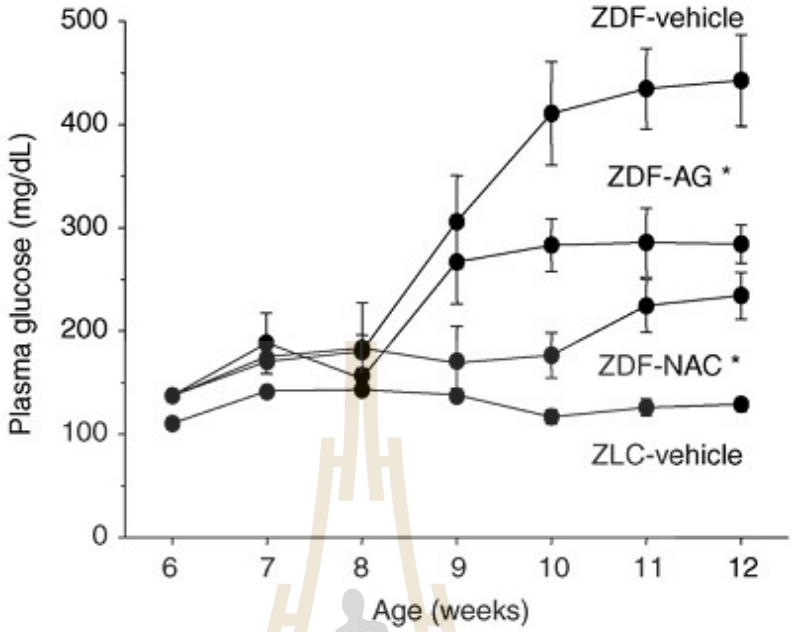


Figure 2.8 Protective effect of antioxidants (aminoguanidine (AG) and n-acetylcysteine (NAC) in Zucker Diabetic Fatty (ZDF) rats *versus* Zucker Lean Control (ZLC) rats (Robertson, 2006).

On the other hand, antioxidant activity of a compound can be studied in isolated cells and in animal models. The oxidant and antioxidant markers - ROS, lactate dehydrogenase, intracellular superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione have been determined in the studies (Dias et al., 2012; Fang et al., 2016; López-Alarcón and Denicola, 2013; Martin et al., 2010; Volobоueva, Liu, Suh, Ames, and Miller, 2005).

2.6 Chemical-induced diabetic model in rodents

Alloxan and streptozotocin (STZ) are two common diabetogenic agents for hyperglycemia induction in animals. The cytotoxic pathways of these chemicals are different but the actions on pancreatic β-cells are similar (Table 2.3). Both are glucose analogue, therefore, the chemicals can accumulate in β-cell via GLUT2 transporter quickly (Lenzen, 2008).

Table 2.3 The toxic effects of the glucose analogues alloxan and streptozotocin in β-cells (Lenzen, 2008).

Chemicals	**Alloxan**	**Streptozotocin**
β-cell selective action	Selective β-cell uptake via the GLUT2 glucose transporter	Selective β-cell uptake via the GLUT2 glucose transporter
Mechanism of β-cell death	β-cell toxicity through ROS	β-cell toxicity through alkylation
Mode of β-cell death	Nescrosis	Nescrosis
Beta cell death result	Insulin – dependent diabetes mellitus	Insulin – dependent diabetes mellitus
Chemical diabetes	Alloxan diabetes	Streptozotocin diabetes

A low dose of STZ (40 mg/kg) injected in five consecutive days caused slowly β-cells apoptotic and resulted in type 1 diabetes (O'Brien, Harmon, Cameron, and Allan, 1996). A mild dose of 100 mg/kg STZ developed the progression of type 2 diabetes

(Hayashi, Kojima, and Ito, 2006). STZ worked well in a combination with special diet to induce type 2 diabetic models which animal exhibited the characteristics of type 2 diabetes associated obesity (Reed et al., 2000).

Alloxan produces ROS in redox cycling with dialuric acid resulting in the necrosis of β-cell. It is a very hydrophilic unstable chemical which is required to be freshly and rapid presentation to β-cell. Alloxan selectively reacts with glucokinase, inhibits many important enzymes, reduces glucose oxidation, down regulates ATP generation and trigger insulin secretion (Lenzen, 2008). The dose of alloxan used to induce diabetic models are various (Gargouri, Magné, and El Feki, 2016; Issa and Hussen Bule, 2015; Saleh, El-Darra, and Raafat, 2017; J. Y. Yang, Kang, Nam, and Friedman, 2012).

2.7 *Chrysophyllum cainito* L.

2.7.1 Description

Chrysophyllum cainito L. belongs to the genus *Chrysophyllum* in Sapotaceae family. There are many local names of *C. cainito* which are different in each country (e.g. star apple, caimito, cainito, milk fruit, golden leaf tree, numnom (Thai), Vú sữa (literally: milky breast, Vietnamese). *C. cainito* is an erected tropical tree. The leaves of *C. cainito* are oval in alternative order, green, glossy upper and golden-brown beneath surfaces (Pham, 1999). Small flowers can be purplish white, greenish-yellow or yellow with 5 or 6 sepals. Fruits can be round or ellipsoid with pale-green, red-purple or dark-purple. Ripe fruits are soft and sweet-milky. Seed cells are in the central and form an asterisk shape (Morton, 1987). Figure 2.9 shows fruit, leaves and stem bark of the plant.

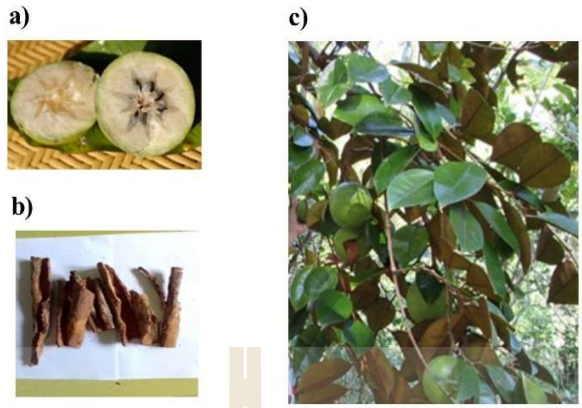


Figure 2.9 *Chrysophyllum cainito* parts: a) fruit, b) stem bark, and c) leaves.

2.7.2 Phytochemicals and biological activities

For pharmacological activity of plant extracts, identification of the active compounds is important. Therefore, the phytochemical study of medicinal plants is of interest among researchers. Phytochemical constituents of *C. cainito* have caught attention because of its potential for using as traditional medicines. The components of *C. cainito* ripe fruits, a part which is commonly used, have been examined by a group of researchers from Cuba. By GC-MS analysis, it was found that the extract contained one hundred and four volatile substances with six major compounds, including (E)-2-hexenal, 1-hexanol, limonene, linalool, α-copaene and hexadecanoic acid (Pino, Marbot, and Rosado, 2002). Fresh fruits were extracted with methanol and partitioned with hexane and ethyl acetate sequentially by a group of researchers from Nigeria. Nine known polyphenolic compounds have been identified. They were (+)-catechin, (+)-gallocatechin, (-)-epigallocatechin, quercetin, quercitrin, isoquercitrin, myricitrin, gallic acid and (-)-epicatechin in the highest concentration (Luo, Basile, and Kennelly, 2002). In addition, the other flavonoids including procatechuic acid, vanillic acid, ferulic acid,

rutin, myricetin, luteolin and kaempferol have been found in both green and ripened fruits of *C. cainito* (Kubola, Siriamornpun, and Meeso, 2011). Moreover, *C. cainito* leaves have also been found to contain ursolic acid, β-sitosterol, lupeol and gallic acid (Shailajan and Gurjar, 2014).

It has been shown that medicinal plants can be a potential source of natural antioxidants due to the phenolic compounds. Since several flavonoids have been found in *C. cainito*, it is not surprising that the extract of fruit peel shows strong anti-oxidative activity (Luo et al., 2002).

It is widely known that phenolic compounds, responsible for antioxidant activity of medicinal plants, exhibit a wide range of pharmacological activities. In the case of *C. cainito*, the extract of the pulp and seed extracts possess antimicrobial properties against various pathogens such as *Escherichia coli*, *Staphylococcus spp*., *Pseudomonas spp*., *Candida spp*. and *Aspergillus spp.* (Oranusi, Braide, and Umeze, 2015). It has also been demonstrated that the extract from the pulp of *C. cainito* has potential in the treatment of hypertension. The extract was shown to relax isolated tissue of aorta and reduce the rise of arterial blood pressure of salt-induced hypertensive rats, probably through an inhibition of angiotensin converting enzyme (Mao et al., 2015).

Not long ago, the crude extract, fractions and pure compounds (lup-20(29)-en-3β-O-hexanoate and 3β-lup-20(29)-en-3-yl acetate) obtained from *C. cainito* leaves possess anti-hypersensitive activity against inflammatory pain in mice, with unidentified mechanism (Meira et al., 2014). The ethanolic extract of *C. cainito* leaves has been established to possess wound healing activity, associated with the increase in hydroxyproline, hexosamine and protein level (Shailajan and Gurjar, 2016). In term of antidiabetic activity, *C. cainito* stem bark has been traditionally used to treat diabetic

patient by the group Dida people in Côte-d'Ivoire (Koffi, Konan Édouard et al., 2009) and the people in the South of Vietnam. However, scientific evidence is very limited. It was claimed that the aqueous extract of *C. cainito* leaves possessed the antihyperglycemic effect on alloxan-induced diabetic rabbits (Koffi, Ernest et al., 2009). The hydro-alcoholic extract from fruits showed the antidiabetic effect against alloxan and streptozoticin induced diabetic rats (Hegde, Arathi, and Mathew, 2016). It was also shown in the same study that the therapeutic dose for anti-postprandial hyperglycemic effect was 3 times lower than the lethal dose. Another safety data of this plant has been recently reported by skin irritation test and acute oral toxicity study (Shailajan and Gurjar, 2016). The dose up to 2 g/kg body weight did not produce any acute toxicity both in short-term and long-term observation. The extract did not either alter the primary irritation index compared to control in the skin test.

2.8 Toxicology of medicinal plants

2.8.1 The importance of safety in using medicinal plants

In pharmacovigilance system introduced by World Health Organisation (2004), safety is a fundermental principal in the elemental provision of medicinal herbs or health care products originated from herbs. Pharmacovigilance is the science and activities taken to detect, estimate, understand, and to limit the adverse effect of drugs and related-drug issues. Recently, the concern has involved the use of herbals, folk and complementary medicines, blood products, biologicals, medicine devices and vaccines. The use of herbals and herbal products has risen over decades. Although medicinal plants have been used since our ancestors and it is believed to be safe with promising good efficiency, many of them have remained unknown in the activity, adverse effect

potentials, cross interaction between herbals and drugs, herbals and food. Hence, safety of the use medicinal plants continues to be a major issue (Ekor, 2014).

In a study conducted by Bussmann et al. (2011), they found that 24% of the species out of 341 medicinal plant species used in Northern Peru showed the toxicity to brine-shrimp, whereas, ethanol extract of 76% of the species revealed the toxicity effect in the comparison with water extract. Medicinal plants widely used in the treatment of many ailments such as antidiabetic, anticancer, antibacterial, cytogenetic effects, etc. also contain latent toxicological impacts (Tamilselvan, Thirumalai, Shyamala, and David, 2014). Many food and plant medicines are potentially toxic, mutagenic and carcinogenic (Fennell et al., 2004).

2.8.2 *In vivo* assessment of medicinal plants toxicology by acute toxicity test

Toxicology is a branch of science that concern with the potential of deleterious effect of materials on organisms (Sass, 2000). To evaluate toxicological activity of the plant medicines, acute and sub-chronic toxicity tests are commonly established. These *in vivo* toxicity examinations were assessed by using mice and rats models (Mukinda and Eagles, 2010; Ping, Darah, Chen, Sreeramanan, and Sasidharan, 2013; Silva et al., 2011). Acute toxicity test is performed to determine the effect of a single exposure to the toxicant whilst subchronic or chronic aim to demonstrate the adverse effect of tested toxicant in a long-term exposure duration. Of two tests, acute lethal toxicity is to detect the adverse effect in a short-time exposure immediately after a single dose or multiple dose within 24 hours of administration. The targets of acute toxicity test include the defining of harzard of the test substance, determining the susceptible populations or species, diagnosing target organs or systems, and providing the important information

of an acute treatment to clinicians. Rats and mice are common choices for acute toxicity evaluation (Sass, 2000).

The Organization for Economic Cooperation and Development (OECD) has produced the guidline for acute toxicity test in rat or mice (OECD, 2002). The substance is orally administered to a group of experimental animals at the defined doses. The substance is tested using a stepwise procedure, in each step three animals of a single sex were used. The absence or presence of mortality of the animals caused by the compound dosed at one step will determine the next step. Data from acute toxicity can help to identify the mode of toxic action of a test substance. In addition, data can be used to contribute to the dose level setting for studies of repeated doses (P. K. Gupta, 2016).

2.8.3 Acute toxicity test in zebrafish embryonic model

Zebrafish (*Danio rerio*) has become an ideal model for toxicology research due to many advantages of this model such as small size, reducing husbandry cost, early morphology, and big data observed. Moreover, small zebrafish embryos allow the formation of high-throughput screens for toxicity testing, small-molecule screening, and for drug discovery. Besides, the transparent of embryos and high fecundity of fish are prominent. The transparent and rapid development of embryos meet the requirement for mutagenesis screening, establishing transgenic lines, and assessing chemicals for teratogenicity. The dark developing pigment can be minimized by treatment with 0.003% phenylthiourea or removed by bleaching after fixation. This allows the researcher to unobstructed observations of the morphological changes up to and beyond pharyngulation. Therefore, adverse effects of chemicals on development of the brain, notochord, heart, and jaw, trunk segmentation, and measurements of size can be

assessed quantitatively (Hill, Teraoka, Heideman, and Peterson, 2005; Yang et al., 2009).

People can identify the developing stage of embryos by visualizing under a stereomicroscope. The major developmental changings of embryo were recorded in the first 3 days after fertilaztion occurred. The stages of zebrafish embryonic development were clearly described by Kimmel and his colleagues (Kimmel, Ballard, Kimmel, Ullmann, and Schilling, 1995). In brieft, seven periods of embryogenesis were defined including zygote (0-¾ hour), cleavage (¾-2¼ hours), blastula (2¼ -5¼ hours), gastrula (5¼ -10 hours), segmentation (10-24 hours), pharyngula (24-48 hours), and hatching periods (48-72 hours). In each period, the development of embryo was devided in many different stages relying on the morphological features. Table 2.4 is a review of different periods of the early zebrafish embryonic progression.

The application of acute toxicity in zebrafish embryos in the safety evaluation of medicinal plant has been increasing lately (Kumar, Kar, Dolai, and Haldar, 2013; Majewski, Kasica, Jakubowski, and Lepczyńska, 2017; Santos et al., 2017; Syahbirin and Mohamad, 2017). Figure 2.10 presents the morphological changes caused by matrine and sophocarpine, two major matrine-type alkaloids included in the traditional Chinese medicine (Lu et al., 2014).

Table 2.4 The periods of early development of zebrafish embryo (Kimmel et al., 1995).

Periods	Hour	Description
Zygote	0-¾	The newly fertilized egg through the completion of the first zygotic cell cycle
Cleavage	¾-2¼	Cell cycles 2 through 7 occur rapidly and synchronously
Blastula	2¼-5¼	Rapid, metasynchronous cell cycles (8, 9) give way to lengthened, asynchronous ones at the midblastula transition; epiboly then begins
Gastrula	5¼-10	Morphogenetic movements of involution, convergence, and extension form the epiblast, hypoblast, and embryonic axis; through the end of epiboly
Segmentation	10-24	Somites, pharyngeal arch primordia, and neuromeres develop; primary organogenesis; earliest movements; the tail appears
Pharyngula	24-48	Phylotypic-stage embryo; body axis straightens from its early curvature about the yolk sac; circulation, pigmentation, and tins begin development
Hatching	48-72	Completion of rapid morphogenesis of primary organ systems; cartilage development in head and pectoral tin; hatching occurs asynchronously
Early larva	72	Swim bladder inflates; food-seeking and active avoidance behaviors

Figure 2.10 Microscope images of zebrafish embryos at 48, 72, 96 hours post fertilization (hpf). A1-A3: normal at 48, 72, 96 hpf; B1-B5: exposed to matrine at 48, 72, 96 hpf; C1-C5: exposed to sophocarpine at 48, 72, 96 hpf (T: tail malformation; S: scoliosis; N: notochord malformation; Y: yolk edema; Black asterisk marks the cardiac edema (Lu et al., 2014).

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and instruments

Major chemicals and instruments used in this study were listed in the Appendix A.

3.2 Plant collection and extraction

Chrysophyllum cainito L. stem barks were collected from Mo Cay Nam district, Ben Tre Province, Vietnam. Plant verification was conducted by Dr Santi Watthana, School of Biology. Suranaree University of Technology. Voucher specimens of leaf, fruit, flowers, and stem were stored at Suranaree University of Technology Botanical Garden under collected number H.DOAN-1.

Collected-stem barks were shade dried for a week, then chopped and ground by the fruit blender before extraction. Fifty grams of the ground stem barks were shaken with 200 ml of deionized water for 2 hours. The process was repeated 4 times. The combined supernatant was filtered by cotton gauze and centrifuged at 5000 rpm for 15 minutes to remove the remaining residue. The extract was concentrated by rotary evaporator and freeze-dried by lyophilizer for 2 days. The dried extract (CE) was kept at -20^{o}C for further use. Yield of the extract was recorded.

3.3 Phytochemical screening and total phenolic content

Phytochemical screening for tannin, phenols, alkaloids, flavonoids, saponin, steroids and glycosides were performed according to the methods described previously (Yadav and Agarwala, 2011). The contents of terpenoids was examined following the method described by Sharma and Paliwal (Sharma and Paliwal, 2013).

Test for phenols and tannin: A 0.1 g of CE was mixed with 2% of $FeCl_3$. The formation of dark-blue or black color indicates the presence of phenols and tannin.

Test for alkaloids: A 0.1 g of CE was mixed with 2 ml of 1% HCl and heated gently. Then, a few drops of Mayer's reagents were added to the mixture. The turbidity precipitate indicates the presence of alkaloids.

Test for flavonoids (Alkaline reagent test): A 0.1 g of CE was mixed with 2ml of 2% NaOH. The mixture color was intense yellow. The yellow color becomes colorless after adding a few drops of 2% HCl indicates the presence of flavonoids.

Test for saponin: Foam test was applied to screen the presence of saponin in the extract. A 0.1 g of CE was dissolved in 5 ml of deionized water. The solution was shaken vigorously and the stable foam formation indicates the presence of saponin.

Test for glycoside (Keller-kilani test): A 0.1 g of CE was mixed with 2 ml of glacial acetic acid containing 2 drops of 2% $FeCl_3$ solution. The mixture was transferred into another tube containing 2 ml of concentrated H_2SO_4. Brown ring formed at the interphase indicates the presence of cardiac glycosides.

Test for steroid: A 0.1 g of CE was mixed with 2 ml of chloroform and concentrated H_2SO_4 was added slowly. The red color in chloroform layer indicates the presence of steroids.

Test for terpenoids (Salkowski test): A 0.1 g of CE was mixed with 2 ml of chloroform and 3 ml of H_2SO_4. A reddish-brown color in the interface indicates the presence of terpenoids.

Total phenolic content: total phenolic content in CE was determined by Folin - Ciocalteu method described previously with some modification (Kumkrai, Weeranantanapan, and Chudapongse, 2015). One hundred microliter of CE were mixed with 2 ml of 2% Na_2CO_3. The mixture was left to stand for 2 minutes at room temperature before adding 100 µl of Folin-Ciocalteu's reagent (diluted in deionized water 1:1 v/v). The absorbance of the sample was measured at 750 nm after 30 minutes. Gallic acid was used to draw the standard curve. Total phenolic content in CE was expressed as mg of Gallic acid equivalents (GAE) per gram of dried extract.

3.4 Determination of antioxidant activity

3.4.1 DPPH radical scavenging assay

One milliliter of different concentrations of CE (0.78, 1.56, 3.125, 6.25, 12.5 and 25 µg/ml) was mixed with 2 ml of 0.1 mM DPPH in methanol. The reaction was left to stand for 1 hour at room temperature in the dark. The absorbance was measured at 515 nm (Abdennacer et al., 2015). Ascorbic acid (AA) and butylated hydroxytoluene (BHT) were used as positive controls. Percent inhibition was calculated based on the absorbance (A) value. The DPPH radical scavenging activity was expressed as IC50 (the concentration required for 50% of scavenging of free radical). % inhibition = $[(A_{control} - A_{sample}) / A_{control}] \times 100$.

