ANTIHYPERGLYCEMIC ACTIVITY OF THE AQUEOUS EXTRACT OF *DERRIS RETICULATA* CRAIB. STEMS AND ITS MECHANISMS OF ACTION

Pakarang Kumkrai

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

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ฤทธิ์ลดระดับน้ำตาลในเลือดของสารสกัดน้ำจากลำต้นชะเอมเหนือและกลไกการออกฤทธิ์

นางสาวปะการัง คำไกร

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

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ปะการัง คำไกร : ฤทธิ์ลดระดับน้ำตาลในเลือดของสารสกัดน้ำจากลำต้นชะเอมเหนือและกลไกการออกฤทธิ์ (ANTIHYPERGLYCEMIC ACTIVITY OF THE AQUEOUS EXTRACT OF *DERRIS RETICULATA* CRAIB. STEMS AND ITS MECHANISMS OF ACTION) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.นวลน้อย จูฑะพงษ์, 113 หน้า.

Derris reticulata Craib. (ชะเอมเหนือ) เป็นพืชสมุนไพรในวงศ์ Leguminosae ในประเทศไทยมีการใช้ชะเอมเหนือเพื่อเป็นทางเลือกในการรักษาโรคเบาหวาน อย่างไรก็ตามยังไม่มีรายงานข้อมูลทางวิทยาศาสตร์เกี่ยวกับฤทธิ์ต้านเบาหวานของชะเอมเหนือมาก่อน วัตถุประสงค์ของการศึกษานี้คือ ตรวจสอบฤทธิ์ลดระดับน้ำตาลในเลือดของสารสกัดน้ำจากลำต้นชะเอมเหนือและกลไกการออกฤทธิ์ นอกจากนี้ได้ทำการตรวจสอบพฤกษเคมีเบื้องต้น ฤทธิ์การต้านอนุมูลอิสระ รวมทั้งศึกษาพิษเฉียบพลันและพิษกึ่งเรื้อรังของสารสกัด

การตรวจสอบพฤกษเคมีเบื้องต้นของสารสกัดชะเอมเหนือพบว่ามีเทอร์ปีนอยด์ ฟลาโวนอยด์ ซาโปนิน แทนนิน และสารประกอบฟีนอลิก สารสกัดชะเอมเหนือและสารมาตรฐาน butylated hydroxytoluene มีฤทธิ์กำจัดอนุมูลอิสระ ABTS โดยมีค่า IC_{50} เท่ากับ 515.05 ± 0.13 µg/ml และ 83.05 ± 0.13 µg/ml ตามลำดับ ในการทดสอบโดยใช้วิธี DPPH พบว่าสารสกัดชะเอมเหนือมีค่า IC_{50} เท่ากับ 239.85 ± 0.13 µg/ml ในขณะที่ ascorbic acid มีค่า IC_{50} เท่ากับ 1.23 ± 0.18 µg/ml ค่า FRAP ของสารสกัดชะเอมเหนือ คือ 0.23 ± 0.05 µmol Fe^{2+}/mg dried extract สำหรับการศึกษาโดยใช้เซลล์มะเร็ง RINm5F เพื่อตรวจสอบฤทธิ์การปกป้องเซลล์ที่ถูกทำลายให้เสียหายด้วยสารอัลลอกแซน พบว่าก่อนและหลังการให้สารสกัดชะเอมเหนือขนาด 250 และ 500 µg/ml สามารถป้องกันความเสียหายที่เกิดขึ้นได้อย่างมีนัยสำคัญ นอกจากนี้ยังได้ทำการศึกษาฤทธิ์ลดระดับน้ำตาลในเลือดของสารสกัดชะเอมเหนือในหนูที่ถูกชักนำให้เป็นเบาหวานด้วยสารอัลลอกแซน พบว่าระดับน้ำตาลในเลือดลดลงและน้ำหนักของหนูเพิ่มขึ้นอย่างมีนัยสำคัญในหนูเบาหวานที่ได้รับสารสกัดชะเอมเหนือ 250 mg/kg เป็นระยะเวลา 15 วัน อีกทั้งค่า aspartate transaminase (AST) และ alanine transaminase (ALT) มีระดับลดลงอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับหนูเบาหวานกลุ่มควบคุม ผลการศึกษาด้านจุลพยาธิวิทยาแสดงให้เห็นว่าตับอ่อนของหนูเบาหวานที่ได้รับสารสกัดชะเอมเหนือมีความเสียหายน้อยกว่าเมื่อเปรียบเทียบกับหนูเบาหวานกลุ่มควบคุม การศึกษาฤทธิ์ของสารสกัดชะเอมเหนือต่อการดูดซึมกลูโคสโดยใช้วิธีกลับลำไส้ส่วนเจจูนัมของหนู ผลการศึกษาพบว่าสารสกัดชะเอมเหนือสามารถยับยั้งการดูดซึมกลูโคสได้ การศึกษาฤทธิ์ของสารสกัดชะเอมเหนือมีความแตกต่างจากไกลเบนคลาไมด์ซึ่งเป็นสารกระตุ้นการหลั่งอินซูลิน โดยสารสกัดชะเอมเหนือนั้นไม่ทำให้เกิดการหลั่งอินซูลินเพิ่มขึ้น จากการทดสอบพิษเฉียบพลันโดยการป้อนสารสกัดชะเอมเหนือเพียงครั้งเดียวให้กับหนูเพศผู้และเพศเมียนั้นไม่พบทั้งอาการพิษและการตาย

โดยมีค่า LD_{50} มากกว่า 2000 mg/kg ส่วนการศึกษาความเป็นพิษกึ่งเรื้อรังด้วยการป้อนสารจนถึงระดับ 2000 mg/kg เป็นระยะเวลา 42 วัน ผลการศึกษาพบว่าไม่เกิดความเป็นพิษต่อการเจริญเติบโต พฤติกรรม ค่าทางโลหิตวิทยา ตับและไตของสัตว์ทดลอง

จากการศึกษาในครั้งนี้สรุปได้ว่าสารสกัดชะเอมเหนือมีฤทธิ์ในการลดระดับน้ำตาลในเลือดและค่อนข้างมีความปลอดภัยสูง ซึ่งกลไกการออกฤทธิ์นั้นเป็นไปได้ว่าเกิดจากสารสกัดชะเอมเหนือมีฤทธิ์ปกป้องความเสียหายของเซลล์ตับอ่อนและมีฤทธิ์ในการยับยั้งการดูดซึมกลูโคส สารสกัดไม่มีฤทธิ์กระตุ้นการหลั่งอินซูลิน สำหรับสารออกฤทธิ์นั้นมีความจำเป็นที่จะต้องทำการศึกษาต่อไป

สาขาวิชาเภสัชวิทยา ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษา _____

PAKARANG KUMKRAI : ANTIHYPERGLYCEMIC ACTIVITY OF THE AQUEOUS EXTRACT OF *DERRIS RETICULATA* CRAIB. STEMS AND ITS MECHANISMS OF ACTION. THESIS ADVISOR : ASSOC. PROF. NUANNOI CHUDAPONGSE, Ph.D. 113 PP.

DERRIS RETICULATA/ANTIOXIDANT/ACUTE TOXICITY/SUB-CHRONIC TOXICITY/ANTIHYPERGLYCEMIC

Derris reticulata Craib. (Cha-em-nuea) is a medicinal plant in the Leguminosae family. In Thailand, *D. reticulata* has been employed as alternative diabetes treatment. However, the scientific data for antidiabetic activity of *D. reticulata* has never been reported. The objectives of this study were to investigate an antihyperglycemic activity of the aqueous extract of *D. reticulata* stems and its mechanisms of action. Furthermore, phytochemical screening, antioxidant activity, acute and sub-chronic toxicities were examined as well.

The phytochemical screening of *D. reticulata* extract showed the presence of terpenoids, flavonoids, saponins, tannins, and phenolic compounds. *D. reticulata* extract and the standard butylated hydroxytoluene exhibited ABTS radical scavenging activity with IC_{50} values 515.05 ± 0.13 µg/ml and 83.05 ± 0.13 µg/ml, respectively. In DPPH assay, the IC_{50} value of *D. reticulata* extract was 239.85 ± 0.13 µg/ml, whereas the IC_{50} value of ascorbic acid was 1.23 ± 0.18 µg/ml. The FRAP value of *D. reticulata* extract was 0.23 ± 0.05 µmol Fe^{2+}/mg dried extract. The insulin-secreting cell lines RINm5F was used to examine cytoprotective effect on alloxan-induced cell damage. Both pre- and post-treatments with *D. reticulata* extract at the doses of 250 and 500 µg/ml were found to have significant protective action *in vitro*. The antihyperglycemic activity of *D. reticulata* extract was further studied in alloxan-

induced diabetic rats. A significant reduction in blood glucose level and an increase in body weight were observed in diabetic rats treated with *D. reticulata* extract at daily dose of 250 mg/kg for 15 days. Aspartate transaminase (AST) and alanine transaminase (ALT) levels were also decreased significantly compared to diabetic control group. Histopathological examination revealed that pancreatic cells of the extract-treated diabetic rat were less damage than those of the diabetic control group. The effect of the *D. reticulata* extract on glucose absorption was studied using everted sac of rat jejunum. The result showed that *D. reticulata* extract suppressed glucose absorption. The result showed that unlike the insulin secretagogue glibenclamide, *D. reticulata* extract did not stimulate insulin secretion. In the acute toxicity, single dose oral administration of *D. reticulata* extract to male and female rats did not induce signs of toxicity or mortality with LD_{50} greater than 2000 mg/kg. Sub-chronic toxicity at the doses up to 2000 mg/kg were examined for 42 days. The result showed that the repeated oral administration of *D. reticulata* extract did not produce any toxicities on animal growth, behavior, hematology, liver, and kidney.