3.4.2 ABTS radical scavenging assay

ABTS• cation radical was produced by the reacting of 14 mM ABTS and 4.9 mM potassium persulphate (1:1 (v/v) for 16 hours in the dark at room temperature. Prior to the assay, the ABTS• solution was diluted with ethanol (approximately 1:90 v/v) to give an absorbance of 0.70 ± 0.02 at 734 nm. The one hundred and fifty microliters of the extract at various concentrations (0.78, 1.56, 3.125, 6.25, 12.5 and 25 µg/ml) were added to 2850 µl of ABTS• solution in the tube and incubated for 6 minutes in the dark. The absorbance was determined at 734 nm (Kumkrai et al., 2015). AA and BHT were used as the positive controls. The results were expressed by IC50 value.

3.4.3 Ferric reducing antioxidant power assay

FRAP activity of CE was measured according to the previous report (Sudan, Bhagat, Gupta, Singh, and Koul, 2014). In brief, working reagent including 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM HCl and 20 mM $FeCl_3$ at the ratio 10:1:1 (v/v/v) was prepared. Five-hundreds microliters of the extract was mixed with 3 ml of the working FRAP reagent and mixed. After 30 minutes of incubation, the absorbance was measured at 593 nm. The standard calibration curve was drawn using different concentrations of $FeSO_4.7H_2O$ solutions (0 - 250 µM). The relative activity was expressed as µM $FeSO_4.7H_2O$/g dried extract.

3.5 Animal husbandry

Male and female rats were obtained from Laboratory Animal Facility, Suranaree University of Technology for acute toxicity test. Male Jcl:ICR mice of 6-week old (28-34 g) were used in antidiabetic study. The animals were obtained from Nomura Siam International Co., Ltd., Bangkok, Thailand. The animals were housed in

stainless steel cages lined with wood shavings at Laboratory Animal Facility, Suranaree University of Technology, under standard condition of 25 ± 2ºC, 45% - 50% relative humidity and 12-hour light/dark cycle. Normal food and water were given *ad libitum*. The experiments were performed after 7 days of acclimatization. The extract was dissolved in distill water and was administered to animal using oral gavage method. All animals were sacrificed by CO_2 inhalation at the end of experiment and for tissue collection. The sample sizes were calculated based on the "resource equation" method (Charan and Kantharia, 2013). All procedures were approved and conducted following the guidelines of the Institutional Animal Care and Use Committee, Suranaree University of Technology, Thailand (1/2559).

3.6 Acute toxicity

Healthy male (120-150 g) and female Wistar rats (100-140 g) were used in the acute toxicity examination based on the guideline number 423 of the Organisation for Economic Co-operation and Development (OECD) (OECD, 2002). Overnight fasted rats were divided into 5 groups (n=3 of each sex): group 1 were received given deionized water as negative control, group 2-5 were received (po) a single dose of CE at 500, 1000, 2000 and 4000 mg per kg of rat body weight.

The behavior of rats was monitored during the first 0.25, 0.5, 1, 4 and 24 hours and the observation was continued daily for 14 days. Rat body weights were noticed at day 0, 7th and day 14th. At the end of the experiment, rats were fasted overnight and anesthetized by carbon dioxide inhalation. Then, rats in all groups were sacrificed. Blood samples were collected for haematological analysis (by Mindray BC-6800 Auto Hematology Analyzer) and for biochemical analysis of liver and kidney functional

markers (alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), blood urea nitrogen, total protein and albumin; by Mindray BS-800 Automatic Chemistry Analyzer). Rats organs were isolated and were checked weight.

3.7 Antidiabetic studies

3.7.1 Oral glucose tolerance test

The effect of CE on blood glucose level was first evaluated via oral glucose challenge. Six-hour fasted normal mice were randomly divided into 4 different groups (n = 6) as the following.

Group 1: normal mice + deionized water

Group 2: normal mice + 250 mg/kg CE

Group 3: normal mice + 500 mg/kg CE

Group 4: normal mice + 10 mg/kg glibenclamide (Daonil®, Jakarta, Indonesia)

All mice were pretreated with drugs prior to the oral glucose administration at the dose 2 g/kg (2 hours for CE and deionized water; 30 minutes for glibenclamide based on the onset of action from our preliminary study). Blood glucose level was monitored at 0, 30, 60 and 120 minutes from small incision of tail tip using Medisafe® EX glucose meter. The area under the curve was calculated using the same formula in the previous report (Ibrahim and Islam, 2014).

3.7.2 Acute hypoglycemic activity in alloxan-induced diabetic mice

Overnight fasted mice were received an intraperitoneal injection of 130 mg/kg of alloxan monohydrate dissolved in cold 0.85% saline solution to induce diabetes.

Diabetic induction was checked after 3 days of alloxan injection. Mice showed glucose level greater than 200 mg/dl was considered as type 2 diabetic mice and used for the experiment. Mice were randomly divided into 5 groups as follow.

Group 1: normal mice + deionized water (n = 6)

Group 2: diabetic mice + deionized water (n = 6)

Group 3: diabetic mice + 250 mg/kg CE (n = 6)

Group 4: diabetic mice + 500 mg/kg CE (n = 6)

Group 5: diabetic mice + 10 mg/kg glibenclamide (n = 5)

After the single dose of drug administration, blood glucose levels were determined at 0, 1, 2, 4 and 6 hours to evaluate acute hypoglycemic effect of the extract as described in the previous study (Parra-Naranjo et al., 2017).

3.7.3 Antidiabetic effect of CE

After an acute test, mice were received treatments for further 21 days. Mice received the treatment once per day. Fasting blood glucose level was checked every week. At the end of the experiment, mice were sacrificed by anesthetizing using carbon dioxide inhalation. Blood samples were collected for biochemical evaluation of total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol (auto chemistry analyzer Mindray BS-200) ALT, AST, ALP, blood urea nitrogen, albumin and creatinine (semi-auto chemistry analyzer Mindray BA-88A). The pancreas was isolated and fixed with 10% neutral buffered formaldehyde for histological examination. The tissue was sectioned at 5 μm thickness and mounted on the slides. The tissues were stained with hematoxylin and eosin (HandE).

3.8 Effect of CE on glucose absorption in mouse everted gut sacs

The inhibitory effect of CE on glucose absorption was investigated using isolated mouse jejunum (Widyawati, Yusoff, Asmawi, and Ahmad, 2015). The jejunum was isolated from normal mouse and placed in oxygenated Kreb-Henseleit solution (composition in g/l; NaCl 6.92, KCl 0.35, $MgSO_4.7H_2O$ 0.29, $CaCl_2$ 0.28, KH_2PO_4 0.16, $NaHCO_3$ 2.1, and D-glucose 1.4), pH 7.4. The jejunum was cut into 6 cm long segments, tied edges, everted and filled with Kreb-Henseleit solution. The sacs were incubated in 10 ml of Kreb-Henseleit solution containing each of the following substances CE (25 and 50 µg/ml) or acarbose (1 mg/ml) for 1 hour in the presence of carbogen at 37°C. Glucose concentrations inside the sacs were determined using peroxidase-glucose oxidase (PGO) enzyme. Ten µl of diluted buffer was interacted with 190 µl of PGO enzyme solution in a microtitter plate. The reaction mixture was incubated at 37°C in the dark for 30 minutes. The intensity of the brown color was measured at 450 nm using spectrophotometer. The concentration of glucose was calculated using standard curve of D-glucose. The amount of glucose absorption was calculated using the following formula.

Amount of glucose absorbed = (amount of glucose after – amount of glucose before)/g of jejunum.

3.9 Effect of CE on glucose uptake in mouse abdominal muscle

Glucose uptake by mouse abdominal muscle was measured as previously described (Hassan, Yam, Ahmad, and Yusof, 2010). Briefly, after animals were sacrificed, abdominal muscles were removed and soaked in the Kreb's-Ringer

bicarbonate (KRB) buffer, pH 7.4 with continuously supply of carbogen for 10 minutes. The muscle was then incubated with KRB buffer containing 200 mg/dl of D-glucose, CE 25 or 50 µg/ml with or without insulin (100 mU/ml) for 30 minutes. Then, buffer was collected and analyzed for the remaining glucose using PGO enzyme. The amount of glucose uptake was calculated by the formula below:

Amount of glucose uptake = (amount of glucose before – amount glucose after)/g of muscle.

3.10 α-Glucosidase inhibitory activity

The α-glucosidase inhibitory activity was measured as described previously (Kumkrai et al., 2015). Briefly, a mixture of 10 µl of 0.25 U/ml α-glucosidase (Sigma–Aldrich, USA), 50 µl of 0.1 M potassium phosphate buffer (pH 6.8) and 20 µl of various concentrations of the extract or the α-glucosidase inhibitor acarbose (Fluka, USA) was incubated at 37°C for 10 minutes. Then, 10 µl of 5 mM p-nitrophenyl-α-D-glucopyranoside (PNPG) was added and further incubated for 30 minutes. To terminate the reaction, 50 µl of 0.1 M Na_2CO_3 was added. The absorbance was measured at 405 nm optically by using a spectrophotometer. Results were expressed as the concentration where the activity of α-glucosidase is inhibited by 50% (IC_{50}).

3.11 Cell culture

Human hepatocarcinoma (HepG2) cells were obtained from ATCC (VA, USA). Cells were grown in a humidified atmosphere containing 5% CO_2 at 37°C in DMEM supplemented with 10% fetal bovin serum and 1% penicillin-streptomycin, and 1%

HEPES. These adherent cells were sub-cultured at 80-90% confluence. Cells were detached from culture flask surface by using 0.25% trypsin-EDTA and were colleted by centrifugating at 1200 rpm in 3 minutes.

3.12 Toxicological study in HepG2 cells

3.12.1 MTT assay

MTT is a simple assay to determine cytotoxic effects of an agent, cell proliferation or activation (Mosmann, 1983). In this assay, HepG2 cells ($4x10^4$ cells/well) were plated in 96-well plate and allowed to adhere overnight. Then, cells were exposed to different concentration of CE (0 – 800 µg/ml) for 24 hours. At the end of the treatment, the media were aspirated and cells were washed with PBS. Then, 100 µl of 0.5 mg/ml MTT were added to each well and incubated for further 4 hours. The remaining of MTT was removed. The purple formazan product was dissolved in 100 µl of DMSO for 10 minutes and analyzed spectrophotometrically at 570 nm. The percentage of cell viability was calculated against control.

3.12.2 Trypan blue exclusive assay

Trypan blue exclusive assay was used to determine the number of viable cells (Strober, 2001). The assay was performed according to the procedure described previously (Chudapongse, Kamkhunthod, and Poompachee, 2010) with some modifications. The HepG2 cells were treated similarly to the treatments in MTT assay. Treated cells were collected by incubating with 0.25% trypsin-EDTA and centrifuging at 1,500 rpm for 3 minutes. The supernatants were removed and the cell pellets were resuspended in DMEM. Then cells were stained with 0.25% Trypan blue. The number

of live cell and dead cell were counted by Automated Cell Counter (Bio-Rad). The percentages of viable cells in treated groups were calculated against control.

3.13 Cytoprotective activity on HepG2 cells

3.13.1 Toxicity of tert-butyl hydroperoxide

This experiment was be taken according to the former description with minor modifications (Kumkrai, Kamonwannasit, and Chudapongse, 2014). Tert-butyl hydroperoxide (TBHP) is a direct-acting oxidative stress agent used in various oxidation processes. HepG2 cells ($4x10^4$ cells/well) were exposed to TBHP at different concentrations (0, 62.5, 125, 250, 500 and 1000 µM) for 3 hours after seeding in the 96-well plate 24 hours in DMEM. Cells viability were determined by MTT assay as described earlier.

3.13.2 Cytoprotective activity on HepG2 cells against TBHP

Pre-treat examination: After 16-18 h incubation, HepG2 cells were treated with various concentrations of CE (0, 2.5, 5, 10 and 20 µg/ml). The extracts were removed after 24 h incubation and washed. The cells were exposed to 250 µM TBHP (caused approximately 50% cells lost) for 3 h. After incubation time, Cells were washed with PBS twice and the percentage of viable cell was determined using MTT assay.

Post-treat examination: cells were exposed to TBHP for 3 h. After removing TBHP, cells were washed with PBS twice. Cells were continued incubating with medium alone or with CE (2.5, 5, 10 and 20 µg/ml) for 24 h. Then, the percent of viable cell was determined using MTT assay.

3.14 Apoptosis analysis

In this study, the apoptosis inducing activity of CE on HepG2 was analyzed using Tali™ apoptosis kit. HepG2 cells at $5x10^5$ cells/ml/well in 12-well plate with the presence of difference concentrations of CE at 0 (control), 50, 70, and 100 µg/ml were incubated for 24 hours. Then, cells were harvested using 0.25% trypsin-EDTA. Cells were washed with PBS once before staining with specific apoptotic dye. Tali™ apoptosis kit contains a green-fluorescent annexin V–Alexa Fluor® 488 conjugate to identify apoptotic cells and red-fluorescent propidium iodide (PI) to determine dead/necrotic cells. Stained cells were loaded in the slide and inserted into Tali® Image-Based Cytometer to measure the percent of apoptotic cells.

3.15 Cell cycle analysis

Cell cycle distribution of HepG2 cells were analyzed using Tali™ Cell cycle kit according to company instruction. Prior to experient, cells were synchronized by culture 24 in FBS-free DMEM (Elias et al., 2016). Then, HepG2 cells at $5x10^5$ cells/ml/well in 12-well plate with the presence of difference concentrations of CE at 0, 50, 70, and 100 µg/ml were incubated for 24 hours. Then, cells were harvested using 0.25% trypsin-EDTA and washed with PBS. Cells were fixed with ice-cold 70% ethanol at -20°C overnight. At this step, the cells can be left for weeks before staining with propidium iodide. Cells cycle distributions of different treatments were analyzed by Tali® Image-Based Cytometer.

3.16 Toxicological effect on zebrafish embryo

Adult zebrafish (*Danio rerio*) were raised in RO water at 26-28°C and 12h light/dark cycle. Fish were fed by commercial fish diet twice a day and artemia once a day alternatively. Male and female fish were raised in separated tanks. To stimulate spawning and fertilization, male and female fish were put in the same tank before the dark cycle. Eggs were collected in the next light cycle. Healthy fertilized eggs were chosen under stereomicroscope and maintained in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$).

Acute toxicity of the extract on zebrafish embryos was performed according to OECD guideline 236 (OECD, 2013) and (Hermsen, van den Brandhof, van der Ven, and Piersma, 2011). Fertilized eggs were selected and delivered into 24 well-plate. Each well contains 1 egg, 20 eggs per treatment. Each concentration was carried out in triplication. Four hours past fertilized (hpf) eggs were exposed to different concentrations of CE (0, 15.63, 31.25, 62.5, 125, 250, 500 and 1000 µg/ml) for 72 hours. The development of embryos including hatching rate, death, and abnormally embryonic development were noted at 24, 48, and 72 hours post treatment application (hpta).

3.17 Statistically analysis

Each experiment was repeated at least 3 times and the result values were expressed as mean ± SEM. The comparisons between means were done using One way- or Two way-ANOVA followed by Student-Newman-Keuls (SNK). A value of $P<0.05$ was considered as statistically significant differences.

CHAPTER IV

RESULTS

4.1 Phytochemical screening and total phenolic content

The results of extract yield and phytochemicals screening were presented in Table 4.1. In this study, extract yield of *C. cainito* stem bark prepared by maceration method using water was 11.22 ± 0.54%. The phytochemical screening revealed the presence of phenols, tannin, glycosides, terpenoids, and saponin but the absence of flavonoids, alkaloids, and steroids. Total phenolic compounds found in the extract was 871.75 ± 10.41 mg GAE/g extract (Table 4.2).

Table 4.1 Phytochemical screening and yield of CE extraction.

Test for	**Results**	**Test for**	**Results**
Phytochemistry			
Phenols	+	Flavonoids	-
Tannins	+	Steroids	-
Glycosides	+	Alkaloids	-
Terpenoids	+	**Yield (%)**	11.22 ± 0.54*
Saponin	+		

+ present; - absent; * Value is expressed as mean ± SEM (n=3).

4.2 Antioxidant activity of CE

To access the antioxidant activity of the extract, DPPH, ABTS free radical scavenging and FRAP assays were performed. The concentrations of CE were varied from 0-25 µg/ml. The extract showed the maximum radical scavenging activity in the highest experimental concentration by 92% in DPPH assay and 99% in ABTS assay (Figure 4.1). The IC_{50} values found for CE, ascorbic acid, and BHT from DPPH and ABTS assays were presented in Table 4.2. The reducing potential of CE was determined using $FeSO_4$ standard curve. The FRAP value of CE was 291.56 ± 3.25 mM Fe^{2+} equivalent per gram of dried extract (Table 4.2).

Table 4.2 Total phenolic content and antioxidant activities of CE.

Samples	**DPPH IC_{50} (µg/ml)**	**ABTS IC_{50} (µg/ml)**	**FRAP (mM Fe^{2+}/g extract)**	**Phenolic content (mg GAE/g CE)**
CE	4.66 ± 0.14*	2.10 ± 0.06#	291.56 ± 3.25	871.75 ± 10.41
AA	3.49 ± 0.12	1.86 ± 0.03	-	-
BHT	4.68 ± 0.03	5.07 ± 0.19	-	-

The values are expressed as mean ± SEM, n = 3. CE, AA and BHT were abbreviations of *C. cainito* extract, ascorbic acid and butylated hydroxytoluene, respectively. *P<0.05 compared with AA; #P<0.05 compared with BHT by one-way ANOVA followed by SNK.

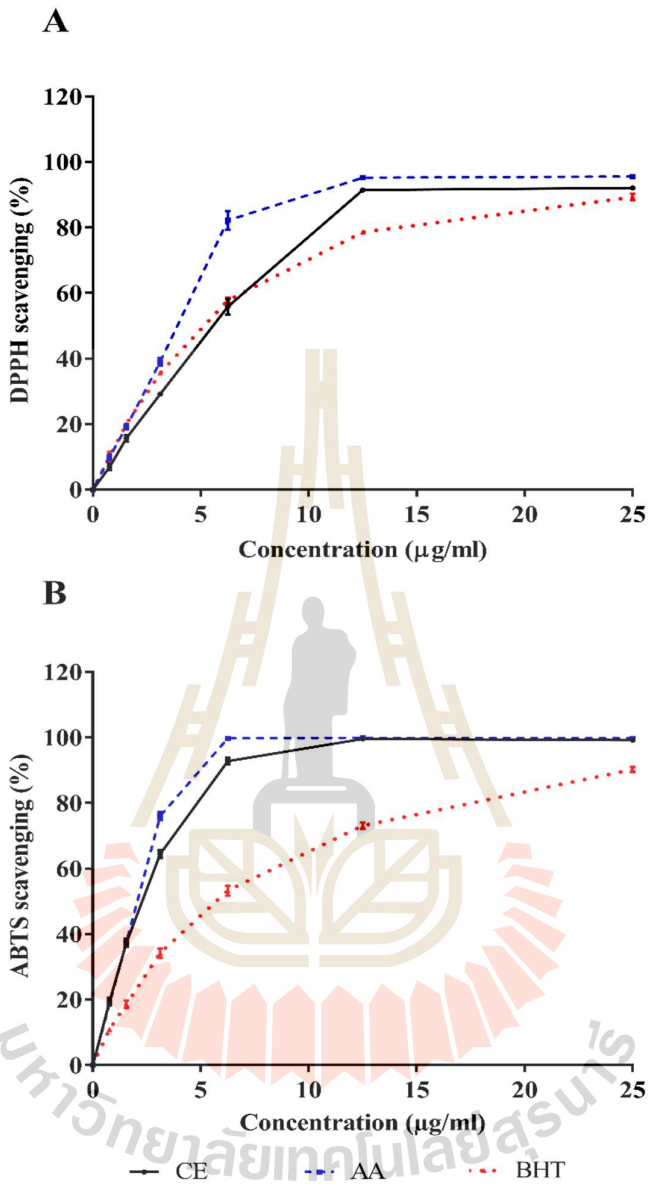


Figure 4.1 Antioxidant activity of *C. cainito* extract. (A) and (B) are the results from DPPH and ABTS) radical scavenging methods, respectively. The values are expressed as mean ± SEM, n = 3. AA: ascorbic acid; BHT: butylated hydroxytoluene; CE*: C. cainito* extract.

4.3 Acute toxicity of CE in rats

A single dose of CE (500 mg/kg to 4000 mg/kg) caused no adverse effect on Wistar rat behavior up to 14 days after administration. No mortality was seen in all treated groups. It was found that weight gain, internal organs relative weights as well as serum biochemical parameters and hematological parameters of rats treated with CE showed no significant difference from control (Table 4.3-4.7).

4.4 Antidiabetic activity of CE

4.4.1 Oral glucose tolerance test

The results of the oral glucose tolerance test in normal mice are shown in Figure 4.2. As seen in Figure 4.2A, the initial blood glucose levels of all groups prior to drug administration were no difference. The blood glucose levels after glucose loading reached a peak at 30 minutes and decreased subsequently over time, in all groups. It was found that CE (250 and 500 mg/kg) or glibenclamide had significantly improved glucose tolerance in normal mice. Mice received CE (500 mg/kg) and glibenclamide (10 mg/kg) noticeably suppressed the elevation of glucose after 30 minutes of glucose load compared to control group ($P<0.05$). In addition, the area under curve (AUC) were significant reduced in all treated groups when compared to control mice (Figure 4.2B).

Table 4.3 Acute toxicity of CE on male rat: body weight, mortality and symptoms of toxicity.

Treatment	Body weight (g)			Mortality	Symptoms of toxicity
	Day 1	Day 7	Day 14		
Control	183.33 ± 6.67	233.33 ± 12.02	263.33 ± 6.67	None	None
CE 500 mg/kg	176.67 ± 3.33	226.67 ± 6.67	263.33 ± 6.67	None	None
CE 1000 mg/kg	160.00 ± 11.55	203.33 ± 17.63	243.33 ± 16.67	None	None
CE 2000 mg/kg	176.67 ± 8.82	220.00 ± 10.00	263.33 ± 12.02	None	None
CE 4000 mg/kg	166.67 ± 3.33	213.33 ± 6.67	253.33 ± 3.33	None	None

Data are expressed as mean ± SEM (n=3). There were no significant differences in rat body weight between groups (P>0.05). None: no mortality as well as no symptoms of toxicity observed during the test.

Table 4.4 Acute toxicity of CE on female rat: body weight, mortality and symptoms of toxicity.

Treatment	Body weight (g)			Mortality	Symptoms of toxicity
	Day 1	Day 7	Day 14		
Control	156.67 ± 3.33	183.33 ± 8.82	200.00 ± 5.77	None	None
CE 500 mg/kg	146.67 ± 8.82	170.00 ± 11.55	186.67 ± 13.33	None	None
CE 1000 mg/kg	150.00 ± 0.00	176.76 ± 3.33	190.00 ± 5.77	None	None
CE 2000 mg/kg	153.33 ± 3.33	176.67 ± 6.67	193.33 ± 3.33	None	None
CE 4000 mg/kg	143.33 ± 8.82	163.33 ± 8.82	180.00 ± 5.77	None	None

Data are expressed as mean ± SEM (n=3). There were no significant differences in rat body weight between groups (P>0.05). None: no mortality as well as no symptoms of toxicity observed during the test.

Table 4.5 Rat serum biochemical parameters in the acute toxicity test of CE.

Parameter	Treatment (mg/ kg BW)				
	Control	**500**	**1000**	**2000**	**4000**
Male					
AST (U/L)	204.67 ± 33.82	364.67 ± 59.74	224.67 ± 36.86	252.00 ± 63.79	241.33 ± 30.07
ALT (U/L)	39.33 ± 2.33	43.33 ± 8.35	40.00 ± 3.46	41.00 ± 5.57	45.33 ± 6.77
ALP (U/L)	107.67 ± 10.40	94.33 ± 5.36	125.00 ± 18.61	99.67 ± 14.15	77.33 ± 15.06
BUN (mg/dl)	20.80 ± 2.15	23.77 ± 1.19	21.43 ± 2.54	23.90 ± 3.46	22.43 ± 2.87
Total Protein (g/dl)	6.27 ± 0.21	6.00 ± 0.20	6.30 ± 0.00	6.07 ± 0.12	6.03 ± 0.31
Albumin (g/dl)	3.73 ± 0.09	3.57 ± 0.03	3.77 ± 0.03	3.90 ± 0.17	3.93 ± 0.12
Female					
AST (U/L)	274.67 ± 14.08	316.33 ± 21.18	251.00 ± 40.28	392.33 ± 58.67	268.00 ± 23.46
ALT (U/L)	48.67 ± 6.89	37.33 ± 2.85	43.33 ± 5.04	53.00 ± 8.08	39.33 ± 0.88
ALP (U/L)	52.67 ± 6.98	48.00 ± 8.89	79.00 ± 8.54	57.33 ± 10.71	74.33 ± 12.72
BUN (mg/dl)	26.20 ± 0.20	28.07 ± 1.62	24.33 ± 3.64	32.57 ± 2.55	23.87 ± 1.74
Total Protein	6.47 ± 0.09	6.00 ± 0.20	6.27 ± 0.19	5.73 ± 0.07	6.13 ± 0.07
Albumin	3.97 ± 0.03	3.77 ± 0.13	3.83 ± 0.07	3.67 ± 0.12	3.67 ± 0.03

Value are expressed as mean ± SEM (n=3). There are no significant differences in any parameters between groups (P>0.05).

Table 4.6 Rat hematological parameters in acute toxicity test of CE.

Parameters	**Treatments (mg/kg)**				
	Control	**CE 500**	**CE 1000**	**CE 2000**	**CE 4000**
Male rats					
RBC ($\times 10^6$ /µl)	7.80 ±0.25	7.76 ± 0.27	8.91 ± 0.43	8.09 ± 0.06	8.03 ± 0.14
HGB (g/dl)	14.87 ± 0.66	13.93 ± 1.48	17.33 ± 0.83	15.85 ± 0.12	16.23 ± 0.38
HCT (%)	52.00 ± 0.58	52.67 ± 1.45	61.67 ± 3.18	55.50 ± 0.58	56.67 ± 1.45
WBC ($\times 10^3$/µl)	4.82 ± 2.29	9.97 ± 2.25	9.69 ± 2.38	8.53 ± 0.50	9.92 ± 2.57
LYM ($\times 10^3$/µl)	84.73 ± 0.64	81.23 ± 3.01	86.30 ± 1.44	86.90 ± 2.16	84.63 ± 1.42
MON ($\times 10^3$/µl)	2.33 ± 0.17	2.77 ± 0.38	1.93 ± 0.19	2.33± 0.20	2.77 ± 0.13
NEU (%)	11.53 ± 0.60	12.10 ± 1.31	10.40 ± 0.95	8.97 ± 1.48	10.87 ± 1.52
PLT ($\times 10^4$/µl)	49.10 ± 13.92	77.50 ± 14.07	79.70 ± 11.11	86.33 ± 4.88	80.03 ± 4.18
MCV (fl)	67.00 ± 1.16	67.67 ± 0.67	69.00 ± 0.58	68.33 ± 0.33	70.33 ± 0.67
MCH (pg)	19.07 ± 0.35	17.87 ± 1.49	19.47 ± 0.12	19.43 ± 0.19	20.20 ± 0.15
MCHC (g/dl)	28.60 ± 1.00	26.50 ± 2.4	28.23 ± 0.34	28.53 ± 0.45	28.80 ± 0.12
RDW-CV (%)	14.33 ± 0.33	14.57 ± 0.29	15.00 ± 0.38	14.63 ± 0.48	14.73 ± 0.27

Data are expressed as mean ± SEM (n=3). There were no significant differences in any parameters between groups (P>0.05).

Abbreviations: RBC: red blood cell, HGB: hemoglobin concentration, HCT: hematocrit, WBC: white blood cell, LYM: lymphocyte, MON: monocyte, NEU: neutrophil, PLT: platelet, MCV: mean cell volume, MCH: mean cell hemoglobin, MCHC: mean cell hemoglobin concentration, RDW-CV: red blood cell distribution width coefficient of variation.

Table 4.6 Rat hematological parameters in acute toxicity test of CE (Continued).

Parameter	Treatment (mg/ kg)				
	Normal control	500	1000	2000	4000
Female rats					
RBC ($\times 10^6$ /µl)	8.61 ± 0.11	9.01 ± 0.50	8.30 ± 0.27	8.40 ± 0.44	8.87 ± 0.13
HGB (g/dl)	17.00 ± 0.06	19.20 ± 0.98	16.83 ± 0.55	15.77 ± 1.47	17.43 ± 0.41
HCT (%)	57.33 ± 0.88	58.67 ± 4.18	54.67 ± 2.03	53.33 ± 3.33	57.00 ± 1.53
WBC ($\times 10^3$/µl)	6.52 ± 1.92	7.45 ± 1.27	5.03 ± 1.67	4.08 ± 0.80	5.29 ± 1.63
LYM ($\times 10^3$/µl)	76.36 ± 5.66	88.50 ± 1.85	85.80 ± 0.57	80.37 ± 0.90	83.67 ± 1.53
MON ($\times 10^3$/µl)	3.80 ± 0.76	1.83 ± 0.48	4.10 ± 0.53	3.77 ± 0.92	2.93 ± 0.69
NEU (%)	13.80 ± 3.10	6.93 ± 1.56	7.80 ± 0.57	11.13 ± 0.90	12.13 ± 0.96
PLT ($\times 10^4$/µl)	103.73 ± 2.89	57.40 ± 34.22	52.33 ± 27.23	43.80 ± 15.55	84.73 ± 9.48
MCV (fl)	66.33 ± 0.33	65.00 ± 1.00	66.00 ± 0.58	63.67 ± 0.88	64.00 ± 1.00
MCH (pg)	19.70 ± 0.23	19.83 ± 1.36	20.30 ± 0.20	18.67 ± 1.13	19.67 ± 0.18
MCHC (g/dl)	29.77 ± 0.46	30.67 ± 2.59	30.63 ± 0.17	29.33 ± 1.45	30.57 ± 0.28
RDW-CV (%)	14.03 ± 0.23	14.13 ± 0.54	12.93 ± 0.07	13.27 ± 0.33	13.33 ± 0.13

Data are expressed as mean ± SEM (n=3). There were no significant differences in any parameters between groups (P>0.05).

Abbreviations: RBC: red blood cell, HGB: hemoglobin concentration, HCT: hematocrit, WBC: white blood cell, LYM: lymphocyte, MON: monocyte, NEU: neutrophil, PLT: platelet, MCV: mean cell volume, MCH: mean cell hemoglobin, MCHC: mean cell hemoglobin concentration, RDW-CV: red blood cell distribution width coefficient of variation.

Table 4.7 Rat relative organs weights in acute toxicity test of CE.

Organs	Treatment (mg/kg)				
	Control	**500**	**1000**	**2000**	**4000**
Male					
Liver	3.86 ± 0.12	3.91 ± 0.04	3.45 ± 0.16	3.75 ± 0.21	3.55 ± 0.09
Heart	0.43 ± 0.03	0.39 ± 0.02	0.43 ± 0.04	0.42 ± 0.02	0.44 ± 0.02
Kidneys	0.77 ± 0.01	0.81 ± 0.02	0.75 ± 0.02	0.79 ± 0.01	0.74 ± 0.02
Lungs	0.57 ± 0.03	0.58 ± 0.09	0.60 ± 0.05	0.54 ± 0.00	0.66 ± 0.07
Spleen	0.26 ± 0.01	0.30 ± 0.03	0.29 ± 0.01	0.26 ± 0.01	0.29 ± 0.03
Testis	1.23 ± 0.01	1.25 ± 0.03	1.26 ± 0.04	1.27 ± 0.04	1.27 ± 0.06
Female					
Liver	3.73 ± 0.35	3.68 ± 0.04	3.42 ± 0.10	3.52 ± 0.11	3.64 ± 0.05
Heart	0.49 ± 0.04	0.41± 0.01	0.46 ± 0.02	0.45 ± 0.01	0.47 ± 0.02
Kidneys	0.79 ± 0.05	0.75 ± 0.01	0.77 ± 0.02	0.69 ± 0.05	0.73 ± 0.04
Lungs	0.67 ± 0.09	0.73 ± 0.09	0.81 ± 0.09	0.79 ± 0.12	0.74 ± 0.05
Spleen	0.34 ± 0.04	0.31 ± 0.05	0.26 ± 0.00	0.29 ± 0.03	0.27 ± 0.02
Ovaries	0.08 ± 0.01	0.06 ± 0.01	0.08 ± 0.00	0.08 ± 0.01	0.09 ± 0.00

Value are expressed as mean ± SEM (n=3). There were no significant differences in weights of any organs betweet groups (P>0.05).

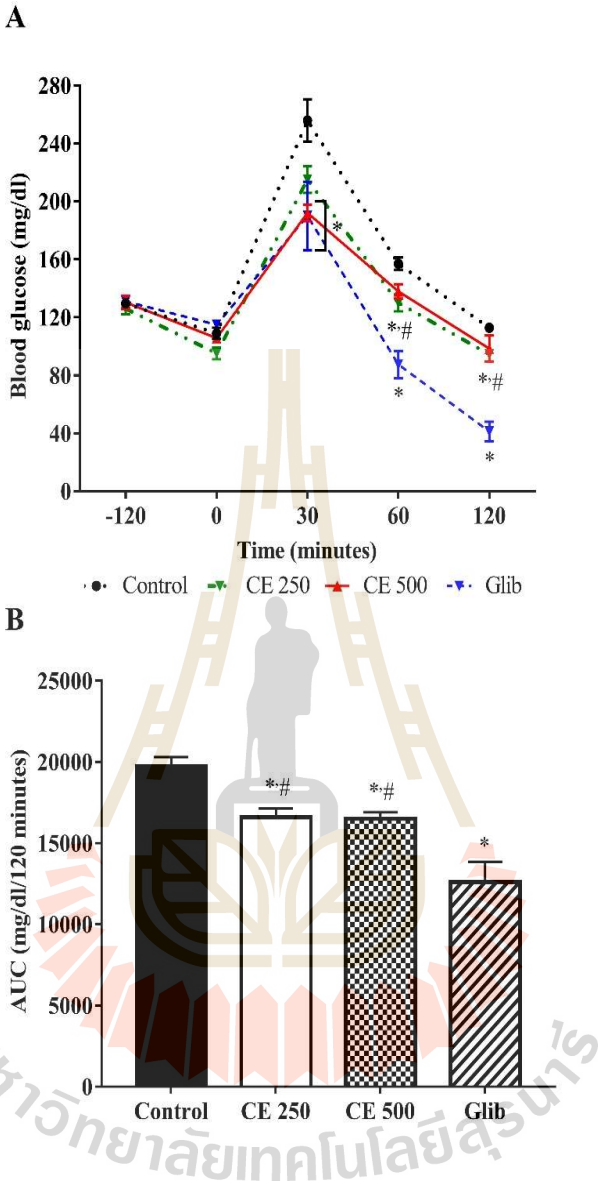


Figure 4.2 Effect of CE on OGTT in normal mice. (A) is blood glucose level during oral glucose challenge whereas (B) is the area under the curve (AUC) of the blood glucose level over time. $^{*}P<0.05$ compared with control mice; $^{\#}P<0.05$ compared with glibenclamide treated mice by one-way ANOVA followed by SNK. The values are expressed as mean ± SEM with n = 6.

4.4.2 Acute hypoglycemic activity of CE on alloxan-induced diabetic mice

Figure 4.3 shows kinetics of blood glucose observed during the period of experiment. In this study, alloxan injection destroyed pancreatic β cells and reduced insulin secretion leading to an elevated blood glucose level compared to normal control mice. The extract and glibenclamide started to suppress the rise of blood glucose in diabetic mice after 2 hours of treatment, but not statistically significant. However, after 4 hours and longer blood glucose levels of the CE and glibenclamide groups declined significantly more than the diabetic control group (P<0.05).

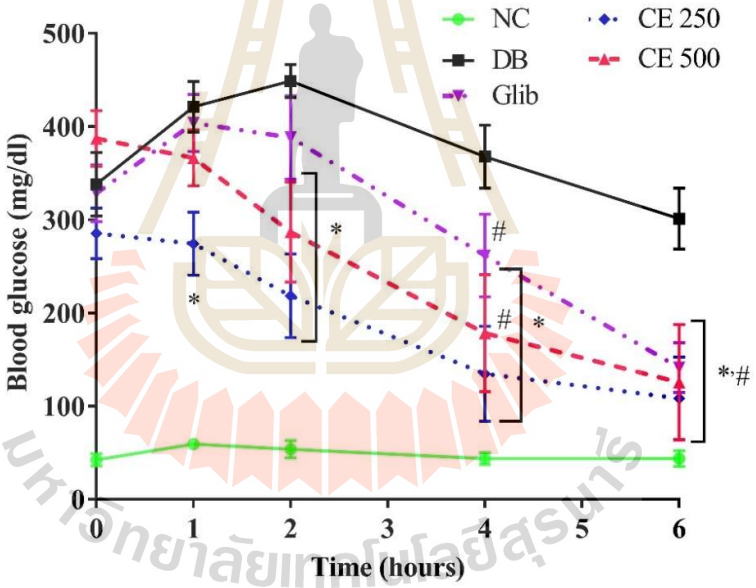


Figure 4.3 Acute effect of CE on alloxan-induced diabetic mice. NC: normal control mice, DB: diabetic control mice, CE250: diabetic mice + CE 250 mg/kg, CE500: diabetic mice + CE 500 mg/kg, Glib: diabetic mice + glibenclamide 10 mg/kg. $^{*}P$<0.05 compared with diabetic control mice at the same time of experiment, $^{\#}P$<0.05 compared to the initial level in the same treatment by one-way ANOVA followed by SNK. The values are expressed as mean ± SEM, n = 5-6.

4.4.3 Effect of CE on diabetic mice after 21 days of treatment

Unexpectedly, the treatments of CE and glibenclamide did not reduce fasting blood glucose after 21 days. As seen in Table 4.8, after treated, fasting blood glucose began to elevate gradually in day 7th, 14th and day 21st. Mice treated with CE showed no difference found in mice serum lipid profiles (total cholesterol, HDL, LDL), liver marker enzymes AST, ALT, and ALP; and kidney functional molecules BUN and creatinine (Table 4.9) in compared with diabetic control mice. Similar result was found in mice treated with glibenclamide. The histology of pancreas showed that the area of islet of Langerhans was markedly reduced in alloxan- induced diabetic mice compared with normal control. The areas of islet of Langerhans of all treated goups were measured using ImageJ software. The size of islets was scarcely improved in mice treated CE and glibenclamide (Figures 4.4 and 4.5).

Table 4.8 Blood glucose in normal and diabetic mice after 21 days of treatments (mg/dl).

Treatments	Day 0	Day 7	Day 14	Day 21
Normal control	42.67 ± 6.72	60.83 ± 4.19	63.50 ± 2.99	67.17 ± 4.98
Diabetic control	338.33 ± 34.13	395.33 ± 32.44	498.33 ± 45.33	538.83 ± 18.50
CE 250 mg/kg	285.50 ± 27.17	431.00 ± 42.54	492.00 ± 53.52	499.00 ± 36.19
CE 500 mg/kg	387.17 ± 29.85	406.83 ± 28.83	495.67 ± 25.54	546.67 ± 9.80
Glibenclamide	328.40 ± 30.44	363.40 ± 40.84	466.20 ± 62.74	498.60 ± 40.64

Value are expressed as mean ± SEM (n=5-6). P>0.05 compared to diabetic control at the same day by one-way ANOVA followed by SNK.