In conclusion, the present study demonstrated that *D. reticulata* extract possessed antihyperglycemic activity with relatively wide margin of safety. The possible underlying mechanisms were cytoprotective effect on pancreatic cells and inhibition of intestinal glucose absorption. The extract did not possess a stimulatory effect on the insulin secretion. Further investigation is needed for an identification of the active ingredients.

School of Pharmacology Student's Signature_____

Academic Year 2013 Advisor's Signature_____

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LIST OF ABBREVIATIONS

μg/ml	=	Microgram per milliliter
μl	=	Microliter
ABTS	=	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate)
BAS	=	Basophil
BHT	=	Butylated hydroxytoluene
Da	=	Dalton
DPPH	=	2,2-diphenyl-1-picrylhydrazyl
EOS	=	Eosinophil
fl	=	Femtoliter
FRAP	=	Ferric reducing antioxidant power
GAE	=	Gallic acid equivalents
HCT	=	Haematocrit
HGB	=	Haemoglobin
LYM	=	Lymphocyte
MCH	=	Mean corpuscular haemoglobin
MCHC	=	Mean corpuscular haemoglobin concentration
MCV	=	Mean corpuscular volume
mg/dl	=	Milligram per deciliter
mg/kg	=	Milligram per kilogram
mg/ml	=	Milligram per milliliter

LIST OF ABBREVIATIONS (Continued)

mM	=	Millimolar
MON	=	Monophil
ng	=	Nanogram
pg	=	Picogram
PLT	=	Platelet cell
RBC	=	Red blood cell
v/v	=	Volume by volume
w/v	=	Weight by volume
WBC	=	White blood cell

CHAPTER I

INTRODUCTION

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia, resulting from defects in insulin secretion, insulin action, or both (American Diabetes Association, 2010). The estimated number of people suffering from diabetes (aged 20-79 years) was 285 million in the year of 2010 and will increase to 439 millions in the year of 2030. People with diabetes are aged over 60 years in developed countries. In contrast, diabetes in developing countries is widely occurring in working age population (aged 40-60 years) (Shaw, Sicree, and Zimmet, 2010). In addition, the prevalence of diabetes is rising 2-3 folds in Asia and Africa (Shabeer, Srivastava, and Singh, 2009). This problem is relevant to urban population in economically developing countries, including Thailand. This group of diabetic patients has significant hospitalization and financial burden (Aekplakorn et al., 2007; Wild, Roglic, Green, Sicree, and King, 2004). It has been estimated that the global health expenditure on diabetes would be at least USD (United States Dollars) 376 billion in 2010 and USD 490 billion in 2030 (Zhang et al., 2010). Hyperglycemia can cause diabetic complication in many organs and tissues, including eyes, kidneys, nerves, and blood vessels (Pandikumar, Babu, and Ignacimuthu, 2009). Furthermore, it has been reported that 50% of patients with diabetes die due to cardiovascular disease, while approximately 10-20% of diabetic patients die from kidney failure. Neuropathy affects up to 50% of patients with diabetes (WHO, 2010). However, the intensive

glycemic control can reduce the progress of diabetic complication and mortality (Stratton et al., 2000). Oral hypoglycemic agents such as sulfonylureas, biguanides, α-glucosidase inhibitors, and thiazolidinediones have been used in the treatment of diabetes mellitus. It has been found that current therapy for diabetes with hypoglycemic agents can produce side effects, including hypoglycemia, gastro-intestinal disturbances, and hepatotoxicity (Patil, Patil, Ahirwar, and Ahirwar, 2011). Therefore, the search for safer and less toxic hypoglycemic agents remains a topic of research interest. World Health Organization (WHO) has recommended the investigation of antidiabetic agents from medicinal plants (WHO Expert Committee on Diabetes Mellitus, 1980). The plants which belong to diverse groups of families such as Leguminoseae, Lamiaceae, Liliaceae, Cucurbitaceae, Asteraceae, Moraceae, Rosaceae, Euphorbiaceae, and Araliaceae which have been shown to posess potent hypoglycemic activities (Patel, Prasad, Kumar, and Hemalatha, 2012). Thus, several plants have been used as an alternative treatment in patients with diabetes (Fatima et al., 2010). Many researchers reported that medicinal plants act by various mechanisms such as increasing insulin secretion, inhibiting the intestinal absorption of glucose, increasing peripheral utilization of glucose, increasing of synthesis of hepatic glycogen or decreasing glycogenolysis (Patel et al., 2012). The combination of alternative medicine and standard modern treatment was used in developing countries (Chotchoungchatchai, Saralamp, Jenjittikul, Pornsiripongse, and Prathanturarug, 2012). In Thailand, the use of traditional medicine plays an important role with medical section in public health (Wiwanitkit, 2011). It is known that knowledge of properties or specific activities of medicinal plants has come from local medicinal plant practitioners (Smirin et al., 2010). In Thailand, *Derris reticulata* Craib. has been traditionally used for diabetic treatment. *D. reticulata*, commonly known in Thai as

Cha-em-nuea, is a climbing plant which belongs to Leguminosae family. There are some studies reporting antidiabetic activities of plants in genus *Derris* such as *D. indica* Lam. and *D. scandens* Benth. The extracts of these plants have been shown to possess strong free radical scavenging activity and inhibit intestinal α-glucosidase (Rao et al., 2009; Rao et al., 2007).

Derris plants have been reported to possess a variety of biological activities. For example, *D. trifoliata* is used for treatment of rheumatism, dysmenorrhea and asthma (Jiang et al., 2012) and *D. scandens* is used to treat arthritis patients (Laupattarakasem, Houghton, Hoult, and Itharat, 2003). In case of *D. reticulata*, it has been used as an expectorant to relieve cough (Vongnam, Wittayalertpanya, Raungrungsi, and Limpanasithikul, 2013). Moreover, all of them have been reported to possess antimicrobial activities (Chivapat, Chavalittumrong, Attawish, and Soonthornchareonnon, 2009; Khan, Omoloso, and Barewai, 2006; Sittiwet and Puangpronpitag, 2009). It has been shown that the extract from *D. scandens* stem significantly reduces myeloperoxide release and inhibits the generation of leukotriene B_4 (Laupattarakasem et al., 2003), which supports its traditional use as an anti-inflammatory agent. Similarly, the ethanolic extract from stem of *D. reticulata* has been demonstrated to exert anti-inflammatory activity by interfering with nitric oxide production of macrophage (Vongnam et al., 2013). However, the data for antidiabetic activity of *D. reticulate* has never been documented. A scientific investigation of antihyperglycemic activity of this plant is very interesting in terms of searching for a new antidiabetic agent from medicinal plants.

The aim of this study was to evaluate an antihyperglycemic potential of the aqueous extract of *D. reticulata* stems. First, an *in vitro* protective effect of *D. reticulata* extract was examined by using the insulin-secreting cell line RINm5F.

Also, antihyperglycemic activity was evaluated in alloxan-induced diabetic rats. The investigation of possible mechanisms of its antihyperglycemic action, such as protective effect on pancreatic cells, inhibition of intestinal glucose absorption and stimulation of insulin secretion *in vitro* were conducted. In addition, the present study was carried out to evaluate acute and sub-chronic toxicities of *D. reticulata* extract. Phenolic compounds and antioxidant activity of the extract, which usually associated with protective effects, were determined as well.

CHAPTER II

LITERATURE REVIEWS

2.1 *Derris reticulata* Craib.

Derris reticulata Craib. (Thai common name: Cha-em-nuea) is a climbing plant (as shown in Figure 2.1) which belongs to Leguminosae family. It is distributed in the tropical regions of Asia and East Africa (Tewtrakul, Cheenpracha, and Karalai, 2009).

Figure 2.1 *D. reticulata* Craib. The picture was taken from Prachinburi province. The arrow indicates the climbing trunk of *D. reticulata* on a wood pole.

The botanical characteristics of *D. reticulata* are described as follows (Songsak, 1995; Bangkok Herbarium, 2009; Siri Ruckhachati Nature Park, 2010). Leaves are odd pinnately compound with 5 leaflets. Leaflets are oblong-ovate, ovate-lanceolate or oblong-lanceolate. The uppermost tips are round, base round or cuneate. The petiole is 1.5-4.0 cm long and rachis with a longitudinal groove is on the upper side. Leaves have 6-8 lateral nerves anastomosing into an intramarginal nerve on both sides. Lateral nerves slightly rise above and below. The thin network veinlet presents on both surfaces. The branchlets cover with ash grey or straw-yellow-ash grey bark and pale brown lenticel. As seen in Figure 2.2A and B, legume is green or straw-yellow and short oblong pod. The seed is thick. The inflorescence flowers are papilonaceous corolla (Figure 2.2C).

Phytochemical studies for some species of *Derris* plants have been reported. Two new pyranoflavanones, 2´´´, 3´´´- epoxylupinifolin and dereticulatin isolated from the stem of *D. reticulata* Benth. were identified (Mahidol et al.,1997). Moreover, new furanoflavanoids were isolated from *D. indica* Lam. (Rango Rao et al., 2009). The study of *D. laxiflora* has revealed that it contains triterpenoids and aromatics (Chiu et al., 2008). Scandenin A, scandenin B and 4´,5´,7´-trihydroxybiprenyliso- flavone have been found in *D. scandens* Benth. (Rao et al., 2007). A benzil and isoflavone derivatives have been also isolated from *D. scandens* Benth. (Mahabusarakam, Deachathai, Phongpaichit, Jansakul, and Taylor, 2004).