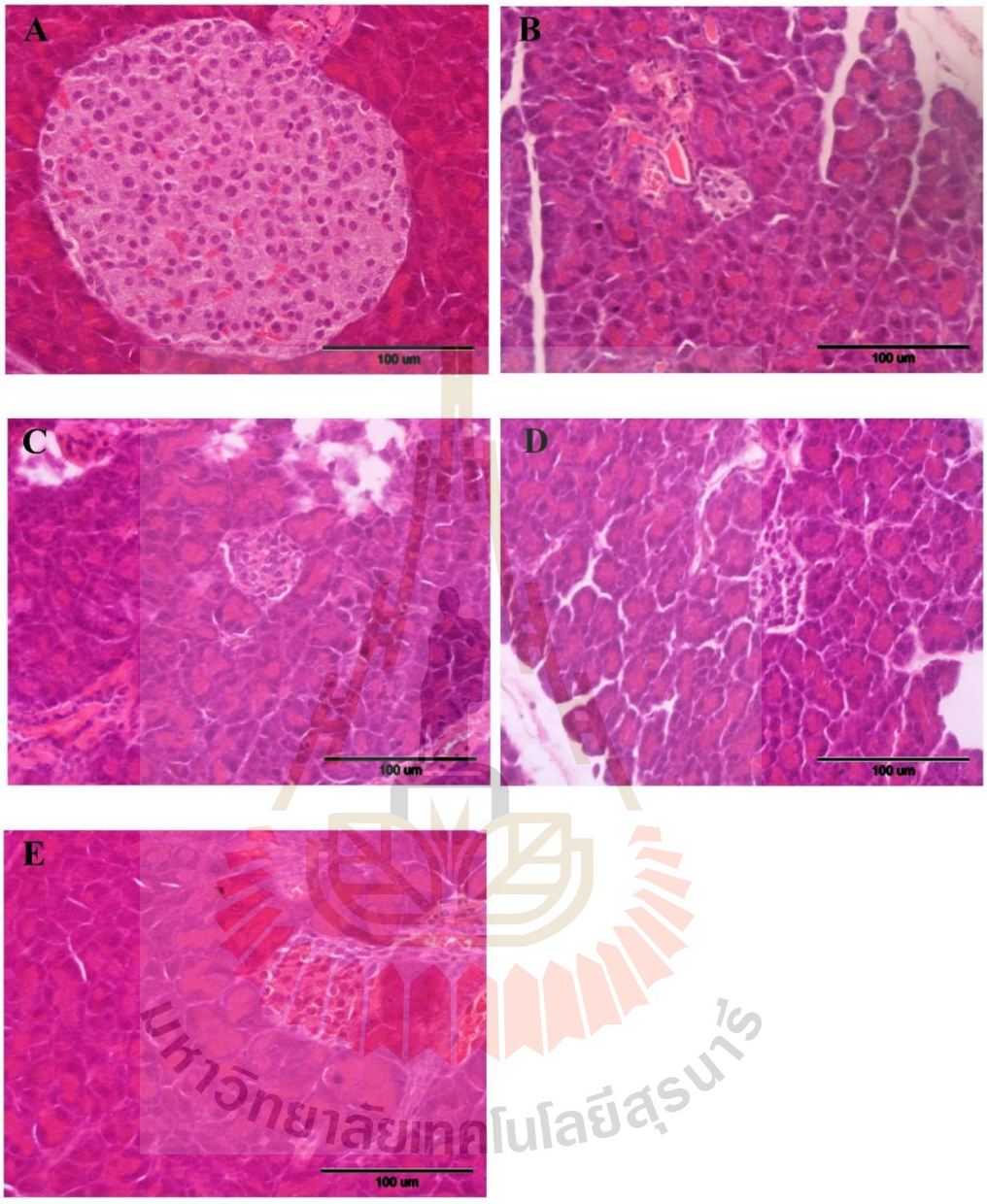


Figure 4.4 Mice pancreatic histology by H and E staining after 21 days of treatments. Images were captured under light microscope Nikon 80i at 400X magnification. A: normal control mice, B: diabetic control mice, C: diabetic mice + CE 250 mg/kg, D: diabetic mice + CE 500 mg/kg, E: diabetic mice + glibenclamide 10 mg/kg. Bar = 100 µm.

The areas of islet of Langerhans of all treated goups were measured using ImageJ software. The result showed that the area of islets of Langerhans in alloxan-injected mice significantly reduced compared with normal control, however that in neither glibenclamide and the extract groups did not increase compared with diabetic control mice (Figure 4.5).

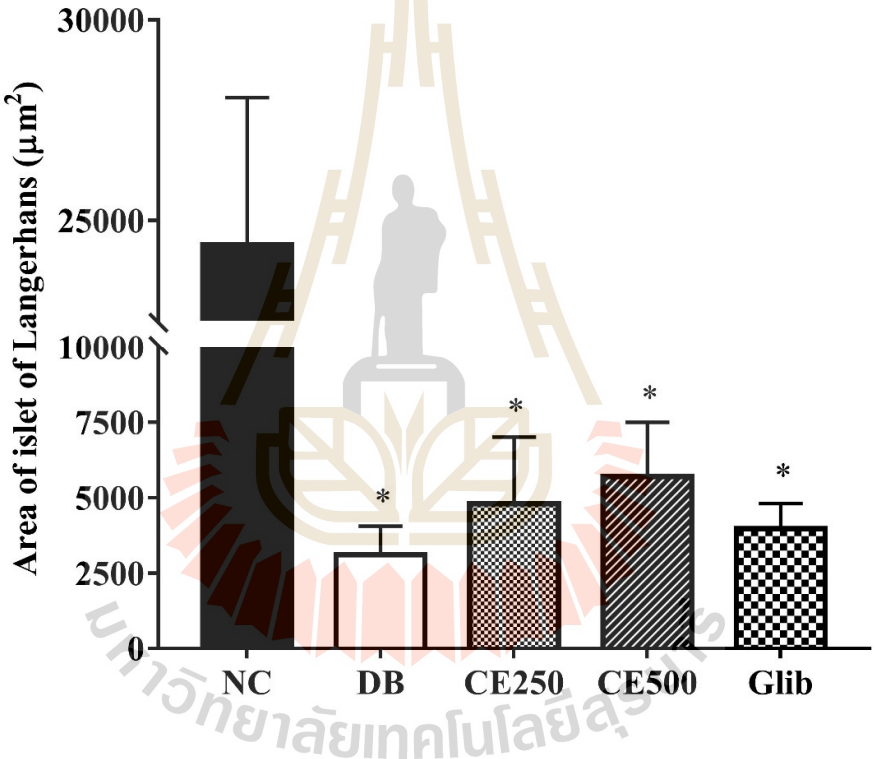


Figure 4.5 The areas of islets of Langerhans measured by ImageJ software. NC: normal control mice, DB: diabetic controlmice, CE250: diabetic mice + CE 250 mg/kg, CE500: diabetic mice + CE 500 mg/kg, Glib: diabetic mice + glibenclamide 10 mg/kg. Value are expressed as mean ± SEM, n=5-6. $^{*}P<0.05$ compared with normal control by one-way ANOVA followed by SNK.

Table 4.9 Alloxan-induced diabetic mice serum analysis after treated with CE 21 days.

Parameters	**Normal**	**CE 250 mg/kg**	**CE 500 mg/kg**	**Glibenclamide**	**Diabetic control**
Total cholesterol (mg/dl)	205.00 ± 8.70	168.33 ± 7.09	169.00 ± 7.05	180.60 ± 18.44	167.17 ± 9.04
HDL (mg/dl)	42.55 ± 1.67	42.06 ± 1.02	45.16 ± 3.12	42.64 ± 4.70	40.92 ± 0.78
LDL (mg/dl)	15.18 ± 2.95	18.68 ± 0.73	19.38 ± 0.99	18.04 ± 1.25	16.65 ± 1.09
AST (U/L)	36.67 ± 2.06	35.33 ± 5.06	35.00 ± 3.32	35.40 ± 3.92	33.50 ± 2.87
ALT (U/L)	37.00 ± 4.20	34.67 ± 3.11	37.60 ± 4.47	31.60 ± 2.38	39.83 ± 3.47
ALP (U/L)	63.00 ± 5.26	59.83 ± 3.60	56.40 ± 3.39	62.80 ± 3.84	61.17 ± 2.15
BUN (mg/dl)	20.50 ± 1.89	21 ± 2.96	26.40 ± 3.82	22.00 ± 2.74	21.83 ± 3.11
Creatinine (mg/dl)	1.08 ± 0.08	1.15 ± 0.18	1.40 ± 0.21	1.18 ± 0.14	1.13 ± 0.17

Value are express as mean ± SEM (n=5-6). $P>0.05$ compared to diabetic control by one-way ANOVA followed by SNK.

4.5 Effect of CE on glucose absorption

The everted sacs of the small intestines from mice were used for investigating the inhibitory effect of CE on glucose absorption *ex vivo*. The results shown in Figure 4.6 indicated that the extract at the experimental concentrations (25 and 50 µg/ml) did not inhibit glucose absorption when compared to control. In contrast, acarbose at 1 mg/ml profoundly suppressed glucose absorption by everted sacs (P<0.05).

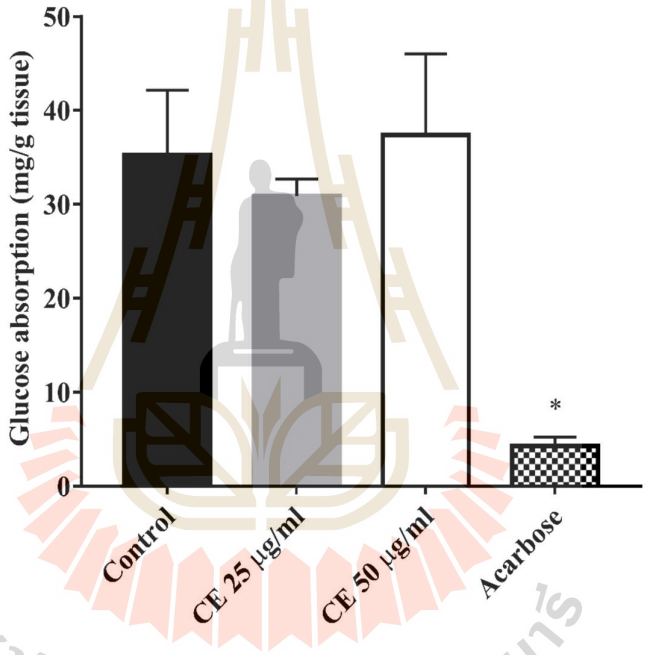


Figure 4.6 The effect of CE on glucose absorption by everted mouse jejunum. The values are expressed as mean ± SEM, n = 5. $^{*}P$<0.05 compared to control by one-way ANOVA followed by SNK.

4.6 Effect of CE on glucose uptake

The effect of CE on glucose uptake is presented in Figure 4.7. In normal group, low glucose uptake was found in the absence of insulin. Addition of insulin to the KRB

buffer increased the glucose uptake significantly (P<0.05). This effect was also observed in all experiments when compared to the non-insulin treated groups. The results showed that treatment of CE at 50 μg/ml with insulin significantly increased glucose uptake from 7.86 ± 0.52 (control) to 9.45 ± 0.82 mg/g tissue. However, without insulin, CE at the doses used had no significant effect on glucose uptake.

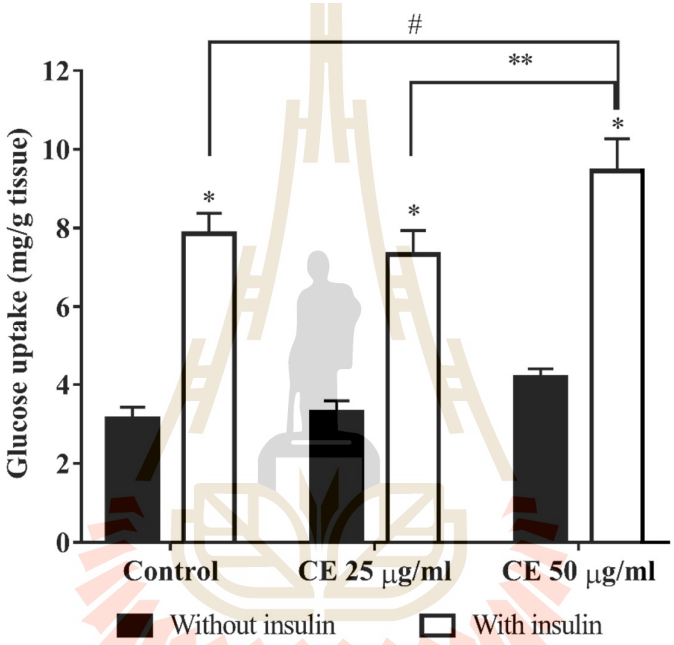


Figure 4.7 The effect of CE on glucose uptake by isolated mouse abdominal muscle. The values are expressed as mean ± SEM, n = 5. $^{*}P$<0.05 compared with non-insulin in the same treated group, $^{\#}P$<0.05 compared with control, $^{**}P$<0.05 compared with CE 25 μg/ml by two-way ANOVA followed by SNK.

4.7 α-Glucosidase inhibitory effect of CE

α-Glucosidase enzyme is one of the medication targets in diabetic management. The enzyme is involved in digestion of polysaccharide into monosaccharide that can be

absorbed by the intestine. In this study, α-glucosidase isolated from *Saccharomyces cerevisiae* was chosen as the target enzyme. The aqueous extract from *C. cainito* exhibited much greater inhibition on α-glucosidase activity compared to acarbose. The IC_{50} of CE was 1.20 ± 0.09 µg/ml whereas that of acarbose was 198.17 ± 4.74 µg/ml (Figure 4.8).

4.8 Cytotoxicity of CE on HepG2 cells

The cytotoxic effect of CE showed significant cell lost in HepG2 cells (Figure 4.9). The results from MTT assay demonstrated that CE treatment inhibited the proliferation of HepG2 cell in dose-dependent manner. HepG2 cell viability significantly dropped at concentration of CE 50 µg/ml. The estimated IC_{50} value of CE was 121.75 ± 7.98 (µg/ml) after 24 hours of incubation. The cytotoxicity effect of the extract on HepG2 cells was also evaluated using trypan blue exclusive assay which resulted in the discrimination of live cell (clear cytoplasm) and dead cell (blue cytoplasm) under light microscope. The IC_{50} value was 169.38 ± 10.40 (µg/ml) which was in accordance with the result from MTT method. The incubation with CE at 100 µg/ml which decreased cell viability of HepG2 cell to approximately 50% was chosen as the highest dose for further investigation.

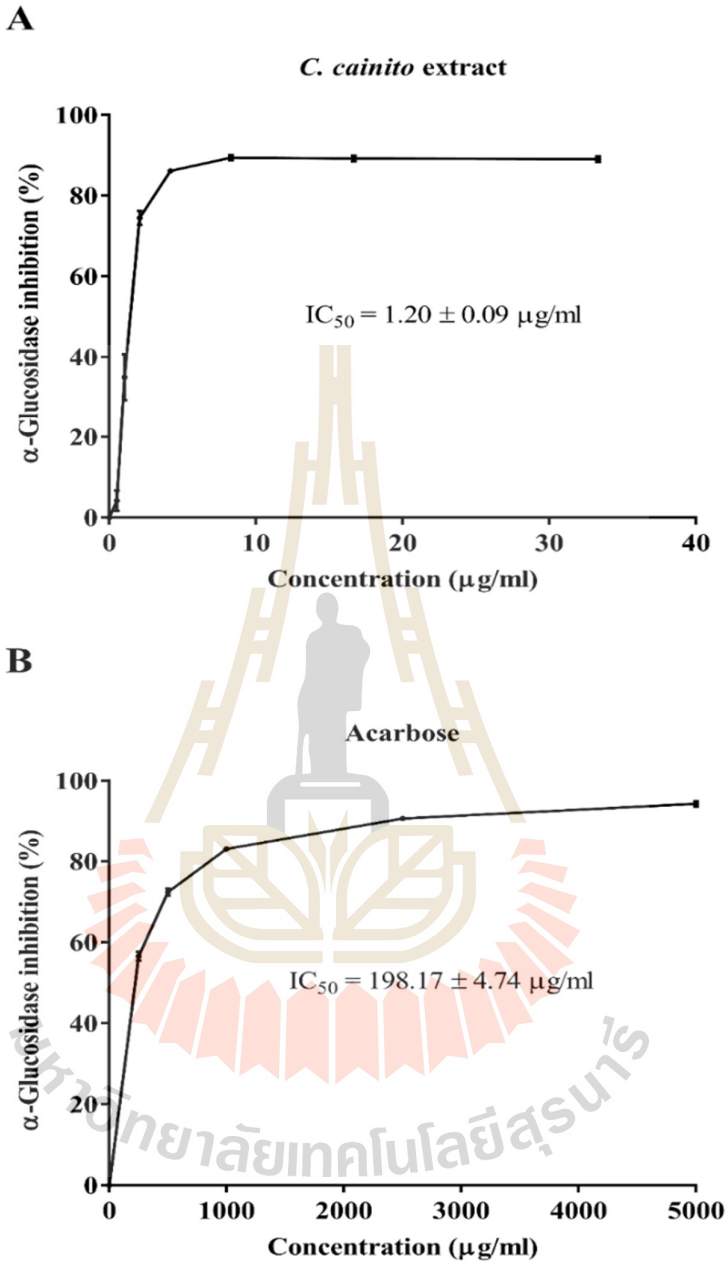


Figure 4.8 The effect of CE on α-glucosidase activity. Values are expressed as mean ± S.E.M (n=3). Acarbose was used as positive control. The calculated IC_{50} of the extract was 1.20 ± 0.09 µg/ml, whereas that of acarbose was 198.17 ± 4.74 µg/ml.

4.9 Cytoprotective effect of CE on TBHP-induced HepG2 cell damage

The proportion of viable cells decrease in dose-dependent manner after 3 hour-incubation of TBHP (Appendix B). The concentration of TBHP at 250 µM (caused about 50% cell death) was chosen for the cytoprotective experiments. In both pretreatment and posttreatment, CE did not show protective activity on cell death induced by TBHP (P>0.05) (Figure 4.10).

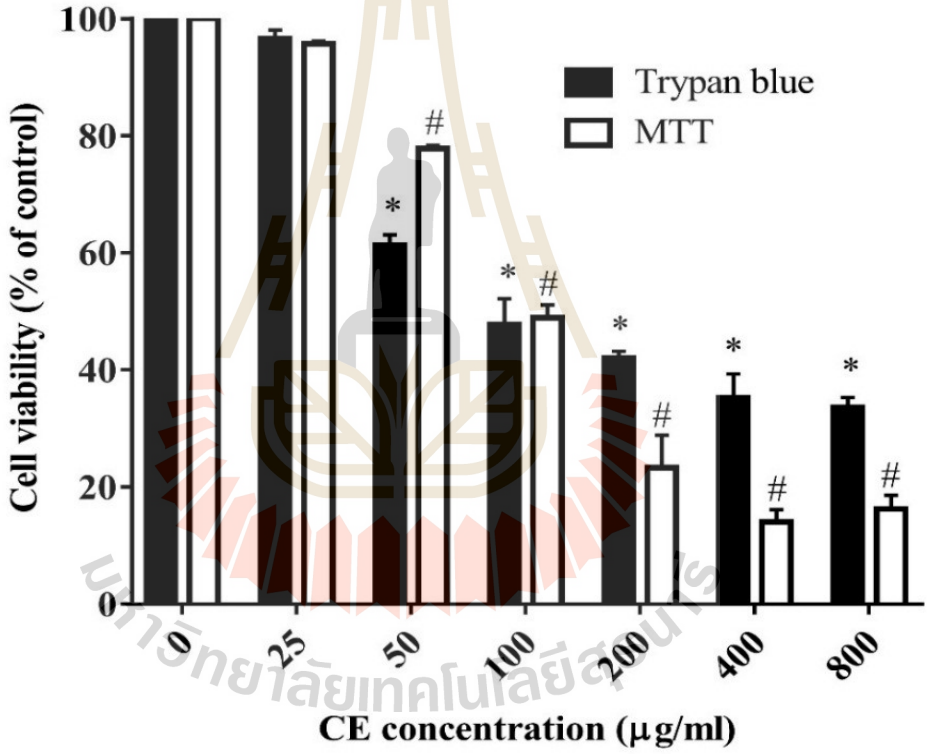


Figure 4.9 Cytotoxicity of CE on HepG2 cells by MTT and trypan blue exclusive assay. Value are expressed as mean ± SEM (n=3). $^{*}P$<0.05 compared with control in MTT assay, $^{\#}P$<0.05 compared with control in Trypan blue exclusive assay by one-way ANOVA followed by SNK.

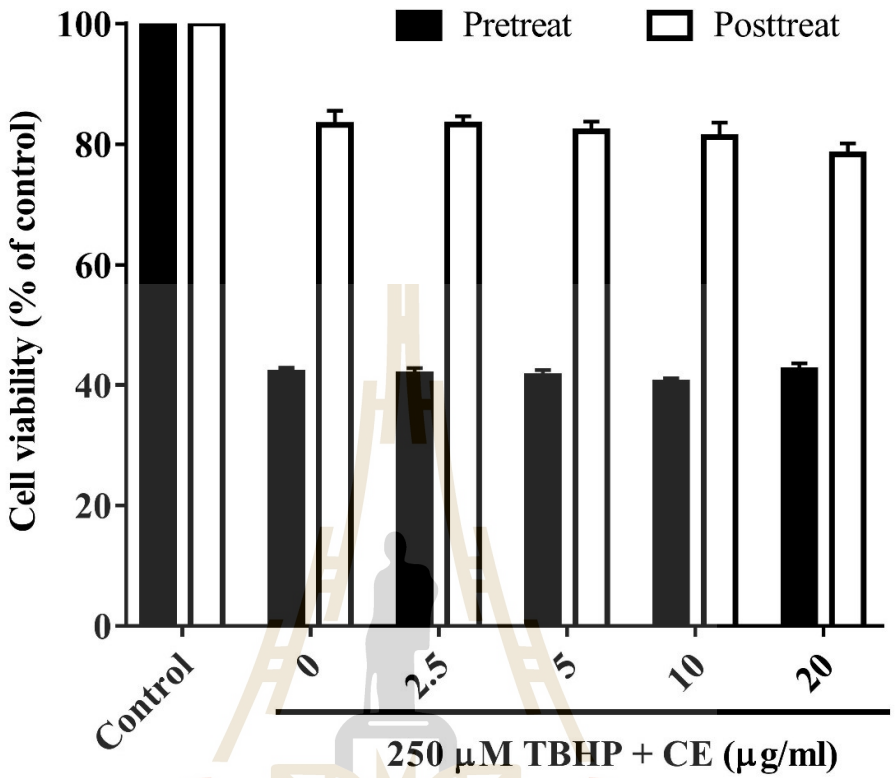


Figure 4.10 Cytoprotective effect of CE in HepG2 cells against TBHP. Values are expressed as mean ± SEM (n=3). TBHP (250 µM) significantly induced cell death compared to control, however there is no statistically significant difference in cell viability between untreated cells and CE-treated cells in both pretreatment and posttreatment (P>0.05).