Several pharmacological activities of plants in this genus have been reported. For example, derrisisoflavone and prenylated flavanones isolated from *D. ferruginea* significantly inhibited the formation of advanced glycation endproducts in protein synthesizing process, whereas prenylated flavanones exhibited strong *in vitro* antiparasitic activity against *Plasmodium falciparum* and *Leishmania major* (Morel et

al., 2013). Lupinfolin which was isolated from the seed pods of *D. trifoliata* showed moderate *in vitro* antiplasmodial activity against the D6 (chloroquine-sensitive) and W2 (chloroquine-resistant) strains of *Plasmodium falciparum* (Yenesew et al., 2009).

D. reticulate, lupinifolin which was also found to be a major compound exhibited an antiviral activity against herpes simplex virus type 1 (Wisetsutthichai, Techamahamaneerat, Junyaprasert, and Soonthornchareonnon, 2005). In addition, this plant has long been traditionally used as anti-coughing and expectorant in Thailand (Siri Ruckhachati Nature Park, 2001). Its antidiabetic application has been performed by some local medicinal plant practitioners in Prachinburi province as alternative medicine. However, to my best knowledge, scientific data to support this use is lacking.

Figure 2.2 Some parts of *D. reticulata* (A): young seed pods; (B): ripen seed pods (Siri Ruckhachati Nature Park, 2001); (C): flowers.

2.2 Antioxidant activity of medicinal plant

Antioxidants in plants have been considered to prevent the oxidative stress caused by free radicals. Free radicals are defined as chemical species containing one or more unpaired electrons. They are highly unstable and can cause damages to other molecules such as DNA, lipids, and proteins (Ali et al., 2008). Free radical species consist of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS includes superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), hydrochlorous acid (HOCl), peroxyl radical ($ROO^{\bullet}$) and hydroperoxyl radical ($HOO^{\bullet}$). Normally, oxygen molecules are used for energy production in aerobic respiration system. During electron transport in mitochondrial respiratory chain, electron can leak out from mitochondria and produces superoxide anion (Ohnishi et al., 2005). Another mechanism to produce ROS is intracellular hydrogen peroxide which can move across cell membrane and react with Fe^{2+}, Cu^{2+} ions to generate hydroxyl radical. Hydroxyl radical is highly reactive molecule and can cause various kinds of damages on biomolecules. RNS are nitric oxide ($NO^{\bullet}$), nitrogen dioxide (NO_2), and peroxynitrite ($ONOO^-$). Nitric oxide has been suggested to involve in an inflammatory condition of many diseases, including type 1 diabetes. The toxicity of nitric oxide is enhanced when it reacts with superoxide radical forming peroxynitrite anion. Environmental factors (e.g., radiation, smoking, pollution, and chemicals) are also sources of free radicals (Hazra, Biswas, and Mandal, 2010; Wang, Melnyk, Tsao, and Marcone, 2011).

The term oxidative stress is defined by an imbalance between oxidants and antioxidants. This imbalance problems contribute to several sickness such as atherosclerosis, stroke, diabetes, and cancer (Valko et al., 2007). Antioxidant defense systems against ROS and RNS mostly are endogenous enzymes such as superoxide

dismutase, catalase, glutathione peroxidase, and glutathione reductase. There are many exogenous substances, for example, vitamin E, carotenoids, and flavonoids that act as antioxidant (Young and Woodside, 2001). WHO estimates that about 80% of people around the world have used plant extracts and their active compounds for oxidative stress therapy (Craig, 1999).

A phenolic compound consists of at least one aromatic ring bearing one or more hydroxyl groups. Their structures are diverse, ranging from simple phenolic molecules to highly polymerized compounds that are referred to as polyphenols (Balasundram, Sundram, and Samman, 2006). Antioxidant properties of polyphenols have been shown to be of health benefits (Patel, Kumar, Laloo, and Hemalatha, 2011) because they are able to (1) intercept radical chain reaction by scavenging initial radicals and preventing hydrogen abstraction from substrates, (2) chelate metal ion catalysts, and (3) decompose primary products to non-radical compound (Tepe, Degerli, Arslan, Malatyali, and Sarikurkcu, 2011). Likewise, plants from Leguminosae family contain phenolic compounds, such as flavonoids and anthocyanin, which are capable of antioxidant activity (Chew, Goh, and Lim, 2009).

2.3 The control of blood glucose

Glucose is the primary source of energy for the body. Neonate and young child require glucose more than adulthood (Beardsall, Yuen, Williams, and Dunger, 2006). The normal blood glucose concentration mainly depends on the actions of two hormones, insulin and glucagon (Figure 2.3). After a meal, the blood glucose concentration is high as glucose is absorbed into the blood. High blood glucose level stimulates insulin secretion from β-cells of pancreas. Insulin exerts hypoglycemic

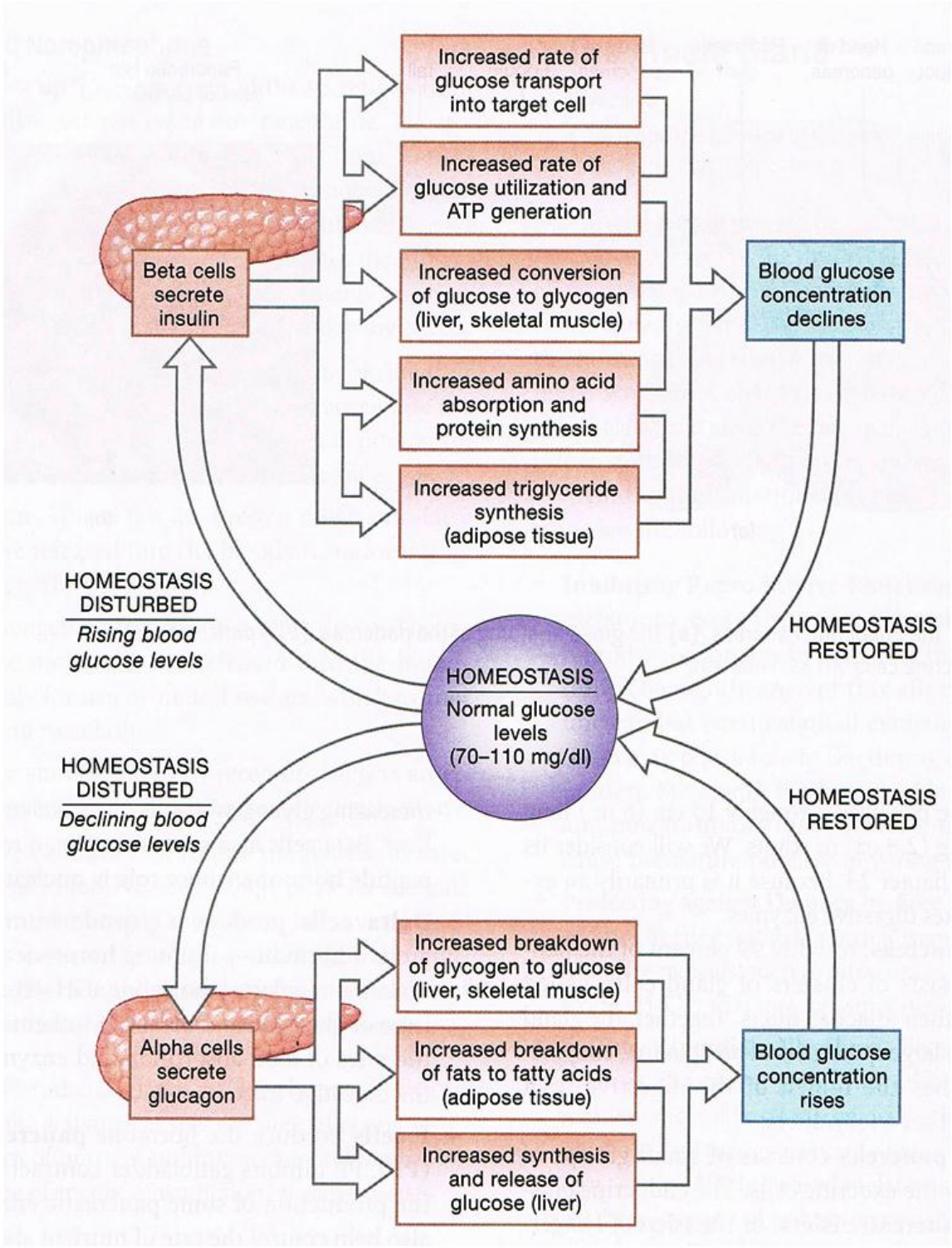

Figure 2.3 The control of blood glucose concentration (Martini, Ober, Garrison, Welch, and Hutchings, 2006).

effect by increasing the number of glucose transporters on plasma membrane. This mechanism causes an acceleration of glucose uptake and subsequently producing adenosine triphosphate (ATP) by the oxidative breakdown of glucose or glycolytic pathway. In addition, insulin promotes the cell uptake of glucose from the blood for conversion to glycogen (glycogenesis). Glycogen is stored in skeletal muscle and liver. Insulin decreases hepatic glucose output by inhibiting gluconeogenesis. The effect of insulin on protein metabolism is to increase translation of messenger RNA, resulting in an increase of protein synthesis and prevention of protein degradation. Insulin exerts effects on fat metabolism by reducing the release of fatty acids from adipose tissue and promoting the use of fatty acids for triglyceride synthesis. Thus, insulin stimulates biosynthetic pathways that lead to an increase of glucose use, carbohydrate and fat storage, and protein synthesis.

Conversely, when glucose level in blood declines, the α-cells of pancreas secrete glucagon. Glucagon causes glycogen breakdown to glucose and stimulates fat breakdown to fatty acids. In liver, glucagon stimulates amino acid uptake and changes amino acid to glucose by gluconeogenesis (Martini et al., 2006). Insulin and glucagon help maintain blood glucose levels. Insulin stimulates the removal of glucose from the blood while glucagon stimulates the release of glucose from storage site into the blood. The abnormal control of blood glucose causes hyperglycemia or hypoglycemia. This abnormality of blood glucose concentration can lead to health problems and subsequently to death (Yeo and Sawdon, 2007). Other hormones influencing the blood glucose levels are summarized in Table 2.1.

Table 2.1 Glucose control mechanisms (Karch, 2011).

Insulin	Decreases blood glucose
	Glycogen storage
	Adipose tissue deposit
	Synthesis of proteins to form amino acids
Glucagon	Increases blood glucose
Somatostatin	Decreases insulin release
	Decreases glucagon release
	Slows gastrointestinal emptying
Growth hormone	Decreases insulin sensitivity
	Increases protein building
	Increases free fatty acid formation
Incretins	Increase insulin release
	Decrease glucagon release
	Stimulate satiety center
	Slow gastrointestinal emptying
Adiponectin	Increases insulin sensitivity
	Decreases glucose output from liver
	Protects vessels from inflammatory reactions
Catecholamines	Decrease insulin release
	Increase glucose output from liver and muscles
	Increase breakdown of fat to free fatty acids
Corticosteroids	Increase glucose output
	Decrease insulin sensitivity
Endocannabinoid system	Increases food intake by blocking satiety signals
	Decreases adiponectin release
	Decrease isnsulin sensitivity
	Increase fats synthesis
	Alters gastric motility

2.4 Insulin

Deficiency of insulin causes hyperglycemia problem. The important role of insulin is to lower high blood glucose concentration to normal level. Insulin is secreted from β-cells of islet of Langerhans. The gene of insulin that encodes the preproinsulin molecule consists of four exons and two introns. Molecular weight of preproinsulin is about 11,500 Da which contains the N-terminal signal peptides, the B chain of insulin, a connecting peptide (C-peptide) and the A chain of insulin (Figure 2.4). After translation, N-terminal signal peptide of preproinsulin is cleaved and converted to proinsulin. The proinsulin molecule, containing the A and B chains of insulin and C-peptide, is then transported to Golgi apparatus where the disulfide links are built for the folded proinsulin. Thereafter, the converting enzymes cleave off C-peptide from proinsulin molecules, leaving insulin structure only the A chain containing 21 amino acids and the B chain containing 30 amino acids, which are connected by disulfide bonds. The molecular weight of insulin is about 6,000 Da (Genuth, 2004).

Half-life of insulin is about 4-6 min. Insulin is removed from the blood by a specific enzyme called insulinase or insulin degrading enzyme. This enzyme is found in liver, muscle, and kidney (Goodman, 2003). Insulin secretion is biphasic phase in both *in vitro* and *in vivo*. The initial rapid phase occurs for 1-3 min and then return towards baseline. Subsequently, the second phase of insulin secretion can be sustained for many hours (Genuth, 2004).

Biological actions of insulin are initiated when insulin binds to its cell surface receptor. The insulin receptor belongs to a family of ligand activated receptor tyrosine kinase. The insulin receptor consists of two extracellular α-subunits and two transmembrane β-subunits linked by disulfide bonds. The β-subunits contain tyrosine kinase activity in the cytosolic domain (Figure 2.5).

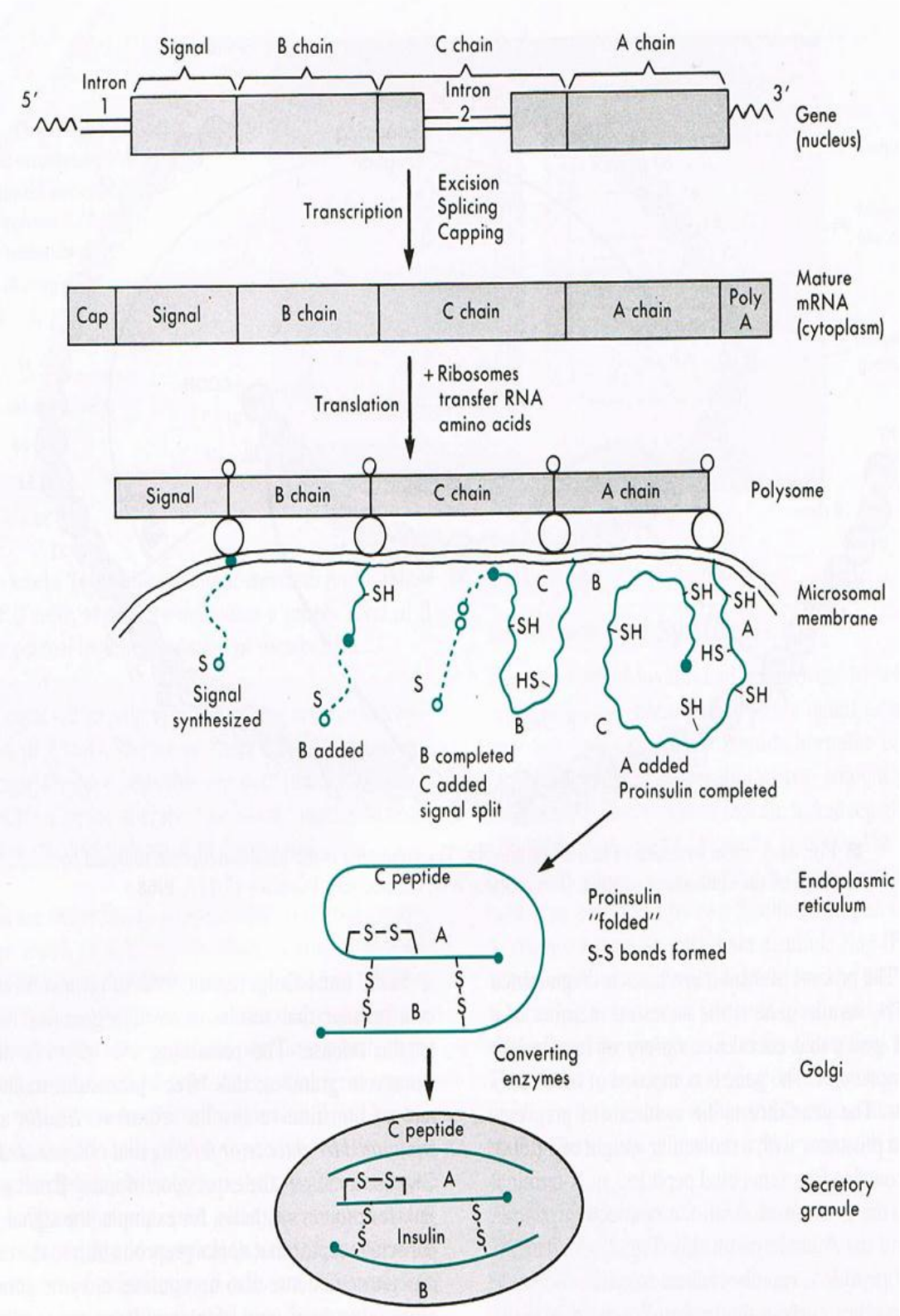

Figure 2.4 Insulin synthesis (Genuth, 2004).

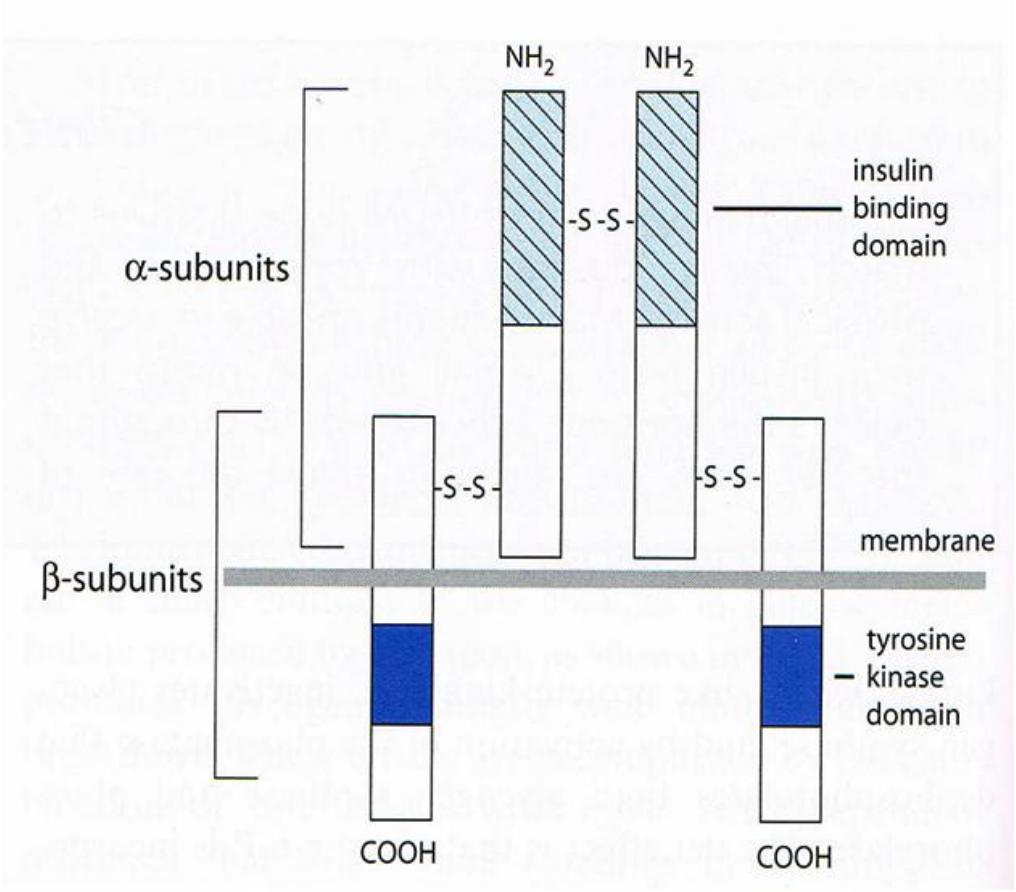

Figure 2.5 Insulin receptor (Goodman, 2003).