4.10 CE induces apoptosis in HepG2 cells

The effect of CE on HepG2 cells apoptosis was analyzed using Tali™ apoptosis kit. Figure 4.11 shows the induction of apoptosis in HepG2 cells by different concentrations of CE. The 100 µg/ml CE which close to inhibit 50% of cells growth

was chosen as a highest concentration in this experiment. The percentage of apoptotic cells accumulated from 1.00 ± 0.00% in control group to 5.60 ± 0.51%, 10.20 ± 1.20%, and 17.40 ± 1.08% of total cells (P<0.05) as the concentrations of CE increased from 0 to 50, 75 and 100 µg/ml, respectively. The result expressed dose-dependent induction of apoptosis by CE in hepatocarcinoma HepG2 cells.

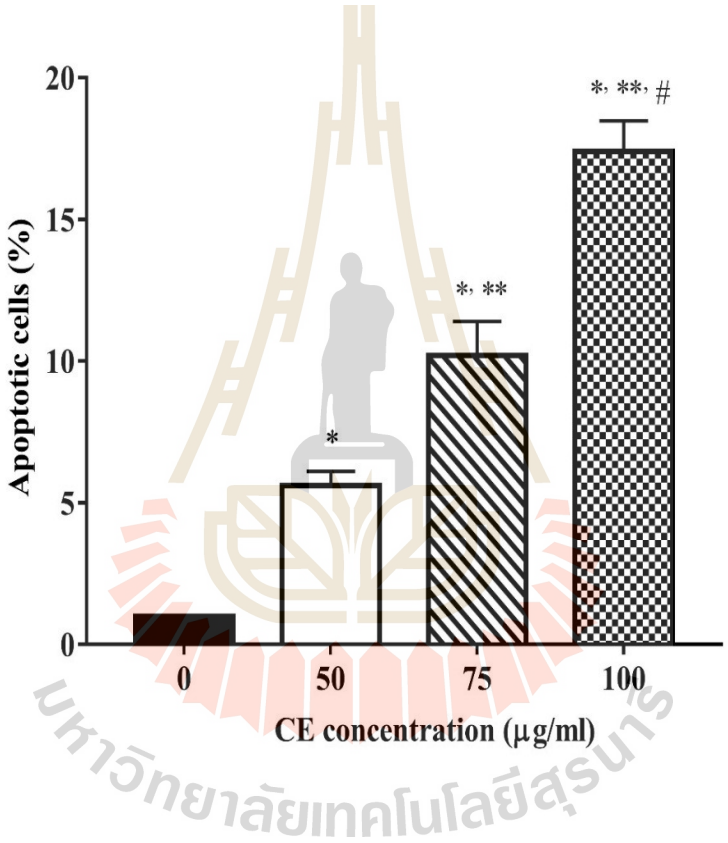


Figure 4.11 Effect of CE on apoptosis of HepG2 cells. HepG2 were treated with CE for 24 hours. Cells were collected and stained by Tali™ apoptosis kit. The values were expressed as mean ± SEM (n=5). $^{*}P$<0.05 compared control; $^{**}P$<0.05 compared with CE 50 µg/ml; $^{\#}P$<0.05 compared with CE 75 µg/ml by one-way ANOVA followed by SNK.

4.11 Effect of CE on cell cycle

Prior to the treatment, HepG2 cells were culture in free-FBS media for 24 hours to synchronized cells at G0 phase. After 24 hours of extract exposure (up to 100 µg/ml), the number of sub-G1 phase as well as G2/M phase significantly increased when compared to non-treated group (P<0.05) (Figure 4.12). The proportion of cells in the sub-G1 phase increased from 7.20% ± 0.37% (control) up to 43.80 ± 0.74% while that in the G2/M phase increased from 5.60% ± 0.68% (control) up to 12.80 ± 0.82%. The results indicated that the cell cycle progress was strongly arrested by CE treatments in the sub-G1 phase.

4.12 Toxicological effect of CE on embryonic development

It is shown in Figure 4.13 that hatching rates of embryos exposed to the CE at 500 µg/ml and 1000 µg/ml were significant dropped compared with the control at 72 hpta. At 24 hpta, there was no significant difference in survial embryos observed. However, after 48 hpta, the mortality of embryos was significantly increased and was remarkedly risen in embryos exposed to 1000 µg/ml of CE. The lowest dose of CE used in this experiment (15.63 µg/ml) did not induce any embryo death (Figure 4.14), whereas the greater doses caused the abnormal development of embryo (yolk sac edema) (Figure 4.15A). CE at the highest concentration (1000 µg/ml) induced a great number of coagulated embryos up to 45% (Figure 4.15B). The concentration that cause a half of embryos death (LC_{50}) was 25.75 ± 1.51 µg/ml.

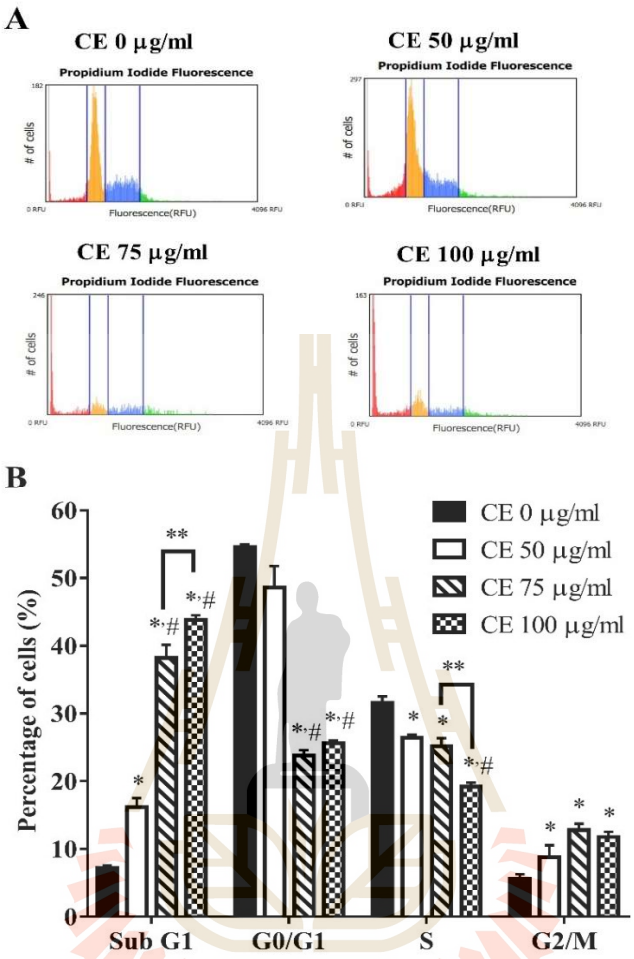


Figure 4.12 Effect of CE on cell cycle of HepG2 cells. HepG2 were treated with CE for 24 hours. Cells were collected and stained by Tali™ cell cycle kit. Cell cycle distribution and its percentage in different cell phases were shown in Panels A and B, respectively. The values were expressed as mean ± SEM (n=5). $^{*}P<0.05$ compared control; $^{**}P<0.05$ compared with CE 50 µg/ml; $^{\#}P<0.05$ compared with CE 75 µg/ml by one-way ANOVA followed by SNK.

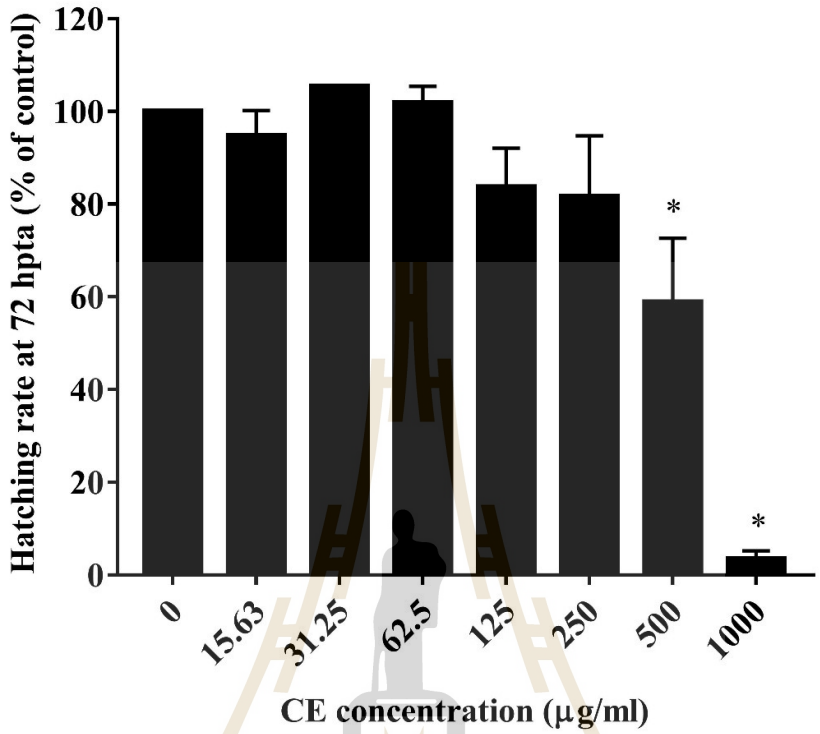


Figure 4.13 Accumulative hatching rate of zebrafish embryos at 72 hpta. Hatching rate of each group was calculated by (no. of hatched individuals)/20 (all individuals of one replicate) x 100%. Value are express as mean ± SEM (n=3). $^{*}P<0.05$ compared with control by one-way ANOVA followed by SNK.

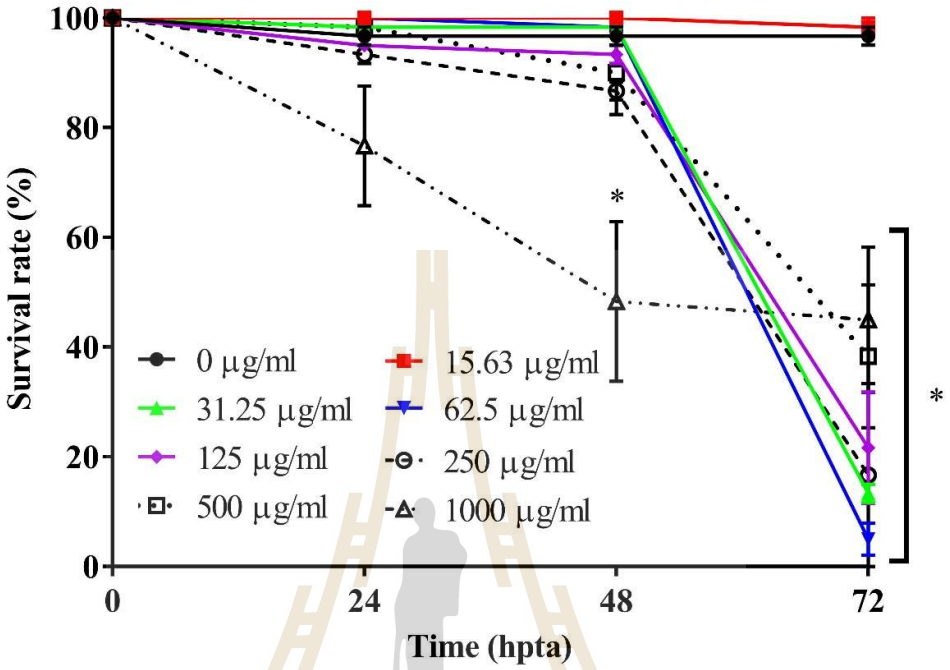


Figure 4.14 Effect of CE on survival rate of zebrafish embryos. Survial rate was calculated by (no. of survial embryos)/ 20 (all embryos of one replicate) x 100%. Value are express as mean ± SEM (n=3). $^{*}P<0.05$ compared with control by one-way ANOVA followed by SNK.

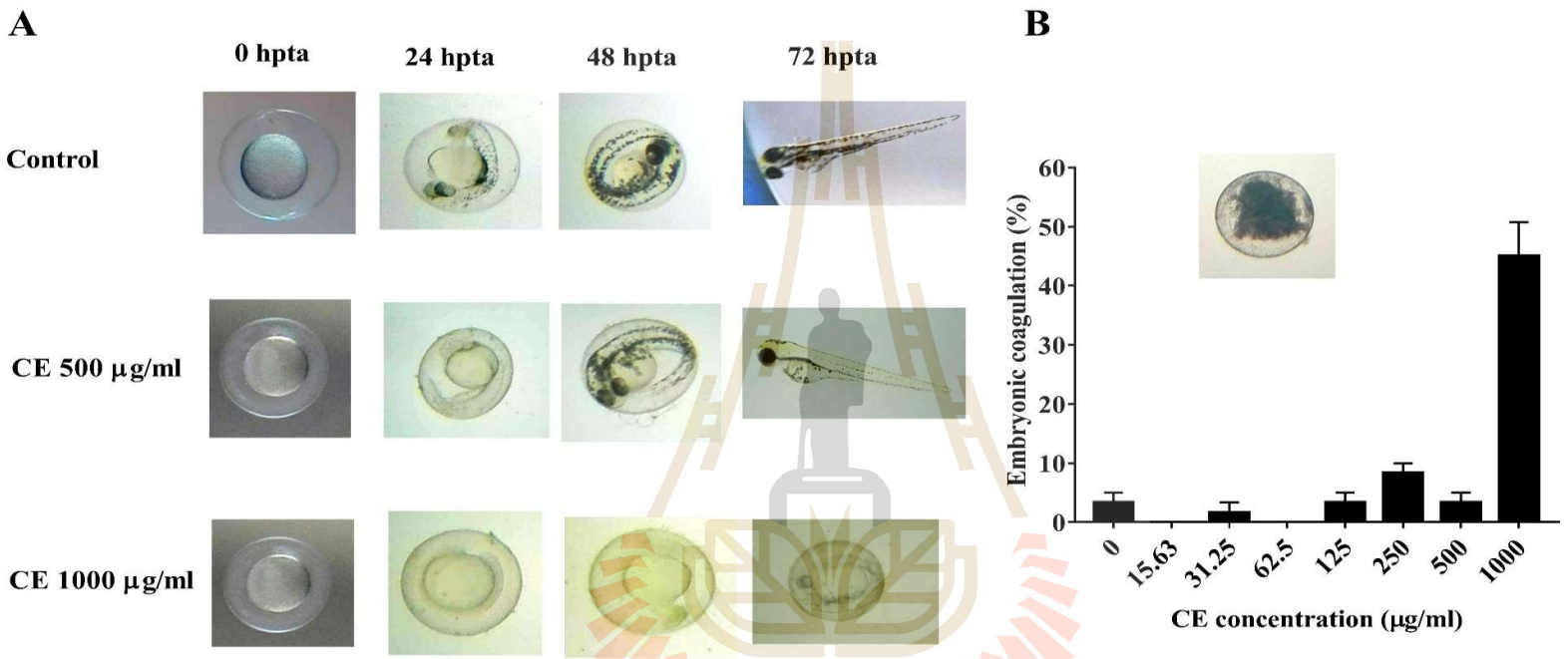


Figure 4.15 Effect of CE on embryonic development. Panel A shows morphology of zebrafish embryos over time of CE exposure. Percentages of embryonic coagulation caused by CE at different concentrations are demonstrated in Panel B. Value are expressed as mean ± SEM (n=3).

CHAPTER V

DISCUSSION AND CONCLUSION

It is widely accepted that the rapidly increasing incidence of diabetes mellitus has become a major health problem worldwide. The modern oral hypoglycemic agents such as sulfonylureas, biguanides, thiazolidinediones and α-glucosidase inhibitors are commonly used for the treatment of type 2 diabetes. However, it is well known that they can produce side effects associated with their applications (Fowler, 2007). Moreover, a progressive decline in their effectiveness, termed secondary failure have been reported (Groop, Pelkonen, Koskimies, Bottazzo, and Doniach, 1986). During the past decade, there is a growing interest in alternative herbal medicine due to their efficacy, less side effects in clinical practice and relatively low costs. It has been estimated that about 800 plants have antidiabetic potentials (Grover, Yadav, and Vats, 2002). Most of them have been used as folk medicines in many countries around the world. *C. cainito*, commonly called Star Apple in English and Vú Sữa (literally: milky breast) in Vietnamese, is one of medicinal plants which has long been prescribed by local practitioners for traditional treatment of diabetes mellitus. However, there is a paucity of scientific evidence that confirms its antidiabetic activity. Herein, the antidiabetic effect of the extract from *C. cainito* stem bark was first evaluated to confirm its benefits according to the use of this plant in Vietnam.

In this study, water was chosen as an extraction solvent because water formulations are safe for human consumption compared to other organic solvents. It

also increases bioavailability of active compounds. Furthermore, water maceration and decoction of this plant have been used by Vietnamese for treatment of diabetic patients. So simple maceration rather than decoction was used to prevent chemical degradation from high temperature.

Phytochemical analysis of CE obtained from this study showed the presence of phenols, tannin, glycosides, terpenoids, and saponin of which the antidiabetic effects have been established (Abdallah, Salama, Abd-elrahman, and El-Maraghy, 2011; Luo et al., 2002; Nazaruk and Borzym-Kluczyk, 2015; Piluzza and Bullitta, 2011). The extract contains great amounts of phenolic compounds (871.75 ± 10.41 mg GAE/g extract) and had high antioxidant potential comparable to antioxidant power of the standard antioxidant ascorbic acid and butylated hydroxytoluene (Table 4.2). Free radicals are found to be associated with many diseases, including diabetes. In diabetes mellitus, the supplement of antioxidant agents show promising effect on the reverse of the oxidative stress biomarkers and diabetic complications (Johansen, Harris, Rychly, and Ergul, 2005). These results agree with previous report that high phenolic content was correlated with strong antioxidant activity (Piluzza and Bullitta, 2011). It can be anticipated that the antioxidant activity of CE may be beneficial in the long-term treatment of diabetic patients. Nine polyphenolic compounds, (+)-catechin, (+)-gallocatechin, (-)-epigallocatechin, quercetin, quercitrin, isoquercitrin, myricitrin, gallic acid and (-)-epicatechin were isolated from fruit of *C. cainito* (Luo et al., 2002). Others including ursolic acid, β-sitosterol, lupeol and gallic acid were extracted from the leaves (Shailajan and Gurjar, 2014). It has been demonstrated that these compounds possess antidiabetic activity (Castro et al., 2015; R. Gupta, Sharma, Dobhal, Sharma, and Gupta, 2011; R. Gupta, Sharma, Sharma, Dobhal, and Gupta, 2012; Oboh,

Ogunsuyi, Ogunbadejo, and Adefegha, 2016). In terms of the compositions of *C. cainito* stem bark, there is lack of information. Phytochemical verification and identification of the active ingredients responsible for its antidiabetic activity need further investigation.

The safety of medicinal plants is important although medicinal plants or herbal remedies were believed to be safe. The *in vivo* safety of CE was accessed via acute toxicity examination in rat and in zebrafish embryos. To conduct the acute toxicity examination of CE in rats, both male and female rats were orally administered a single dose of CE 0, 500, 1000, 2000, or 4000 mg/kg. There was no sign of morbidity occurred at the first 24 hours and no mortality was observed in 14 days. The data revealed that LD_{50} of CE is higher than 4000 mg/kg suggesting that animals are well tolerate to this plant extract. Hematopoietic system is one of the most sensitive target sites of the toxic agents (Mukinda and Eagles, 2010). The consumption of CE at all doses did not affect any significant changes in hematological profile of treated rats. Certainly, liver is the major organ involved in drug metabolism (Sahi, Grepper, and Smith, 2010). Therefore, the level of liver biomarker enzymes in rat serum were measured in order to demonstrate the possible toxicological effect of CE on liver. The result show that the level of AST, ALT and ALP in the serum of rats treated with CE even at the highest dose were not different in comparison with control. It is widely known that kidneys play a crucial role in the excretion of the waste product from system metabolism, especially in the elimination of the drug and xenobiotics (Lohr, Willsky, and Acara, 1998). It was found that CE did not change the functional kidney markers in acute toxicity study. Finally, no significant changes in rat body weights and relative weights of the internal organs were found in the tested rats up to 14 days of the experiment. The

consumption of CE upto 4000 mg/kg is safe in rats. It can be concluded that the lethal dose of CE was greater than 4000 mg/kg. Hence, the aqueous extract of *C. cainito* can be classified as category 5 (less to no toxicity) based on the Globally Harmonized Classification System of OECD. These data were used to consider the dosage of CE for antidiabetic investigation *in vivo*.