Mechanism of glucose stimulated insulin secretion is demonstrated in Figure 2.6. Glucose enters the β-cells via glucose transporter type 2 (GLUT2). The breakdown of glucose by glycolysis provides ATP, which causes the ATP sensitive potassium channel to close. Membrane depolarization resulting from the closure of potassium channel causes calcium channel to open, resulting in an increase of intracellular calcium concentration, which then stimulates the exocytosis of insulin granules (Goodman, 2003; Hinson, Raven, and Chew, 2007). The physiologic role of insulin is to promote storage of metabolic fuel. The effects of insulin on liver, muscle and adipose tissue are summarized in Table 2.2.

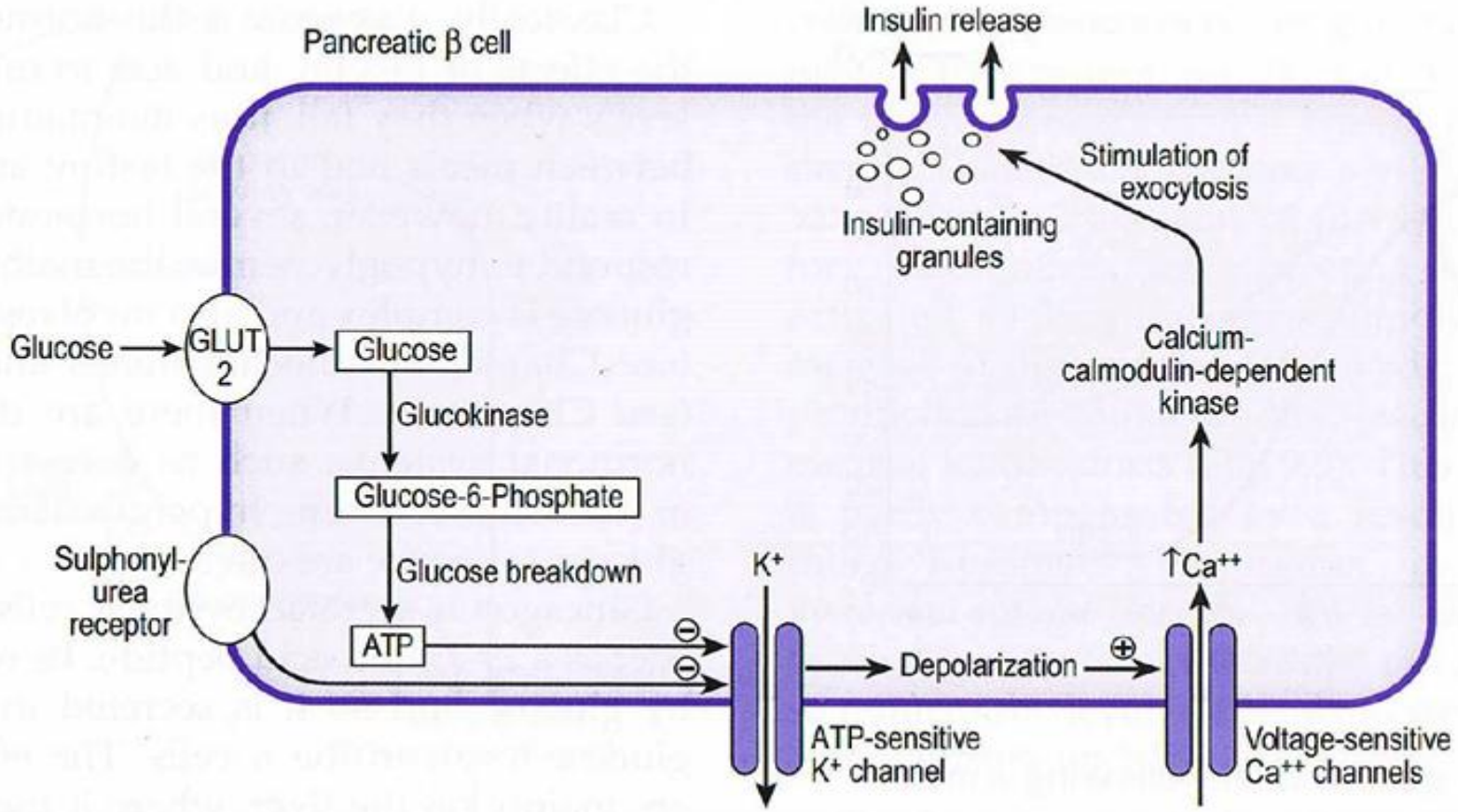

Figure 2.6 Insulin secretions (Hinson et al., 2007).

Table 2.2 Endocrine effects of insulin (Nolte, 2009).

Effects on liver:
Reversal of catabolic features of insulin deficiency
Inhibits glycogenolysis
Inhibits conversion of fatty acids and amino acids to keto acids
Inhibits conversion of amino acids to glucose
Anabolic actions
Promotes glucose storage as glycogen (induces glucokinase and glycogen synthase, inhibits phosphorylase
Increases triglyceride synthesis and very low density lipoprotein formation
Effects on muscle:
Increased protein synthesis
Increases amino acid transport
Increases ribosomal protein synthesis
Increased glycogen synthesis
Increases glucose transport
Induces glycogen synthase and inhibits phosphorylase
Effects on adipose tissue:
Increased triglyceride storage
Lipoprotein lipase is induced and activated by insulin to hydrolyze triglycerides from lipoproteins
Glucose transport into cell provides glycerol phosphate to permit esterification of fatty acids supplied by lipoprotein transport
Intracellular lipase is inhibited by insulin

2.5 Diabetes mellitus

Diabetes mellitus is a group of metabolic disorder characterized by hyperglycemia. High blood glucose concentration causes glycosuria, the presence of glucose in urine. Glycosuria leads to frequent urination and loss of water. The body compensates this condition via increasing thirst (polydipsia). Furthermore, the

inadequate insulin in patient with diabetes results in incapability of utilizing glucose properly. The body shifts to use fat and protein for energy. Fat breakdown or lipolysis causes ketoacidosis with high concentration of ketone bodies which cannot be removed from the body effectively. The protein breakdown results in an increased nitrogen waste and elevated blood urea nitrogen (Karch, 2011). Two major forms of diabetes mellitus are classified as type 1 diabetes previously known as juvenile or insulin dependent diabetes mellitus and type 2 diabetes previously known as adult or non-insulin dependent diabetes mellitus (Notkins, 2002).

2.5.1 Type 1 diabetes

The prevalence of type 1 diabetes is about 5-10%. Type 1 diabetes is caused by destruction of β-cells, resulting in absolute deficiency of insulin. This form of diabetes is associated with autoimmune damage of the β-cells, which is stimulated by environmental and genetic factors. The autoimmune components are linked with major histocompatibility complex (MHC) antigen and autoantibodies. MHC complex antigens, such as DR3 and DR4 are present in type 1 diabetic patients. Autoantibodies in type 1 diabetes include islet cell autoantibodies, autoantibodies to insulin, autoantibodies to glutamic acid decarboxylase (GAD) and autoantibodies to the tyrosine phosphatase IA-2 and IA-2β. The risk of inheriting diabetes mellitus is greater if the family has history of type 1 diabetes. Some of the patients with type 1 diabetes who have no known etiologies and no evidence of autoimmunity are called idiopathic diabetes.

2.5.2 Type 2 diabetes

The prevalence of diabetes type 2 is approximately 90-95%. Type 2 diabetes is caused by diminished insulin secretion and related with increased peripheral tissue resistance to the action of insulin. Most patients in this type are obese. In obese

subjects, insulin resistance often occurs because of down regulation of insulin receptors. When the number of receptors is decreased, the number of insulin molecules that bind to the receptors at given insulin level will be reduced, therefore the effect of insulin is diminished (American Diabetes Association, 2010).

2.5.3 Other specific types of diabetes

2.5.3.1 Genetic defects of the β-cell

Several forms of diabetes are associated with monogenic defects in β-cell function. These forms of diabetes are frequently characterized by onset of hyperglycemia at an early age, usually before 25 years of age. They are referred to as maturity-onset diabetes of the young (MODY), in which impaired insulin secretion with minimal or no defects in insulin action is found. MODY genes have been identified.

MODY-1 is a mutation in the gene-encoding hepatocyte nuclear factor (HNF)-4α, which is a member of the steroid/thyroid hormone receptor superfamily and a regulator of HNF-1α expression. MODY-2 is due to mutations in glucokinase. Glucokinase which is the starting point of glucose metabolism converts glucose to glucose-6-phosphate. ATP which is the endproduct of glucose metabolism stimulates insulin secretion from the β-cell. MODY-3 is due to transcription factor mutations in the gene-encoding HNF-1α. The mutations of this transcription factor alter insulin secretion in the mature β-cell resulting in altered β-cell development, proliferation and cell death. Other specific genetic defects including point mutations in mitochondrial DNA also lead to diabetes (American diabetes association, 2010; Kacsoh, 2000).

2.5.3.2 Genetic defects in insulin action

The genetic defects in insulin action involved the mutations of the insulin receptor resulting in hyperinsulinemia and modest hyperglycemia to severe diabetes. Furthermore, diabetes may result from disease process affecting the pancrease such as pancreatitis, trauma, infection, and pancreatectomy. Pancreatic carcinoma may also involve diabetes. Excess amounts of growth hormones, cortisol, glucagon, and epinephrine can cause diabetes. Certain viruses (congenital rubella, coxsackievirus B, cytomegalovirus, adenovirus, and mumps) have also been associated with β-cell destruction.

2.5.3.3 Gestational diabetes mellitus (GDM)

Gestational diabetes mellitus is defined as any degree of glucose intolerance with onset during pregnancy. Deterioration of glucose tolerance occurs normally during pregnancy, particularly in the 3rd trimester. Diabetes during pregnancy increased risk of developing type 2 diabetes.

2.5.4 Diagnostic criteria for diabetes mellitus

The diagnosis of diabetes mellitus is evaluated by fasting blood glucose level (FBG), oral glucose tolerance test (OGTT), and glycosylated hemoglobin test (A1C). There are criteria for diagnosis of diabetes mellitus which are classified as follows:

1. A1C $\geq$ 6.5%.

2. FBG $\geq$ 126 mg/dl (7.0 mmol/l). Fasting is defined as no caloric intake for at least 8 h.

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

4. In a patient with classic symptoms of hyperglycemic or hyperglycemia crisis, a random plasma glucose ≥ 200 mg/dl (11.1 mmol/l).

* In the absence of unequivocal hyperglycemia, criteria 1-3 should be confirmed by repeat testing (American Diabetes Association, 2010).

2.5.5 Complication of diabetes

Long term hyperglycemia and abnormalities in carbohydrate, fat, and protein metabolisms lead to diabetic complication, including a thickening of the basement membrane and thin layer of collagen filament in blood vessels. The destruction of blood vessels produces narrow vessel, causing a decrease of blood flow and inability to carry oxygen across the membrane to the tissue. These results in diabetic complications such as neuropathy, atherosclerosis, myocardial infarction, and stroke. Moreover, the blood vessels in the eyes are narrowed and closed which lead to blindness. When the basement membrane of the glomerulus is changed, it decreases filtration rate and generates nephropathy (Clayton, Stock, and Cooper, 2009; Karch, 2011).

2.6 Antidiabetic drugs

The aim of diabetic type 1 and type 2 treatments is to maintain blood glucose in normal level. WHO classificaion of diabetes is useful for determination of principle therapy. Treatment for type 1 diabetes relies on exogenous insulin because the β-cells in the pancreas cannot produce insulin. Antidiabetic drugs help to treat type 2 diabetic patients. Administration of insulin and/or oral antidiabetic drugs can decrease mortality associated with diabetes.

2.6.1 Insulin

2.6.1.1 Rapid acting insulin and short acting insulin

This class of insulin preparation includes regular insulin, insulin lispro, insulin aspart, and insulin glulisine. Regular insulin is a short acting, soluble, and crystalline zinc insulin. After injection, regular insulin takes 30 min to start acting. Insulin lispro is insulin whose two amino acids (lysine and proline) at positions 28 and 29 in the B chain are reversed. The reversal results in more rapid onset of action after subcutaneous injection than regular insulin. Insulin lispro and insulin aspart start to act within 10 min after injection and provide 1-2 h duration of action. Also, insulin glulisine is similar in the onset of action to insulin lispro and insulin aspart but has a shorter duration of action. These rapid and short acting insulins are used to control hyperglycemia without having longer lasting effects with hypoglycemia.

2.6.1.2 Intermediate acting insulin

Neutral Protamine Hagedorn (NPH) insulin is an intermediate acting insulin containing a suspension of crystalline zinc insulin and protamine. When administered subcutaneously, NPH insulin is slowly released from the protamine and becomes active, giving the intermediate acting insulin.

2.6.1.3 Long acting insulin

This group consists of insulin glargine and insulin detemir. Insulin glargine has prolonged hypoglycemic effect. Its onset of action is after 90 min. It has a prolonged duration of 24 h or more. Insulin detemir has fatty acid side chain, which enhances its action.

2.6.1.4 Insulin combination

Some preparations of insulin are a mixture of insulin, for instance, 70% NPH insulin mixed with 30% regular insulin or 50% of each.

The symptom of hypoglycemia is a common adverse effect of insulin. Other adverse effects include weight gain, lipodystrophy (less common with human insulin), allergic reaction, and local injection site reactions.

2.6.2 Oral hypoglycemic drugs

Oral antidiabetic agents act by different mechanisms to control hyperglycemia. There are several categories of oral antidiabetic drugs as follows:

2.6.2.1 Insulin secretagogues: Sulphonylureas

Sulphonylureas block the ATP sensitive potassium channels on the plasma membrane of β-cells. These result in depolarization and calcium influx and subsequently release of insulin. An administration of sulphonylureas appears to decrease hepatic glucose output and increase insulin sensitivity on peripheral tissues.

2.6.2.2 Insulin secretagogues: Meglitinide

The mode of action of meglitinide is similar to that of sulphonylureas. Meglitinide binds to a different site on the sulphonylureas receptor of ATP sensitive potassium channels. Side effects of sulphonylureas contain hypoglycemia and weight gain. These drugs should be used with caution in patients with hepatic or renal insufficiency, because delayed excretion of the drug resulting in its accumulation may cause hypoglycemia. However, meglitinide can cause hypoglycemia, the incidence of this adverse effect appears to be lower than that with the sulfonylureas.

2.6.2.3 Biguanides

Metformin is only biguanide that currently available. Its cellular mode of action is glycolysis stimulation, resulting in an increased glucose removal from the blood. It reduces hepatic glucose output and reduces glucose absorption from small intestine and enhances glucose uptake in muscle and adipose tissue. Side effects

are mainly gastrointestinal. Metformin is contraindicated in diabetics with renal and/or hepatic disease, acute myocardial infarction, severe infection, or diabetic ketoacidosis.

2.6.2.4 Thiazolidinediones

There are several members in this class, such as pioglitazone and rosiglitazone. They appear to lower insulin resistance, by stimulating peroxisome proliferator activated receptor gamma. The major action of this receptor stimulation is to increase glucose uptake and insulin sensitivity in adipose tissue, liver, and skeletal muscle. Troglitazone was first approved for treatment of type 2 diabetes but was withdrawn after a number of deaths due to hepatotoxicity were reported. Later, two members of this class, pioglitazone, and rosiglitazone, were launched to the market (Finkel, Clark, and Cubeddu, 2009). However, due to an increased risk of cardiac events, rosiglitazone has been withdrawn from the market in 2010 (North Central London Cluster, 2010). Weight increase can occur, possibly through the ability of thiazolidinediones to increase subcutaneous fat or due to fluid retention.

2.6.2.5 α-glucosidase inhibitors

Acarbose and migitol are competitive inhibitors of the intestinal α-glucosidase. They act by delaying digestion of carbohydrate and lowering potprandial glucose levels. The major side effects are flatulence, diarrhea, and abdominal cramping.

2.6.2.6 Dipeptidyl peptidase-IV inhibitor

Incretin is a peptide that is produced in the gastrointestinal tract. One of these incretins is glucagon like polypeptide-1 (GLP-1). GLP-1 increases insulin secretion and decreases glucagon release. GLP-1 is metabolized by dipeptidyl peptidase-IV (DPP-IV) and has a very short half-life. Thus, DPP-IV inhibitors help increase insulin release in response to meals (Clayton et al., 2009; Finkel et al., 2009;

Nolte, 2009). Adverse effects of dipeptidyl peptidase-IV inhibitor include nasopharyngitis and headache.

2.7 *In vivo* models of diabetes mellitus

2.7.1 Chemical induction of diabetes mellitus in animal model

In *in vivo* studies, chemicals that selectively damage pancreatic β-cells, such as streptozotocin (STZ) and alloxan, are used for diabetic induction. Streptozotocin is synthesized from *Streptomyces achromogenes*. The toxicity of streptozotocin is caused by alklylation of DNA, resulting in β-cell damage. After streptozotocin injection to rats, different methylated purines will be found in tissues of rats. In this research project, alloxan was used because it is less expensive. The chemical names of alloxan are 2,4,5,6-tetraoxypyrimidine or 5,6-dioxyuracil. It was discovered in 1818 by Brugnatelli and synthesized by Wohler and Leibig in 1838. The specific necrotic effect of alloxan on the pancreatic β-cells in rabbits was reported by Dunn and his colleagues in 1943 (Szkudelski, 2001).

Alloxan is a hydrophilic compound and enters β-cells via glucose transporter type 2 (GLUT2). Alloxan is an unstable substance with 1.5 min half-life at pH 7.0 and temperature 37 °C. Nevertheless, its half-life is longer when kept at lower temperature. Alloxan can be dissolved in ice cold saline (0.9% NaCl) but it must be freshly used. The routes of administration which exert diabetogenic action are intravenous, intraperitoneal and subcutaneous injections. The range of diabetogenic dose of alloxan depends on animal species. Usually, the dose for intravenous injection is about 65 mg/kg body weight. However, the effective doses for subcutaneous or intraperitoneal are higher. The dose of alloxan below 150 mg/kg is insufficient to induce diabetes if given by intraperitoneal injection.