Antidiabetic effect of the *C. cainito* extract was first conducted in normal mice. Hypoglycemic effect of the extract was investigated through an oral glucose tolerance test (OGTT). OGTT is a gold standard diagnostic method for diabetic screening (Ito et al., 2000; Phillips, 2012). A six-hour fasting is considered as a best fasting duration for establishing an OGTT in mice (Andrikopoulos, Blair, Deluca, Fam, and Proietto, 2008). The rise in blood glucose after 30 minutes confirmed successful oral glucose loading in every groups (Figure 4.2). The antidiabetic drug glibenclamide used in this study as a positive control reduces the postprandial hyperglycemia by increasing insulin secretion from β cell. Based on our preliminary experiment, the dose at 250 mg/kg and 500 mg/kg was chosen for oral administration. As shown in Figure 4.2A and B, the extract and glibenclamide significantly improved glucose tolerance compared to vehicle control.

Next, antihyperglycemic effect of CE on diabetic model was established using alloxan-injected mice. Alloxan, a toxic glucose analogue, enters pancreatic β-cell via GLUT2 glucose transporter. This chemical plays an important role in hyperglycemic animal model through its specific inhibition of glucokinase and stimulation of reactive oxygen species production, consequently causing necrosis and destruction of β-cells (Lenzen, 2008). The data from Figure 4.3 clearly showed that the administration of CE reduced blood glucose level in alloxan-induced diabetic mice similar to that of

glibenclamide. These *in vivo* experiments provide the first scientific evidence supporting an anti-hyperglycemic activity of *C. cainito* stem bark.

Numerous mechanisms of action have been proposed for medicinal plants used in the treatment of diabetes mellitus. However, none has been postulated for antidiabetic activity of *C. cainito*. In this study, two possible mechanisms underlying its acute antidiabetic activity, an inhibition of glucose absorption and a stimulation of glucose uptake were examined. The glucose absorption was performed using everted jejunal sacs of mice. Acarbose, a well-known α-glucosidase inhibitor currently used for the treatment of diabetic patients, was used as a positive control because it has been shown to additionally inhibit the absorption of D-glucose from the intestinal lumen into the blood stream (Hirsh, Yao, Young, and Cheeseman, 1997; Widyawati et al., 2015). The possible mechanism is that acarbose affect to the subunit or a second-messenger system which is able to phosphorylate a site on SGLT-1. In addition, the interconnection between SGLT-1 and disaccharidase enzymes on the surface of brush body membrane might contribute to that effect. The data in Figure 4.6 shows that unlike acarbose, CE did not inhibit the glucose absorption.

Isolated skeletal muscle such as epitrochlearis muscle (Kleinert et al., 2011; Ma et al., 2009) and abdominal muscle (Boyd et al., 2000; Hassan et al., 2010; O'Harte, Gray, and Flatt, 1998) have been exploited in the glucose uptake study. In the current study, we isolated the abdominal muscle from mice and incubated in a bicarbonate buffer with cabogen supplied constantly. The effect of CE on glucose uptake was evaluated in the absence and presence of insulin. The data in Figure 4.7 shows that in the presence of insulin CE at 50 μg/ml significantly enhanced glucose uptake by the muscle (9.45 ± 0.82 mg/g tissue) compared to control (7.86 ± 0.52 mg/g tissue).

Without insulin, CE treatment also showed an increase of glucose uptake, but not statistical significant. These *in vitro* experiments revealed that the extract promoted glucose transport in the skeletal muscle, especially in the presence of insulin, but no effect on glucose absorption. It is likely that the enhancement on glucose uptake contributes to the antidiabetic effect of CE.

Long-term treatment in 21 days did not show a decrease in BG of diabetic mice treated with CE. In this study, the BG test was established in fasting stage after mice received their treatments about 24 hours. Fasting blood glucose tends to increase gradually after treated 7, 14, and 21 days in all groups (Table 4.8). As seen from histological result of pancreas, the islets of Langerhans were shrunken in all diabetic mice treated and untreated groups. The result in Figure 4.5 showed that the area of islet of Langerhans in alloxan-injected mice significantly reduced compared with normal control. In all diabetic mice received the treatments, the areas of islet did not show any difference compared with diabetic control. The remedies of CE did not show benefit in recovering β-cell. This result was corresponding with the cytoprotective examination of CE. Thus, CE of 2.5 – 20 μg/ml showed no protective activity to the oxidative damage in HepG2 cells in neither pretreatment nor posttreatment (Figure 4.10). Similar result was obtained in glibenclamide treated mice in comparison with diabetic control. The explaination for this treatment failure from both CE and glibenclamide would be the case that late diabetic state induced by the dose of alloxan used in this study is too severe compared to early state of induction.

The influence of CE on BG was mainly observed only in the acute treatment to provide scientific evidence to support the hypoglycemic activity of the extract. One may argue that the postulated action on glucose uptake in this study could not explain

the acute anti-hyperglycemic effect of the extract. Skeletal muscle is recognized as the major site of insulin-mediated glucose uptake after carbohydrate consumption in human (DeFronzo and Tripathy, 2009). Insulin resistance is a hallmark of non-insulin dependent diabetic mellitus. Peroxisome proliferator-activated receptor gamma (PPARγ) is a key factor in insulin sensitivity. The activation of PPARγ by insulin sensitizers (e.g. thiazolidinediones) markedly improve the sensitivity to insulin, however, it requires long-term effect to cause gene expression change (Olefsky, 2000). Moreover, PPARγ is prominently present in adipose tissue and nearly absent in muscle (Ferré, 2004). The enhancement of insulin action in skeletal muscle by CE found in this study tend to be mediated via other mechanism(s).

It is well established that glucose uptake by skeletal muscle is mostly via glucose transporter 4 (GLUT4). It has been shown that GLUT4 recruitment from cytosol to the cell surfaces of muscle can be acutely stimulated by both insulin and exercise independent of transcription or translation (Herman and Kahn, 2006; Rose and Richter, 2005). The translocation of GLUT4 from intracellular vesicles to accumulate in the plasma membrane in the response to insulin was demonstrated to depend on the activation of the insulin receptor substrate 1, PI3K, PDK1 and Akt2 (Satoh, 2014). In addition, the data from a previous study suggested that an acute stimulation effect on insulin-mediated glucose uptake in skeletal muscle was related to the elevation of the phosphorylation and activation of key proteins involving in the translocation of GLUT4 such as Rac1, AS160 and Akt (Röhling, Herder, Stemper, and Müssig, 2016). Other studies suggested that the rising in glucose uptake was due to the increase of AMPK phosphorylation (Ma et al., 2009; Vlavcheski, Naimi, Murphy, Hudlicky, and Tsiani,

2017). This pathway supports the acute anti-hyperglycemic effect of the CE by increasing glucose uptake in the muscle.

α-Glucosidase is a digestive enzyme which catalyzes the breakdown of polysaccharides into monosaccharide, the form of carbohydrate that the intestine can only be able to transport into the blood circulation. Therefore, the inhibition of α-glucosidase is one of the important approaches in oral antidiabetic medication. Reducing postprandial glucose level by delay glucose absorption after meal is the prominent benefit from α-glucosidase inhibitor (Mohamed et al., 2012). The α-glucosidase inhibitory effect of *C. cainito* extract was determined in this study. The IC_{50} value of the extract was found at 1.20 ± 0.09 µg/ml, approximately 200 times lower than that of acarbose which is used clinically as antidiabetic drug. Thus, this action is most likely to be one of the mechanisms underlying the antidiabetic effect of *C. cainito* extract. Moreover, *C. cainito* could be a potential candidate for a search of a new α-glucosidase inhibitory drug for diabetic care.

In addition to acute toxicity in rats, cytotoxicity of CE in HepG2 was also investigated through MTT method and trypan blue exclusive assay. The growth inhibitory response of HepG2 cells to CE was dose-dependence. The viability of cells was dramatically dropped at 50 µg/ml and more severe when the concentration of CE was increasing. The concentrations of CE that caused 50% of cell death (IC_{50}) were 121.75 ± 7.98 µg/ml by MTT assay and 169.30 ± 10.40 µg/ml by trypan blue exclusion. According to Dutra and colleagues, the IC_{50}<10 µg/ml is classified into “very toxic” group; IC_{50} from 50-100 µg/ml is classified into “potentially toxic” group; IC_{50} from 100-1000 µg/ml is classified into “potentially harmful” group; and when IC_{50}>1000

µg/ml is classified into “potentially nontoxic” group (Dutra et al., 2012). Thus, CE is classified in to potential harmful group.

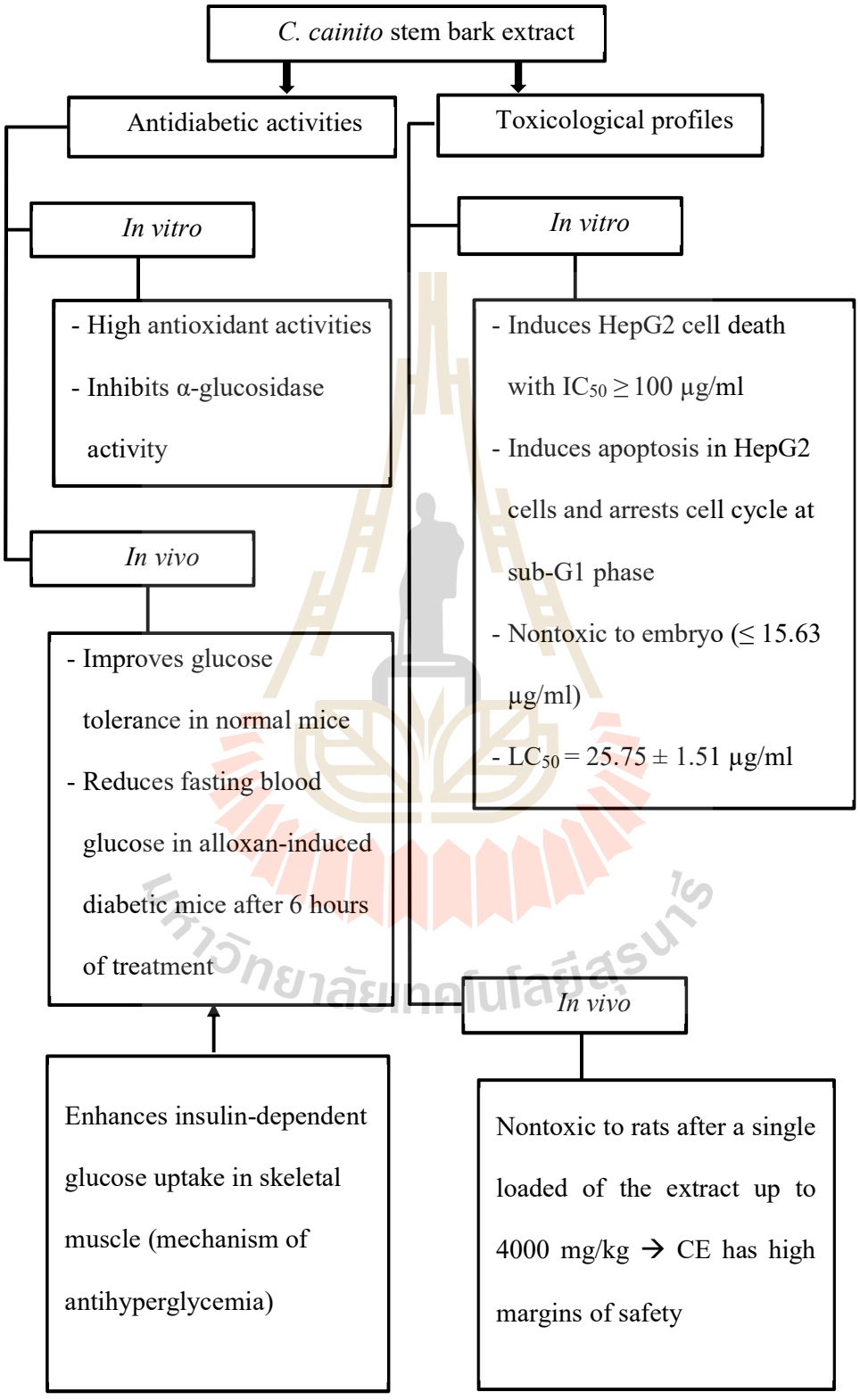
Apoptosis was found as a mechanism underlying action to CE causing cell death. In this study, the apoptotic induction ability of CE was investigated using Tali™ apoptosis kit. The kit contains two dyes annexin-V alexa fluor® 488 and propidium iodide. Under apoptotic condition, phosphatidylserine is converted from inner cell membrane to outer surface of cell membrane. Thus, the fluorescent labeled annexin-V detects apoptotic cells by binding to this exposed protein meanwhile propidium iodide is used to determine the dead cell. We found that CE caused the accumulation of apoptosis in HepG2 cells. The percentage of apoptotic cells markedly increased correlatively with the concentration of CE (Figure 4.11). In addition, the result from cell cycle analysis revealed that CE increased the proportion of HepG2 cells in the sub-G1 phase dose-dependently (Figure 4.12), suggesting that CE-treated HepG2 cells were arrested in the sub-G1 phase (Huang et al., 2009; Pumiputavon et al., 2017; Sun et al., 2010). The increase in sub-G1 peak represented cell apoptosis associated with DNA fragmentation (Malgorzata, Dorota, Juliusz, and Zbigniew, 2007; Riccardi and Nicoletti, 2006).

According to Mandrell and co-workers, the early stage of zebrafish embryonic development is the most sensitive stage of life to chemicals (Mandrell et al., 2012). Nowadays, zebrafish embryo is an attracted model for toxicologist due to its cheap and small size. It is an excellent model for phonotypic and genomic studies. The researchers can observe the entire development of embryos (embryogenesis). It needs short-time and is easy to monitor and evaluate because of the transparency of embryos (Yang et al., 2009). In addition to the acute toxicity test in rats, the toxic effect on development

of CE was also conducted using zebrafish embryos. The effect of CE on the morphology of embryos were monitored under light stereomicroscope. The data in figure 4.14 shows no toxicity on embryonic development when embryos were treated with the lowest dose (15.63 µg/ml) of CE. The estimated value of LC_{50} was 25.75 ± 1.51 µg/ml. The higher concentrations induced larval mortality only after hatched from 48 to 72 hpta. This outcome may occurred from the embryonic protection of chorion which inhibit the absorption of chemical into the embryos (Johnson, Carew, and Sloman, 2007). The delaying of hatching time is due to the inhibition of some hatching enzymes such as zebrafish hatching enzyme 1 (ZHE1) (Muller, Lin, and Nisbet, 2015). The highest concentration used in this study (1000 µg/ml) strongly caused the death of embryos (coagulation) and the delay of hatching with abnormal development (yolk sac edema). Therefore, the application of CE in pregnancy must be carefully considered. However, the clarification in this matter, such as the ability of active ingredients to cross the placenta, should be further investigated.

In conclusion, the aqueous extract from *C. cainito* stem bark possesses a strong *in vitro* antioxidant activity and *in vivo* antidiabetic effects. It is postulated that the mechanism of action contributing the acute anti-hyperglycemic effect of the extract is the enhancement of glucose uptake by the muscles. Moreover, the extract showed strong α-glucosidase inhibitory effect which supports its postprandial anti-hyperglycemic action in diabetic patients. No serious toxic effect of extract was found in rats. Based on OECD classification, the extract is considered as category 5 (less to no toxicity). However, since the extract can cause developmental abnormality to zebrafish embryos and cell apotosis in high doses. The results obtained from this study suggest a precaution when used in pregnancy patients.

The summarization of this study is presented in the diagram below.



REFERENCES

REFERENCES

Abdallah, H. M., Salama, M. M., Abd-elrahman, E. H., and El-Maraghy, S. A. (2011). Antidiabetic activity of phenolic compounds from Pecan bark in streptozotocin-induced diabetic rats. **Phytochemistry Letters.** 4(3): 337–341.

Abdennacer, B., Karim, M., Yassine, M., Nesrine, R., Mouna, D., and Mohamed, B. (2015). Determination of phytochemicals and antioxidant activity of methanol extracts obtained from the fruit and leaves of Tunisian *Lycium intricatum* Boiss. **Food Chemistry.** 174: 577–584.

Adeghate, E., and Kalász, H. (2011). Amylin Analogues in the treatment of diabetes mellitus: medicinal chemistry and structural basis of its function. **The Open Medicinal Chemistry Journal.** 5(2): 78–81.

Afkhami-Ardekani, M., and Shojaoddiny-Ardekani, A. (2007). Effect of vitamin C on blood glucose, serum lipids and serum insulin in type 2 diabetes patients. **The Indian Journal of Medical Research.** 126(5): 471–474.

Alañón, M. E., García-Ruíz, A., Díaz-Maroto, M. C., Pérez-Coello, M. S., and Moreno-Arribas, M. V. (2015). Antimicrobial and antioxidant activity of pressurized liquid extracts from oenological woods. **Food Control.** 50: 581–588.

Andrikopoulos, S., Blair, A. R., Deluca, N., Fam, B. C., and Proietto, J. (2008). Evaluating the glucose tolerance test in mice. **American Journal of Physiology. Endocrinology and Metabolism.** 295(6): E1323–E1332.

Boyd, A. C., Abdel-Wahab, Y. H., McKillop, A. M., McNulty, H., Barnett, C. R.,

O'Harte, F. P., and Flatt, P. R. (2000). Impaired ability of glycated insulin to regulate plasma glucose and stimulate glucose transport and metabolism in mouse abdominal muscle. **Biochimica et Biophysica Acta.** 1523(1): 128–134.

Bursell, S. E., Clermont, A. C., Aiello, L. P., Aiello, L. M., Schlossman, D. K., Feener, E. P., Laffel, L., and King, G. L. (1999). High-dose vitamin E supplementation normalizes retinal blood flow and creatinine clearance in patients with type 1 diabetes. **Diabetes Care.** 22(8): 1245–1251.

Bussmann, R. W., Malca, G., Glenn, A., Sharon, D., Nilsen, B., Parris, B., Dubose, D., Ruiz, D., Saleda, J., Martinez, M., Carillo, L., Walker, K., Kuhlman, A., and Townesmith, A. (2011). Toxicity of medicinal plants used in traditional medicine in Northern Peru. **Journal of Ethnopharmacology.** 137(1): 121–140.

Cantley, J., and Ashcroft, F. M. (2015). QandA: insulin secretion and type 2 diabetes: why do beta-cells fail? **BMC Biology.** 13: 33.

Cariou, B., Charbonnel, B., and Staels, B. (2012). Thiazolidinediones and PPARγ agonists: Time for a reassessment. **Trends in Endocrinology and Metabolism.** 23(5): 205–215.

Castro, A. J. G., Frederico, M. J. S., Cazarolli, L. H., Mendes, C. P., Bretanha, L. C., Schmidt, É. C., Bouzon, Z. L., de Medeiros Pinto, V. A., da Fonte Ramos, C., Pizzolatti, M. G., and Silva, F. R. M. B. (2015). The mechanism of action of ursolic acid as insulin secretagogue and insulinomimetic is mediated by cross-talk between calcium and kinases to regulate glucose balance. **Biochimica et Biophysica Acta.** 1850(1): 51–61.

Charan, J., and Kantharia, N. D. (2013). How to calculate sample size in animal studies? **Journal of Pharmacology and Pharmacotherapeutics.** 4(4): 303–306.

Chaudhury, A., Duvoor, C., Reddy Dendi, V. S., Kraleti, S., Chada, A., Ravilla, R., Marco, A., Shekhawat, N., Montales, M. T., Kuriakose, K., Sasapu, A., Beebe, A., Patil, N., Musham, C. K., Lohani, G,P., and Mirza, W. (2017). Clinical review of antidiabetic drugs: implications for type 2 diabetes mellitus management. **Frontiers in Endocrinology.** 8: 6.

DeFronzo, R. A., and Tripathy, D. (2009). Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. **Diabetes Care.** 32(2): S157–S163.

Derosa, G., and Maffioli, P. (2012). α-Glucosidase inhibitors and their use in clinical practice. **Archives of Medical Science.** 8(5): 899–906.

Dey, P., Saha, M. R., Chowdhuri, S. R., Sen, A., Sarkar, M. P., Haldar, B., and Chaudhuri, T. K. (2015). Assessment of anti-diabetic activity of an ethnopharmacological plant *Nerium oleander* through alloxan induced diabetes in mice. **Journal of Ethnopharmacology.** 161: 128–137.

Dias, T., Liu, B., Jones, P., Houghton, P. J., Mota-Filipe, H., and Paulo, A. (2012). Cytoprotective effect of *Coreopsis tinctoria* extracts and flavonoids on tBHP and cytokine-induced cell injury in pancreatic MIN6 cells. **Journal of Ethnopharmacology.** 139(2): 485–492.

Dutra, R. C., Pittella, F., Dittz, D., Marcon, R., Pimenta, D. S., Lopes, M. T. P., and Raposo, N. R. B. (2012). Chemical composition and cytotoxicity activity of the essential oil of *Pterodon emarginatus* . **Revista Brasileira de Farmacognosia.** 22(5): 971-978.

Ekor, M. (2014). The growing use of herbal medicines: Issues relating to adverse reactions and challenges in monitoring safety. **Frontiers in Neurology.** 4: 1–10.

Elias, S. T., Macedo, C. C. S., Simeoni, L. A., Silveira, D., Magalhaes, P. O., Lofrano-

Porto, A., Coletta, R. D., Neves, F. A., and Guerra, E. N. S. (2016). Cytotoxic effect of *Erythroxylum daphnites* extract is associated with G1 cell cycle arrest and apoptosis in oral squamous cell carcinoma. **Cell Cycle.** 15(7): 948–956.

Ezuruike, U. F., and Prieto, J. M. (2014). The use of plants in the traditional management of diabetes in Nigeria: pharmacological and toxicological considerations. **Journal of Ethnopharmacology.** 155(2): 857–924.

Fang, Y., Su, T., Qiu, X., Mao, P., Xu, Y., Hu, Z., Zhang, Y., Zheng, X., Xie, P., and Liu, Q. (2016). Protective effect of alpha-mangostin against oxidative stress induced-retinal cell death. **Science Reports.** 6: 21018.

Fennell, C. W., Lindsey, K. L., McGaw, L. J., Sparg, S. G., Stafford, G. I., Elgorashi, E. E., Grace, O. M., and van Staden, J. (2004). Assessing African medicinal plants for efficacy and safety: pharmacological screening and toxicology. **Journal of Ethnopharmacology.** 94(2): 205–217.

Ferré, P. (2004). The biology of peroxisome proliferator-activated receptors. **Diabetes.** 53(1): S43–S50.

Florkowski, C. (2013). HbA(1c) as a diagnostic test for diabetes mellitus – reviewing the evidence. **The Clinical Biochemist Reviews.** 34(2): 75–83.

Forbes, J. M., and Cooper, M. E. (2013). Mechanisms of diabetic complications. **Physiological Reviews.** 93(1): 137–188.

Fowler, M. J. (2007). Diabetes treatment, Part 2: Oral agents for glycemic management. **Clinical Diabetes.** 25(4): 131–134.

Fowler, M. J. (2008). Microvascular and macrovascular complications of diabetes. **Clinical Diabetes.** 26(2): 77–82.

Garber, A. J. (2011). Long-acting Glucagon-Like Peptide 1 receptor agonists. **Diabetes**

Care. 34(2): S279–S284.

Gargouri, M., Magné, C., and El Feki, A. (2016). Hyperglycemia, oxidative stress, liver damage and dysfunction in alloxan-induced diabetic rat are prevented by Spirulina supplementation. **Nutrition Research.** 36(11): 1255–1268.

Geerlings, S., Fonseca, V., Castro-Diaz, D., List, J., and Parikh, S. (2014). Genital and urinary tract infections in diabetes: Impact of pharmacologically-induced glucosuria. **Diabetes Research and Clinical Practice.** 103(3): 373–381.

Gomez-Perez, F. J., and Rull, J. A. (2005). Insulin therapy: current alternatives. **Archives of Medical Research.** 36(3): 258–272.

Groop, L. C., Pelkonen, R., Koskimies, S., Bottazzo, G. F., and Doniach, D. (1986). Secondary failure to treatment with oral antidiabetic agents in non-insulin-dependent diabetes. **Diabetes Care.** 9(2): 129–133.

Grover, J. K., Yadav, S., and Vats, V. (2002). Medicinal plants of India with anti-diabetic potential. **Journal of Ethnopharmacology.** 81(1): 81–100.

Guardado-Mendoza, R., Prioletta, A., Jiménez-Ceja, L. M., Sosale, A., and Folli, F. (2013). The role of nateglinide and repaglinide, derivatives of meglitinide, in the treatment of type 2 diabetes mellitus. **Archives of Medical Science.** 9(5): 936–943.

Guillausseau, P.-J., Meas, T., Virally, M., Laloi-Michelin, M., Medeau, V., and Kevorkian, J.-P. (2008). Abnormalities in insulin secretion in type 2 diabetes mellitus. **Diabetes and Metabolism.** 34(2): S43–S48.

Gupta, P. K. (2016). Chapter 12 - Toxicological testing: In vivo systems BT. **In: Fundamentals of toxicology** (pp. 131–150). Academic Press. Retrieved from https://doi.org/10.1016/B978-0-12-805426-0.00012-3.

Gupta, R., Sharma, A. K., Dobhal, M. P., Sharma, M. C., and Gupta, R. S. (2011). Antidiabetic and antioxidant potential of beta-sitosterol in streptozotocin-induced experimental hyperglycemia. **Journal of Diabetes.** 3(1): 29–37.

Gupta, R., Sharma, A. K., Sharma, M. C., Dobhal, M. P., and Gupta, R. S. (2012). Evaluation of antidiabetic and antioxidant potential of lupeol in experimental hyperglycaemia. **Natural Product Research.** 26(12): 1125–1129.

Hassan, Z., Yam, M. F., Ahmad, M., and Yusof, A. P. M. (2010). Antidiabetic properties and mechanism of action of *Gynura procumbens* water extract in streptozotocin-induced diabetic rats. **Molecules.** 15(12). 9008–9023.

Hayashi, K., Kojima, R., and Ito, M. (2006). Strain differences in the diabetogenic activity of streptozotocin in mice. **Biological and Pharmaceutical Bulletin.** 29(6): 1110–1119.

Hegde, K., Arathi, A., and Mathew, A. (2016). Evaluation of antidiabetic activity of hydro alcoholic extract of *Chrysophyllum cainito* fruits. **International Journal of Pharmaceutical Sciences and Research.** 7(11): 4422–4428.

Henriksen, E. J., Diamond-Stanic, M. K., and Marchionne, E. M. (2011). Oxidative stress and the etiology of insulin resistance and type 2 diabetes. **Free Radical Biology and Medicine.** 51(5): 993–999.

Herman, M. A., and Kahn, B. B. (2006). Glucose transport and sensing in the maintenance of glucose homeostasis and metabolic harmony. **The Journal of Clinical Investigation.** 116(7): 1767–1775.

Hermsen, S. A. B., van den Brandhof, E.-J., van der Ven, L. T. M., and Piersma, A. H. (2011). Relative embryotoxicity of two classes of chemicals in a modified zebrafish embryotoxicity test and comparison with their in vivo potencies.

Toxicology in Vitro. 25(3): 745–753.

Hernandez, A. V, Usmani, A., Rajamanickam, A., and Moheet, A. (2011). Thiazolidinediones and risk of heart failure in patients with or at high risk of type 2 diabetes mellitus: a meta-analysis and meta-regression analysis of placebo-controlled randomized clinical trials. **American Journal of Cardiovascular Drugs.** 11(2): 115–128.

Hill, A. J., Teraoka, H., Heideman, W., and Peterson, R. E. (2005). Zebrafish as a model vertebrate for investigating chemical toxicity. **Toxicological Sciences.** 86(1): 6–19.

Hirsh, A. J., Yao, S. Y., Young, J. D., and Cheeseman, C. I. (1997). Inhibition of glucose absorption in the rat jejunum: a novel action of alpha-D-glucosidase inhibitors. **Gastroenterology.** 113(1): 205–211.

Huang, S. P., Chen, J. C., Wu, C. C., Chen, C. T., Tang, N. Y., Ho, Y. T., Lo, X., Lin, J. P., Chung, J. G., and Lin, J. G. (2009). Capsaicin-induced apoptosis in human hepatoma HepG2 cells. **Anticancer Research.** 29(1): 165–174.

Ibrahim, M. A., and Islam, M. S. (2014). Butanol fraction of *Khaya senegalensis* root modulates beta-cell function and ameliorates diabetes-related biochemical parameters in a type 2 diabetes rat model. **Journal of Ethnopharmacology.** 154(3): 832–838.

Iino, K., Fukui, T., Anzai, K., Iwase, M., Kogawa, K., Ogimoto, M., Ono, J., Asano, T., Lida, M., and Tamura, K. (2005, November). Serum vitamin C levels in type 2 diabetic nephropathy. **Diabetes Care.** 28(11): 2808–2809.

International Diabetes Federation. (2015). **IDF Diabetes Atlas.** International Diabetes Federation. Retrieved from https://doi.org/10.1289/image.ehp.v119.i03.

Issa, I. A., and Hussen Bule, M. (2015). Hypoglycemic effect of aqueous and methanolic extract of *Artemisia afra* on alloxan induced diabetic swiss albino mice. **Evidence-Based Complementary and Alternative Medicine.** 2015: 752486.

Ito, C., Maeda, R., Ishida, S., Harada, H., Inoue, N., and Sasaki, H. (2000). Importance of OGTT for diagnosing diabetes mellitus based on prevalence and incidence of retinopathy. **Diabetes Research and Clinical Practice.** 49(2): 181–186.

Johansen, J. S., Harris, A. K., Rychly, D. J., and Ergul, A. (2005). Oxidative stress and the use of antioxidants in diabetes: Linking basic science to clinical practice. **Cardiovascular Diabetology.** 4: 5.

Johnson, A., Carew, E., and Sloman, K. A. (2007). The effects of copper on the morphological and functional development of zebrafish embryos. **Aquatic Toxicology.** 84(4): 431–438.

Jovanovic, L., Hassman, D. R., Gooch, B., Jain, R., Greco, S., Khutoryansky, N., and Hale, P. M. (2004). Treatment of type 2 diabetes with a combination regimen of repaglinide plus pioglitazone. **Diabetes Research and Clinical Practice.** 63(2): 127–134.

Kaneto, H., Katakami, N., Matsuhisa, M., and Matsuoka, T. (2010). Role of reactive oxygen species in the progression of type 2 diabetes and atherosclerosis. **Mediators of Inflammation.** 2010: 453892.

Kawamori, R., Kaku, K., Hanafusa, T., Oikawa, T., Kageyama, S., and Hotta, N. (2014). Effect of combination therapy with repaglinide and metformin hydrochloride on glycemic control in Japanese patients with type 2 diabetes mellitus. **Journal of Diabetes Investigation.** 5(1): 72–79.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. **Developmental Dynamics.** 203(3): 253–310.

Kleinert, M., Liao, Y.-H., Nelson, J. L., Bernard, J. R., Wang, W., and Ivy, J. L. (2011). An amino acid mixture enhances insulin-stimulated glucose uptake in isolated rat epitrochlearis muscle. **Journal of Applied Physiology.** 111(1): 163–169.

Koffi, N., Ernest, A. K., Marie-Solange, T., Beugre, K., Noeuml, Z. G., and L. (2009). Effect of aqueous extract of *Chrysophyllum cainito* leaves on the glycaemia of diabetic rabbits. **African Journal of Pharmacy and Pharmacology.** 3(10): 501–506.

Koffi, N., Konan Édouard, K., and Kouassi, K. (2009). Ethnobotanical study of plants used to treat diabetes, in traditional medicine, by Abbey and Krobou People of Agboville (Côte-d'Ivoire). **American Journal of Scientific Research.** 2009(4): 45–58.

Kubola, J., Siriamornpun, S., and Meeso, N. (2011). Phytochemicals, vitamin C and sugar content of Thai wild fruits. **Food Chemistry.** 126(3): 972–981.

Kumar, R. B. S., Kar, B., Dolai, N., and Haldar, P. K. (2013). Study on developmental toxicity and behavioral safety of *Streblus asper* Lour. bark on zebrafish embryos. **Indian Journal of Natural Products and Resources.** 4(3): 255–259.

Kumkrai, P., Kamonwannasit, S., and Chudapongse, N. (2014). Cytoprotective and anti-diabetic effects of *Derris reticulata* aqueous extract. **Journal of Physiology and Biochemistry.** 70(3): 675–684.

Kumkrai, P., Weeranantanapan, O., and Chudapongse, N. (2015). Antioxidant, α-glucosidase inhibitory activity and sub-chronic toxicity of *Derris reticulata*

extract: its antidiabetic potential. **BMC Complementary and Alternative Medicine.** 15(1): 35.

Lenzen, S. (2008). The mechanisms of alloxan- and streptozotocin-induced diabetes. **Diabetologia.** 51(2): 216–226.

Liu, J., Li, L., Li, S., Jia, P., Deng, K., Chen, W., and Sun, X. (2017). Effects of SGLT2 inhibitors on UTIs and genital infections in type 2 diabetes mellitus: a systematic review and meta-analysis. **Scientific Reports.** 7(1): 2824.

Lohr, J. W., Willsky, G. R., and Acara, M. A. (1998). Renal drug metabolism. **Pharmacological Reviews.** 50(1): 107–142.

López-Alarcón, C., and Denicola, A. (2013). Evaluating the antioxidant capacity of natural products: A review on chemical and cellular-based assays. **Analytica Chimica Acta.** 763: 1–10.

Lu, Z.-G., Li, M.-H., Wang, J.-S., Wei, D.-D., Liu, Q.-W., and Kong, L.-Y. (2014). Developmental toxicity and neurotoxicity of two matrine-type alkaloids, matrine and sophocarpine, in zebrafish (Danio rerio) embryos/larvae. **Reproductive Toxicology.** 47: 33–41.

Luo, X.-D., Basile, M. J., and Kennelly, E. J. (2002). Polyphenolic antioxidants from the fruits of *Chrysophyllum cainito* L. (star apple). **Journal of Agricultural and Food Chemistry.** 50(6): 1379–1382.

Ma, X., Iwanaka, N., Masuda, S., Karaike, K., Egawa, T., Hamada, T., Toyoda, T., Miyamoto, L., Nakao, K., and Hayashi, T. (2009). Morus alba leaf extract stimulates 5′-AMP-activated protein kinase in isolated rat skeletal muscle. **Journal of Ethnopharmacology.** 122(1): 54–59.

Majewski, M., Kasica, N., Jakubowski, P., and Lepczyńska, M. (2017). Fresh garlic

juice versus aged garlic extract. Determination of lethal concentration (LC20 and LC50) values on zebrafish (*Danio rerio*) embryos and larvae. **Journal of Elementology.** 22(4): 1495–1505.

Malgorzata, K., Dorota, H. H., Juliusz, P., and Zbigniew, D. (2007). Discontinuous fragmentation of nuclear DNA during apoptosis revealed by discrete "sub-G1" peaks on DNA content histograms. **Cytometry Part A.** 71A(3): 125–131.

Malviya, N., Jain, S., and Malviya, S. (2010). Antidiabetic potential of medicinal plants. **Acta Poloniae Pharmaceutica.** 67(2): 113–118.

Mandrell, D., Truong, L., Jephson, C., Sarker, M. R., Moore, A., Lang, C., Simonich, M. T., and Tanguay, R. L. (2012). Automated zebrafish chorion removal and single embryo placement: optimizing throughput of zebrafish developmental toxicity screens. **Journal of Laboratory Automation.** 17(1): 66–74.

Mao, L. M., Qi, X. W., Hao, J. H., Liu, H. F., Xu, Q. H., and Bu, P. L. (2015). In vitro, ex vivo and in vivo anti-hypertensive activity of *Chrysophyllum cainito* L. extract. **International Journal of Clinical and Experimental Medicine.** 8(10): 17912–17921.

Martin, M. A., Ramos, S., Mateos, R., Izquierdo-Pulido, M., Bravo, L., and Goya, L. (2010). Protection of human HepG2 cells against oxidative stress by the flavonoid epicatechin. **Phytotherapy Research.** 24(4): 503–509.

Meira, N. A., Klein, L. C. J., Rocha, L. W., Quintal, Z. M., Monache, F. D., Cechinel Filho, V., and Quintao, N. L. M. (2014). Anti-inflammatory and anti-hypersensitive effects of the crude extract, fractions and triterpenes obtained from *Chrysophyllum cainito* leaves in mice. **Journal of Ethnopharmacology.** 151(2): 975–983.

Mohamed, E. A. H., Siddiqui, M. J. A., Ang, L. F., Sadikun, A., Chan, S. H., Tan, S. C., Asmawi, M.Z., and Yam, M. F. (2012). Potent α-glucosidase and α-amylase inhibitory activities of standardized 50% ethanolic extracts and sinensetin from *Orthosiphon stamineus* Benth as anti-diabetic mechanism. **BMC Complementary and Alternative Medicine.** 12(1): 176.

Moodley, K., Joseph, K., Naidoo, Y., Islam, S., and Mackraj, I. (2015). Antioxidant, antidiabetic and hypolipidemic effects of *Tulbaghia violacea* Harv. (wild garlic) rhizome methanolic extract in a diabetic rat model. **BMC Complementary and Alternative Medicine.** 15: 408.

Moreira, D. de L., Teixeira, S. S., Monteiro, M. H. D., De-Oliveira, A. C. A. X., and Paumgartten, F. J. R. (2014). Traditional use and safety of herbal medicines. **Revista Brasileira de Farmacognosia.** 24(2): 248–257.

Morton, J. F. (1987). The star apple. **In: Fruits of Warm Climates** (pp. 408–410). Julia F. Morton, Miami, FL.

Mukinda, J. T., and Eagles, P. F. K. (2010). Acute and sub-chronic oral toxicity profiles of the aqueous extract of Polygala fruticosa in female mice and rats. **Journal of Ethnopharmacology.** 128(1): 236–240.

Muller, E. B., Lin, S., and Nisbet, R. M. (2015). Quantitative adverse outcome pathway analysis of hatching in zebrafish with CuO nanoparticles. **Environmental Science and Technology.** 49(19): 11817–11824.

Nasri, H., and Shirzad, H. (2013). Toxicity and safety of medicinal plants. **Journal of HerbMed Pharmacology.** 2(2): 21–22.

National institute of diabetes and digestive and kidney diseases. (2013). Your guide to diabetes; type 1 and type 2. **U.S. Department of Health and Human Services.**

14(4016), 1–67.

Nazaruk, J., and Borzym-Kluczyk, M. (2015). The role of triterpenes in the management of diabetes mellitus and its complications. **Phytochemistry Reviews.** 14(4): 675–690.

Nesto, R. W., Bell, D., Bonow, R. O., Fonseca, V., Grundy, S. M., Horton, E. S., Le Winter, M., Porte, D., Semenkovich, C. F., Smith, S., Young, L., H., and Kahn, R. (2003). Thiazolidinedione use, fluid retention, and congestive heart failure: a consensus statement from the American Heart Association and American Diabetes Association. October 7, 2003. **Circulation.** 108(23): 2941–2948.

Oboh, G., Ogunsuyi, O. B., Ogunbadejo, M. D., and Adefegha, S. A. (2016). Influence of gallic acid on α-amylase and α-glucosidase inhibitory properties of acarbose. **Journal of Food and Drug Analysis.** 24(3): 627–634.

O'Brien, B. A., Harmon, B. V., Cameron, D. P., and Allan, D. J. (1996). Beta-cell apoptosis is responsible for the development of IDDM in the multiple low-dose streptozotocin model. **The Journal of Pathology.** 178(2): 176–181.

OECD. (2002). Test No. 423: Acute Oral toxicity - Acute Toxic Class Method. **OECD Guidelines for the Testing of Chemicals**. Section 4, No. 630. OECD Publishing. Paris.

OECD. (2013). Test No. 236: Fish Embryo Acute Toxicity (FET) Test. **OECD Guidelines for the Testing of Chemicals.** Section 2, No. 55. OECD Publishing. Paris.

Ogurtsova, K., da Rocha Fernandes, J. D., Huang, Y., Linnenkamp, U., Guariguata, L., Cho, N. H., Cavan, D., Shaw, J. E., and Makaroff, L. E. (2017). IDF Diabetes Atlas: Global estimates for the prevalence of diabetes for 2015 and 2040. **Diabetes**

Research and Clinical Practice. 128: 40–50.

O'Harte, F. P., Gray, A. M., and Flatt, P. R. (1998). Gastric inhibitory polypeptide and effects of glycation on glucose transport and metabolism in isolated mouse abdominal muscle. **The Journal of Endocrinology.** 156(2): 237–243.

Olefsky, J. M. (2000). Treatment of insulin resistance with peroxisome proliferator–activated receptor γ agonists. **The Journal of Clinical Investigation.** 106(4): 467–472.

Oranusi, S. U., Braide, W., and Umeze, R. U. (2015). Antimicrobial activities and chemical compositions of *Chrysophyllum cainito* (star apple) fruit. **Ambit Journal.** 1(2): 8–24.

Ozougwu, O. (2013). The pathogenesis and pathophysiology of type 1 and type 2 diabetes mellitus. **Journal of Physiology and Pathophysiology.** 4(4): 46–57.