Tetraphasic of blood glucose concentration response to alloxan injection is shown in Figure 2.7. The first phase of alloxan-induced effects is hypoglycemic effect which appears in 30 min after alloxan injection. The first phase is caused by temporarily stimulation of insulin secretion. The second phase is hyperglycemic phase which occurs 1 h after administration. Morphological damages such as intracellular vacuolization, extension of rough endoplasmic reticulum, reduction of Golgi area and insulin granules, and swollen mitochondria, can be observed during the second phase. Thereafter, the hypoglycemic phase returns with the third phase. This phase is the critical period as animal may die without glucose administration. This severe phase results from alloxan-induced disruption of β-cell membrane, leading to high insulin level in circulation. The ultimate loss of β-cells is correlated with permanent hyperglycemic phase in the fourth phase.

The mechanism of action of alloxan is illustrated in Figure 2.8. First, alloxan binds to sulfhydryl (-SH) groups in the sugar binding site of glucokinase, causing enzyme inactivation. Inactivation of glucokinase results in a reduction of glucose oxidation and ATP formation, therefore decreasing insulin secretion. Secondly, dialuric acid is formed as a result of alloxan reduction. The reaction between alloxan and dialuric acid produces alloxan radicals ($HA^{\bullet}$) and unidentified compound 305 (maximum absorption at 305 nm). The superoxide radicals generated by alloxan are able to liberate ferric ions from ferritin and reduce them to ferrous ions. The reaction between ferrous ion and hydrogen peroxide can form hydroxyl radical which is responsible for β-cell damage. The third mechanism is to induce calcium influx, increase calcium mobilization from the internal store and limit calcium elimination from cytoplasm. In conclusion, alloxan induces diabetic mellitus by oxidizing -SH group, inhibiting glucokinase, generating free radical and disturbing intracellular

calcium homeostasis, resulting in its toxic action on pancreatic β-cells (Fröde and Medeiros, 2008; Lenzen, 2008; Szkudelski, 2001).

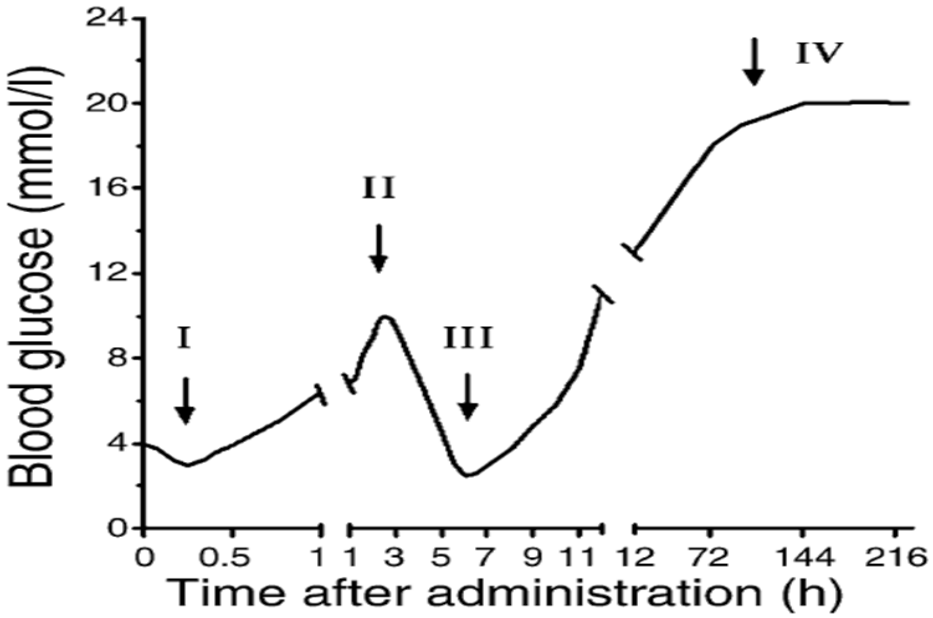

Figure 2.7 Tetraphasic of blood glucose response to alloxan injection (Lenzen, 2008).

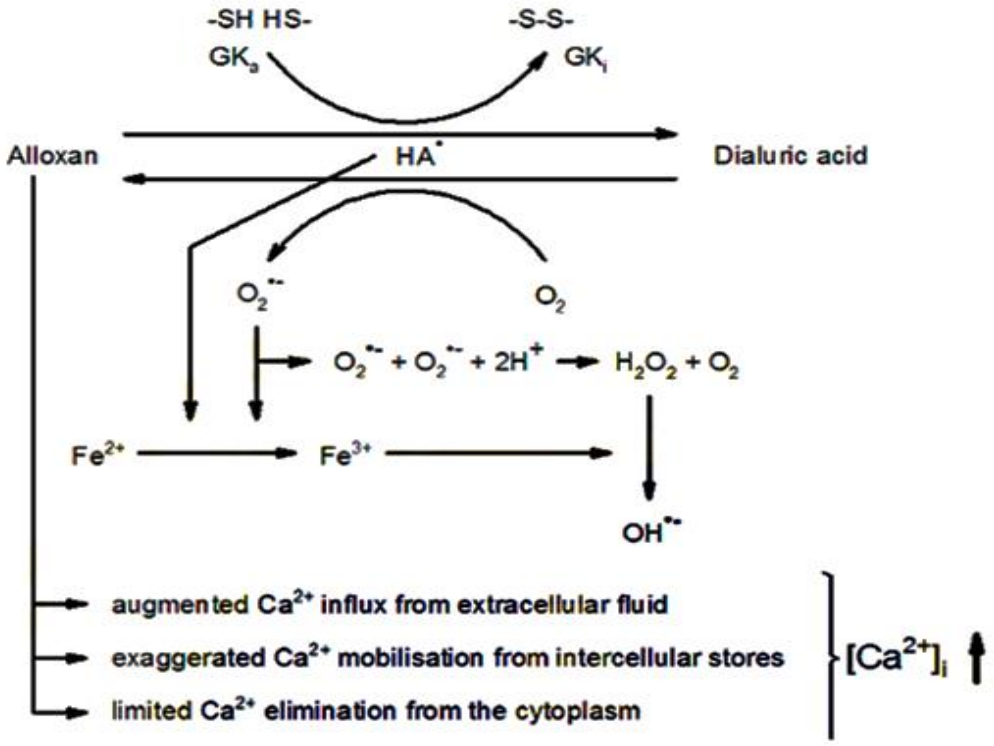

Figure 2.8 The mechanism of action of alloxan (Szudelski, 2001).

2.7.2 Surgical models of diabetes

Pancreatectomy can be used for induction of diabetes mellitus. This method is conducted by the complete removal of the pancreas. However, this model contains several limitations. First, high level of technical expertise and adequate surgical room environment are required. Secondly, animal infection often occurs with this major surgery. Thirdly, adequate post-operative analgesia and antibiotic administration are absolutely necessary to comfort all animals. Moreover, supplementation with pancreatic enzymes is needed to prevent malabsorption. Finally, after surgery animal will lose pancreatic counter regulatory response to hypoglycemic state. With these disadvantages, this surgical model is not popular for studying diabetes in animal. Instead, partial pancreatectomy has been employed. However, it has been found that 80% of animals are needed to receive resurgery to obtain mild to moderate state of diabetes (Fröde and Medeiros, 2008).

2.7.3 Genetic models of diabetes

2.7.3.1 Animal strains that spontaneously develop diabetes

Animal strains that spontaneous develop diabetes exhibit complex and heterogeneous characteristics similar to human condition. For example, the non-obese diabetic (NOD) mouse and bio breeding laboratories (BB) rat consider as type 1 diabetes models because the NOD mouse shows hyperglycemia between 12 and 30 weeks of age, whereas in BB rats it occurs approximately in 12^{th} week. Animal models associated with type 2 diabetes such as Goto-Kakizaki rat is a model of mild type 2 diabetes. It is obtained by selective breeding over many generations of glucose intolerance non-diabetic Wistar rats. Db/db mouse-monogenic model of obesity (leptin resistant) develops hyperglycemia since their pancreatic β-cells are unable to maintain the high levels of insulin secretion. In contrast, Ob/Ob mouse, monogenic model of

obesity (leptin deficient) is lack of leptin, a hormone released by fat cells and which acts on brain to suppress feeding and stimulate metabolism (Fröde and Medeiros, 2008; Chen and Wang, 2005).

2.7.3.2 Genetically engineered diabetic mice

Molecular biological techniques are able to be used for the study of diabetes. Transgenics refers to the incorporation of modified genes into the pronucleus of a zygote. The transferred gene are randomly incorporated into the host genome and some offsprings will therefore express the modified gene, producing transgenic animals. Knockout animals are created from embryonic stem cell which has been injected with disrupted version of a gene. Once inside the cell, the engineered gene will recombine with the homologous target gene. With interrupted sequence, the target gene is knocked out (Rees and Alcolado, 2004). Some of transgenic and knockout animals in diabetes research are presented in Table 2.3 and 2.4, respectively.

Table 2.3 Transgenic animals in diabetes research (Rees and Alcolado, 2004).