Parra-Naranjo, A., Delgado-Montemayor, C., Fraga-López, A., Castañeda-Corral, G., Salazar-Aranda, R., Acevedo-Fernández, J. J., and Waksman, N. (2017). Acute hypoglycemic and antidiabetic effect of teuhetenone a isolated from *Turnera diffusa*. **Molecules**. 22(4): pii: E599.

Pecoits-Filho, R., Abensur, H., Betônico, C. C. R., Machado, A. D., Parente, E. B., Queiroz, M., Salles, J. E. N., Tatin, S., and Vencio, S. (2016). Interactions between kidney disease and diabetes: dangerous liaisons. **Diabetology and Metabolic Syndrome.** 8: 50.

Pham, H. H. (1999). **An illustrated flora of Vietnam (Vietnamese)**. Youth Publisher. Vietnamese book.

Phillips, P. (2012). Oral glucose tolerance testing. **Australian Family Physician.** 41: 391–393.

Piluzza, G., and Bullitta, S. (2011). Correlations between phenolic content and antioxidant properties in twenty-four plant species of traditional ethnoveterinary use in the Mediterranean area. **Pharmaceutical Biology.** 49(3): 240–247.

Ping, K. Y., Darah, I., Chen, Y., Sreeramanan, S., and Sasidharan, S. (2013). Acute and subchronic toxicity study of *Euphorbia hirta* L . methanol extract in rats. **BioMed Research International.** 2013: 182064.

Pino, J., Marbot, R., and Rosado, A. (2002). Volatile constituents of star apple (*Chrysophyllum cainito* L.) from Cuba. **Flavour and Fragrance Journal.** 17(5): 401–403.

Proks, P., Reimann, F., Green, N., Gribble, F., and Ashcroft, F. (2002). Sulfonylurea stimulation of insulin secretion. **Diabetes.** 51(3): S368–S376.

Pumiputavon, K., Chaowasku, T., Saenjum, C., Osathanunkul, M., Wungsintaweekul, B., Chawansuntati, K., Wipasa, J., and Lithanatudom, P. (2017). Cell cycle arrest and apoptosis induction by methanolic leaves extracts of four Annonaceae plants. **BMC Complementary and Alternative Medicine.** 17(1): 294.

Raskin, P., McGill, J., Saad, M. F., Cappleman, J. M., Kaye, W., Khutoryansky, N., and Hale, P. M. (2004). Combination therapy for type 2 diabetes: repaglinide plus rosiglitazone. **Diabetic Medicine.** 21(4): 329–335.

Reed, M. J., Meszaros, K., Entes, L. J., Claypool, M. D., Pinkett, J. G., Gadbois, T. M., and Reaven, G. M. (2000). A new rat model of type 2 diabetes: the fat-fed, streptozotocin-treated rat. **Metabolism.** 49(11): 1390–1394.

Rena, G., Hardie, D. G., and Pearson, E. R. (2017). The mechanisms of action of metformin. **Diabetologia.** 60(9): 1577–1585.

Riccardi, C., and Nicoletti, I. (2006). Analysis of apoptosis by propidium iodide

staining and flow cytometry. **Nature Protocols.** 1: 1458.

Robertson, R. P. (2006). Oxidative stress and impaired insulin secretion in type 2 diabetes. **Current Opinion in Pharmacology.** 6(6): 615–619.

Röder, P. V, Wu, B., Liu, Y., and Han, W. (2016). Pancreatic regulation of glucose homeostasis. **Experimental and Molecular Medicine.** 48(3): e219.

Röhling, M., Herder, C., Stemper, T., and Müssig, K. (2016). Influence of acute and chronic exercise on glucose uptake. **Journal of Diabetes Research.** 2016: 2868652.

Rose, A. J., and Richter, E. A. (2005). Skeletal muscle glucose uptake during exercise: how is it regulated? **Physiology.** 20: 260–270.

Sahi, J., Grepper, S., and Smith, C. (2010). Hepatocytes as a tool in drug metabolism, transport and safety evaluations in drug discovery. **Current Drug Discovery Technologies.** 7(3): 188–198.

Saini, V. (2010). Molecular mechanisms of insulin resistance in type 2 diabetes mellitus. **World Journal of Diabetes.** 1(3): 68–75.

Sakai, K., Matsumoto, K., Nishikawa, T., Suefuji, M., Nakamaru, K., Hirashima, Y., Kawashima, J., Shirotani, T., Ichinose, K., Brownlee, M., and Araki, E. (2003). Mitochondrial reactive oxygen species reduce insulin secretion by pancreatic β-cells. **Biochemical and Biophysical Research Communications.** 300(1): 216–222.

Saleh, F. A., El-Darra, N., and Raafat, K. (2017). Hypoglycemic effects of *Prunus cerasus* L. pulp and seed extracts on alloxan-induced diabetic mice with histopathological evaluation. **Biomedicine and pharmacotherapy.** 88: 870–877.

Samuel, V. T., and Shulman, G. I. (2012). Mechanisms for insulin resistance: Common

threads and missing links. **Cell.** 148(5): 852–871.

Santos, M. M. R., Agpaoa, A. R., Sayson, A. D., Castro, M. E. G. De, Dulay, M. R., Mari, M., and Santos, R. (2017). Toxic and teratogenic effects of water leaf extract of *Momordica charantia* in zebrafish (*Danio rerio*) embryos. **Der Pharma Chemica.** 9(6): 119–122.

Sass, N. (2000). Humane endpoints and acute toxicity testing. **ILAR Journal.** 41(2): 114–123.

Satoh, T. (2014). Molecular mechanisms for the regulation of insulin-stimulated glucose uptake by small guanosine triphosphatases in skeletal muscle and adipocytes. **International Journal of Molecular Sciences.** 15(10): 18677–18692.

Schmitz, O., Brock, B., and Rungby, J. (2004). Amylin agonists: a novel approach in the treatment of diabetes. **Diabetes.** 53(3): S233–S238.

Shailajan, S., and Gurjar, D. (2014). Pharmacognostic and phytochemical evaluation of *Chrysophyllum cainito* Linn. leaves. **International Journal of Pharmaceutical Sciences Review and Research.** 26(1): 106–111.

Shailajan, S., and Gurjar, D. (2016). Wound healing activity of *Chrysophyllum cainito* L. leaves: Evaluation in rats using excision wound model. **Journal of Young Pharmacists.** 8(2): 96–103.

Sharma, V., and Paliwal, R. (2013). Preliminary phytochemical investigation and thin layer chromatography profiling of sequential extracts of *Moringa oleifera* pods. **International Journal of Green Pharmacy.** 7: 41–45.

Silva, M. G. B., Aragão, T. P., Vasconcelos, C. F. B., Ferreira, P. A., Andrade, B. A., Costa, I. M. A., Costa-Silva, J. H., Wanderley, A. G., and Lafayette, S. S. L. (2011). Acute and subacute toxicity of *Cassia occidentalis* L. stem and leaf in

Wistar rats. **Journal of Ethnopharmacology.** 136(2): 341–346.

Skotti, E., Anastasaki, E., Kanellou, G., Polissiou, M., and Tarantilis, P. A. (2014). Total phenolic content, antioxidant activity and toxicity of aqueous extracts from selected Greek medicinal and aromatic plants. **Industrial Crops and Products.** 53: 46–54.

Strober, W. (2001). Trypan blue exclusion test of cell viability. **Current Protocols in Immunology.** Appendix 3: Appendix 3B.

Stumvoll, M., Goldstein, B. J., and van Haeften, T. W. (2005). Type 2 diabetes: principles of pathogenesis and therapy. **Lancet.** 365(9467): 1333–1346.

Sudan, R., Bhagat, M., Gupta, S., Singh, J., and Koul, A. (2014). Iron (FeII) chelation, ferric reducing antioxidant power, and immune modulating potential of *Arisaema jacquemontii* (Himalayan cobra lily). **BioMed Research International.** 2014: 179865.

Sun, P.-C., Tzao, C., Chen, B.-H., Liu, C.-W., Yu, C.-P., and Jin, J.-S. (2010). Suberoylanilide hydroxamic acid induces apoptosis and sub-G1 arrest of 320 HSR colon cancer cells. **Journal of Biomedical Science.** 17(1): 76.

Syahbirin, G., Mumuh, N., and Mohamad, K. (2017). Curcuminoid and toxicity levels of ethanol extract of javanese ginger (*Curcuma xanthorrhiza*) on brine shrimp (*Artemia salina*) larvae and zebrafish (*Danio rerio*) embryos. **Asian Journal of Pharmaceutical and Clinical Research.** 10(4): 169–173.

Tahrani, A. A., Bailey, C. J., Del Prato, S., and Barnett, A. H. (2011). Management of type 2 diabetes: new and future developments in treatment. **Lancet.** 378(9786): 182–197.

Tamilselvan, N., Thirumalai, T., Shyamala, P., and David, E. (2014). A review on some

poisonous plants and their medicinal values. **Journal of Acute Disease.** 3(2): 85–89.

Taylor, R. (2012). Insulin resistance and type 2 diabetes. **Diabetes.** 61(4): 778–779.

Vlavcheski, F., Naimi, M., Murphy, B., Hudlicky, T., and Tsiani, E. (2017). Rosmarinic acid, a rosemary extract polyphenol, increases skeletal muscle cell glucose uptake and activates AMPK. **Molecules.** 22(10): pii: E1669.

Voloboueva, L. A., Liu, J., Suh, J. H., Ames, B. N., and Miller, S. S. (2005). (R)-alpha-lipoic acid protects retinal pigment epithelial cells from oxidative damage. **Investigative Ophthalmology and Visual Science.** 46(11): 4302–4310.

Widyawati, T., Yusoff, A. N., Asmawi, Z. M., and Ahmad, M. (2015). Antihyperglycemic effect of methanol extract of *Syzygium polyanthum* (Wight.) leaf in streptozotocin-induced diabetic rats. **Nutrients.** https://doi.org/doi: 10.3390/nu7095365.

Word Health Organization. (2016). **WHO diabetes country profiles 2016.** Geneva: World Health Organization. Retrieved from http://www.who.int/diabetes/country-profiles/en/.

World Health Organisation. (2004). **WHO guidelines on safety monitoring of herbal medicines in pharmacovigilance systems.** Geneva: World Health Organization. Retrieved from http://www.who.int/iris/handle/10665/43034.

World Health Organization. (2011). **Use of glycated haemoglobin (HbA1c) in the diagnosis of diabetes mellitus: abbreviated report of a WHO consultation.** Geneva: World Health Organization. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK304267/.

Yadav, R. N. S., and Agarwala, M. (2011). Phytochemical analysis of some medicinal

plants. **Journal of Phytology.** 3(12): 10–14.

Yang, J. Y., Kang, M. Y., Nam, S. H., and Friedman, M. (2012). Antidiabetic effects of rice hull smoke extract in alloxan-induced diabetic mice. **Journal of Agricultural and Food Chemistry.** 60(1): 87–94.

Yang, L., Ho, N. Y., Alshut, R., Legradi, J., Weiss, C., Reischl, M., Mikut, R., Leibel, U., Müller, F., and Strähle, U. (2009). Zebrafish embryos as models for embryotoxic and teratological effects of chemicals. **Reproductive Toxicology.** 28(2): 245–253.

Zhang, P., Zhang, X., Brown, J., Vistisen, D., Sicree, R., Shaw, J., and Nichols, G. (2010). Global healthcare expenditure on diabetes for 2010 and 2030. **Diabetes Research and Clinical Practice.** 87(3): 293–301.

Zheng, Y., Ley, S. H., and Hu, F. B. (2017). Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. **Nature Reviews Endocrinology.** 14: 88.

APPENDICES

APPENDIX A

MAIJOR CHEMICALS AND INSTRUMENTS

Table A.1 List of major chemicals used in this study.

Chemical name	**Source**
Folin-Ciocalteu reagent	Carlo-Ebar, Val de Reuil Cedex, France
2,2-diphenyl-1-picrylhydrazyl (DPPH) radical	Sigma, MO, USA
2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS)	Sigma, MO, USA
2,4,6-tri(2-pyridyl)-s-triazine (TPTZ)	Sigma, MO, USA
2,4,6-tripytidyl-s-triazine (TPTZ)	Sigma, MO, USA
Acarbose	Fluka, ND, USA
Ascorbic acid	Carlo-Ebar, Val de Reuil Cedex, France
Butylated hydroxytoluene	Fluka, ND, USA
Gallic acid	Sigma, MO, USA
Cell culture media and components	Gibco, NY, USA
MTT	Invitrogen, OR, USA
Trypan blue	Sigma, MO, USA
Tali™ apoptosis kit	Invitrogen, OR, USA
Tali™ cell cycle kit	Invitrogen, OR, USA
α-Glucosidase	Sigma, MO, USA
Tert-butyl hydroperoxide	Sigma, MO, USA
Alloxan monohydrate	Sigma, MO, USA
Peroxidase-glucose oxidase capsule	Sigma, MO, USA
Blood glucose test strip	Terumo, Tokyo, Japan

Table A.2 List of instruments used in this study.

Instrument name	**Source**
Cell culture well plate and flash	Nunc
Autoclave	Hirayama
Centrifuge Universal 320 R	Hettich
Centrifuge CT15RT	Techcom
Benchmark Plus microplate spectrophotometer	Bio-rad
Haemocytometer	Fisher Scientific
Cell counter TC20	Bio-rad
Lyophilizer	Labconco
Rotary evaporator	Buchi
Microscope CKX4	Olympus
Everted phase contract microscope IX51	Olympus
Stereo microscope	Olympus
Microscope Eclipse 80i	Nikon
Varioskan Lux spectrophotometer	Thermo scientific
Tali® image-based cytometer	Thermo scientific
Scale BP3100 S	Satorious
Scale ADAM PW254	ADAM
Medisafe® Glucometer	Terumo

APPENDIX B

TOXICOLOGICAL EFFECT OF TBHP

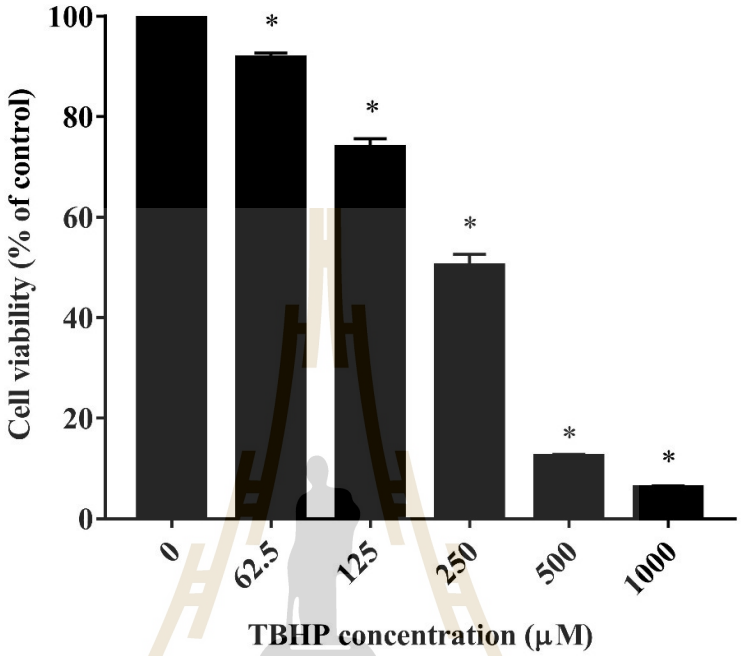
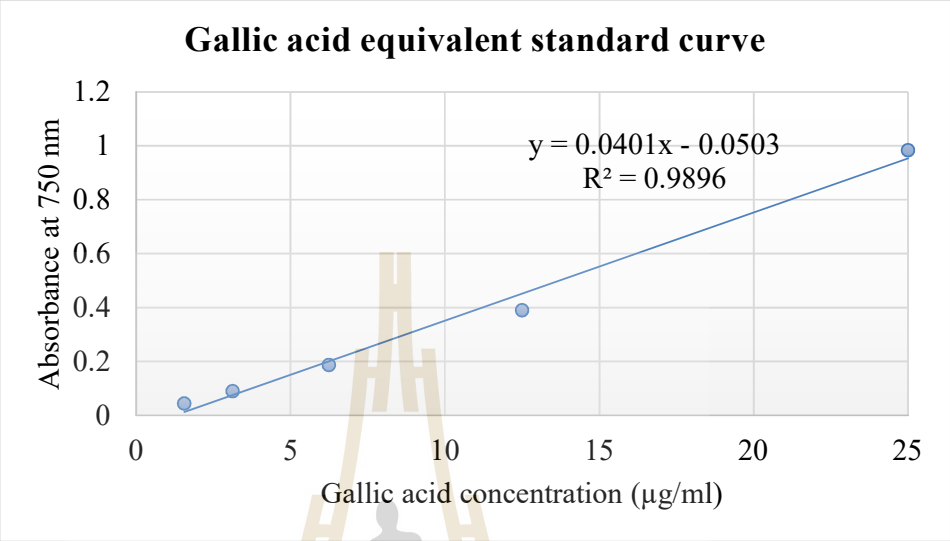


Figure B Effect of TBHP on HepG2 cells viability. HepG2 cells ($4x10^4$ cells/well) were treated with TBHP for 3 hours. Values are expressed as mean ± SEM (n=3). $^*P<0.05$ compared with control by one-way ANOVA followed by SNK.

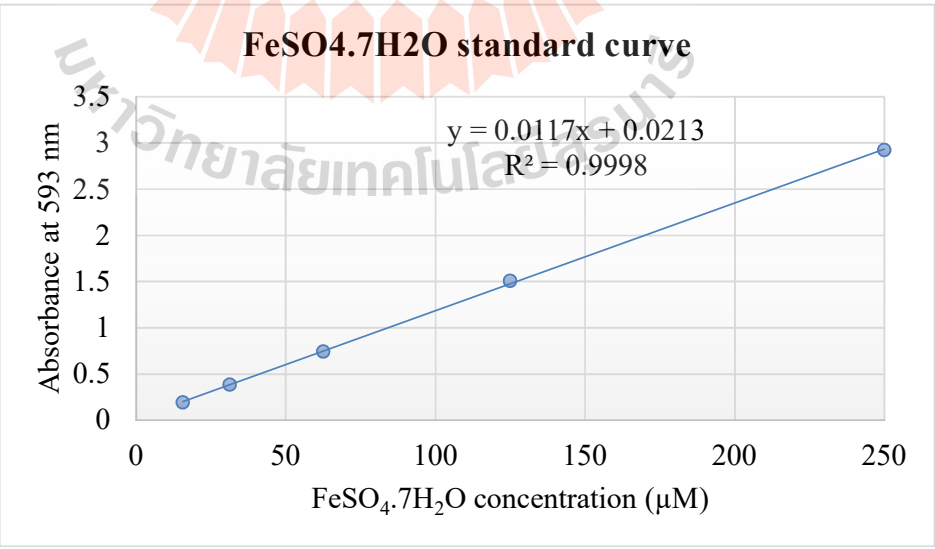
APPENDIX C

STANDARD CURVES

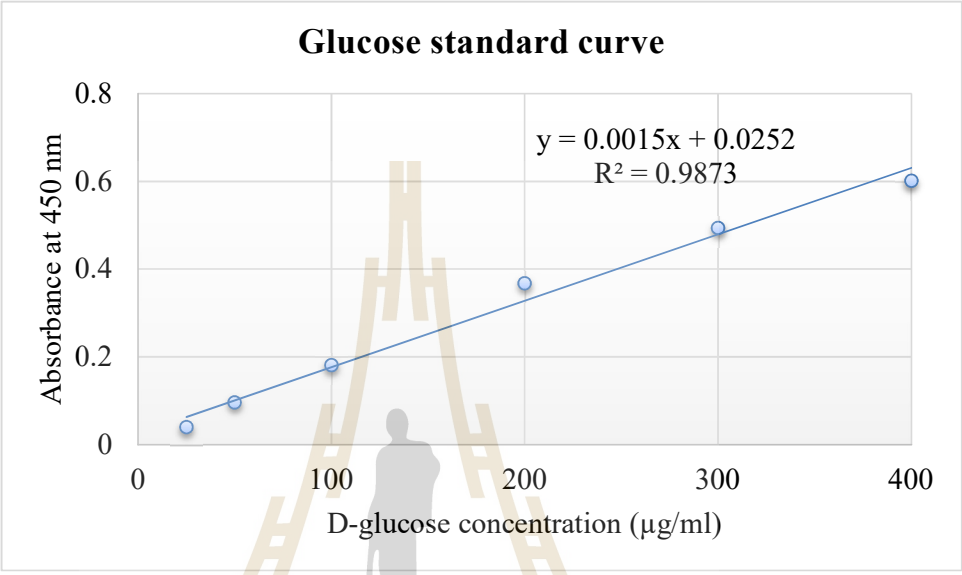
C.1 Standard Gallic acid



C.2 Standard $FeSO_4$



C.3 Glucose standard curve



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