Gene	Copy number	Phenotype	References
Insulin receptor	0	Severe hyperglycemia, neonatal death from ketoacidosis	Joshi et al., 1996
Insulin receptor	1 (in muscle)	Mild insulin resistance	Chang, Benecke, Le Marchand-Bruste, Lawitts, and Moller, 1994
IRS1	0	Mild insulin resistance Normoglycemia Growth retardation	Tamemoto et al., 1994

Table 2.3 Transgenic animals in diabetes research (Rees and Alcolado, 2004) (Continuted).

Gene	Copy number	Phenotype	References
IRS2	0	Hyperglycemia Insulin resistance Reduced β-cell mass	Kubota et al., 2000; Withers et al., 1998
Glucokinase	0	Severe hyperglycemia Perinatal death	Grupe et al., 1995
Glucokinase	1	Non-progressive glucose intolerance	Grupe et al., 1995
GLUT4	>2	Increased insulin sensitivity	Liu et al., 1993

Table 2.4 Tissue-specific knockouts in diabetes (Rees and Alcolado, 2004).

Genetic knockout	Tissue specificity	References
Insulin receptor	β-cells, brown fat, liver, muscle, brain	Kulkarni et al., 1999
PPARγ	β-cells	Okamoto, 2003
GLUT4	Muscle	Rosen et al., 2003
IGF1	Liver	Minokoshi, 2003

2.8 *In vitro* study of diabetes mellitus

2.8.1 Studies using isolated pancreatic islet

There are two methods for isolating islets from rodent pancreatic tissue. In the first method, pancreas is isolated from euthanized animal. Then, the pancreas are cut into 1-2 mm pieces followed by digestion with collagenase solution. The second method has been described by Gotoh and colleagues (Gotoh, Maki, Kiyoizumi, Satomi,

and Monaco, 1985). In the second method, collagenase solution is injected through the common bile duct into pancreas without cutting into pieces. After isolation, islet cells are seeded in culture media supplemented with fetal bovine serum and antibiotics. Cells are incubated in a humidified atmosphere with 5% CO_2 at 37 °C. Islet morphology is evaluated under microscope (Carter, Dula, Corbin, Wu, and Nunemaker, 2009) for confirmation. The feature of islet appears spherical, golden-brown, and about 50-250 µm in diameter (Figure 2.9).

2.8.2 Studies using insulin-secreting cell lines

The most widely used insulin-secreting cell lines are rat insulinoma cell line (RIN), hamster pancreatic beta cell line (HIT), beta tumor cell line (beta TC), transgenic C57BL/6 mouse insulinoma cell line (MIN6), and insulinoma cell line (INS-1) (Poitout, Olson, and Robertson, 1996). RINm5F cells were used in this study.

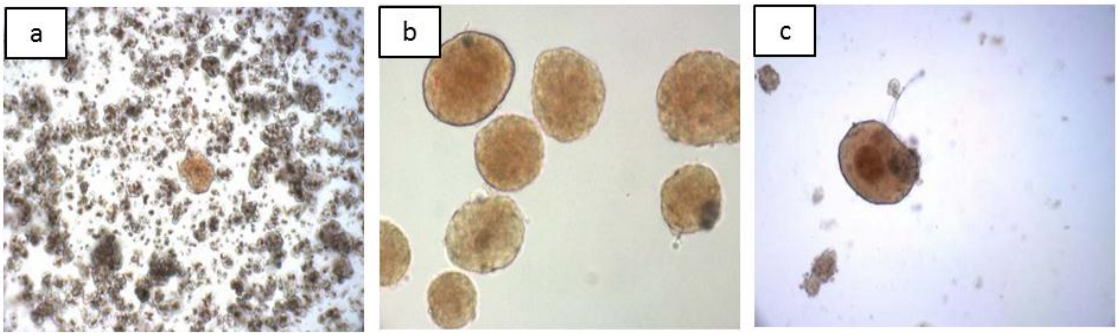

Figure 2.9 Morphology of isolated istets isolation. a: A freshly isolated islet in pancreatic acinar tissue on the day of isolation. b: Islets purified from acinar tissue, after incubation at 37 °C and 5% CO_2 for 18-20 h. c: Islet incubated for 18-20 h with darkened, hypoxic center (Carter et al., 2009).

RIN were derived from a transplantable islet cell tumor. The initial tumor had been induced by high-dose irradiation in an inbred NEDH (New England Deaconess

Hospital) rat strain (Chick et al., 1977). This initial tumor established two cell lines. The cell lines were named RIN-r and RIN-m which were derived from tumors maintained in NEDH rats and in nude mice, respectively (Gazdar et al., 1980). The RINm5F cells are derived from the RIN-m line which is subcloned to release high rate of insulin (Gazdar et al., 1980). It has been reported that along with insulin RINm5F also secretes glucagon and somatostatin (Praz et al., 1983). Morphological features of RINm5F cells are illustrated in Figure 2.10.

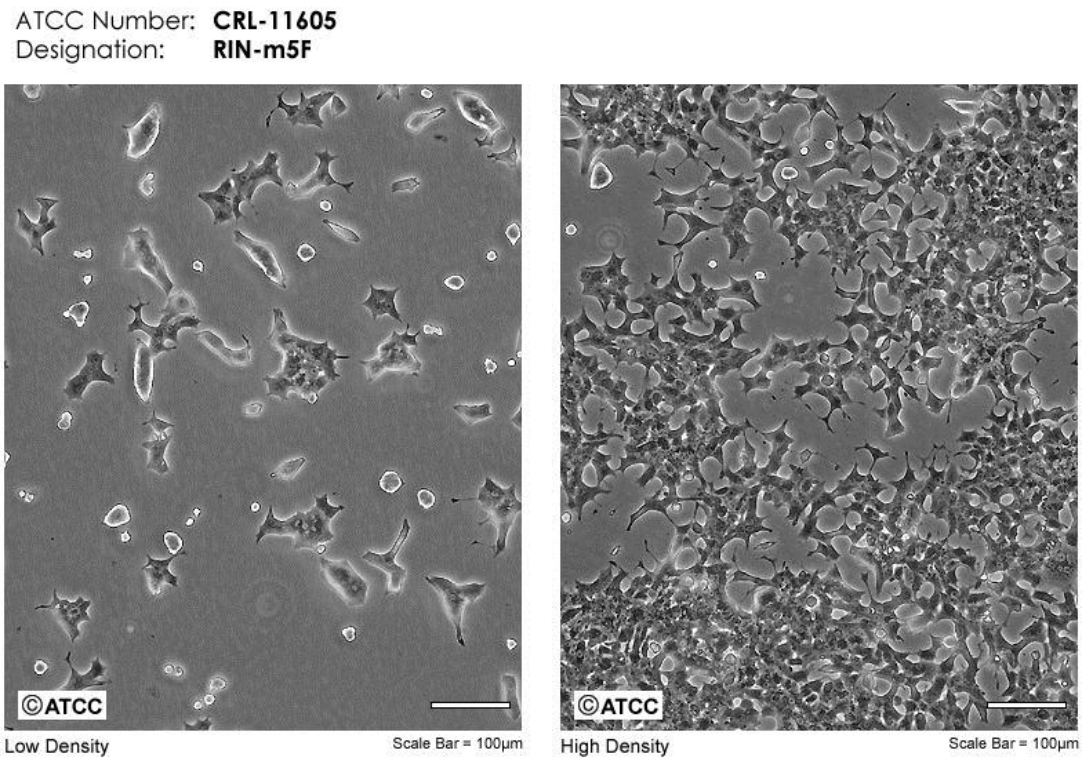

Figure 2.10 RINm5F cell line is a clone derived from the RIN-m rat islet cell line (American Type Culture Collection, 2014).

CHAPTER III

MATERIALS AND METHODS

The *in vivo* model was used for evaluation of acute and sub-chronic toxicities by oral administration of *Derris reticulata* extract. Both *in vivo* and *in vitro* antihyperglycemic activities of *D. reticulata* extract were investigated. In this study, phytochemical screening, antioxidant compounds and antioxidant activity in *in vitro* were also determined.

3.1 Chemicals and instruments

All chemicals and instruments used in this study are listed in Table 3.1 and 3.2.

Table 3.1 List of chemicals used in the study.

Name	**Source**
2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS)	Sigma
2,2-diphenyl-1-picrylhydrazyl (DPPH) radical	Sigma
2,4,6-tripyridyl-s-triazine (TPTZ)	Sigma
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma
Acetone	Carlo
Alloxan	Sigma

Table 3.1 List of chemicals used in the study (Continued).

Name	**Source**
Aluminum chloride hexahydrate ($AlCl_3.6H_2O$)	Sigma
Antibiotic-antimycotic solution	Gibco
Ascorbic acid	Carlo
Butylated hydroxytoluene (BHT)	Sigma
Capture antibody (Anti-Insulin antibody)	Abcam
Catechin	Fluka
Chloroform	Carlo
Detection antibody	Abcam
Anti-Insulin + Proinsulin antibody labeled with biotin	
D-Glucose	Himedia
Dimethylsulphoxide (DMSO)	Carlo
Eosin	Bio-Optica
Ethyl alcohol	Carlo
Ferric chloride ($FeCl_3$)	Sigma
Ferrous sulfate ($FeSO_4$)	Sigma
Fetal bovine serum	Gibco
Folin-Ciocalteau reagent	Carlo
Formaldehyde	Carlo
Gallic acid	Fluka
Glibenclamide	Sigma
Glibenclamide tablets	Daonil
Haematoxylin	Bio-Optica

Table 3.1 List of chemicals used in the study (Continued).

Name	**Source**
Insulin	Sigma
Peroxidase-glucose oxidase (PGO) enzyme	Sigma
Potassium persulphate ($K_2S_2O_8$)	Carlo
RPMI-1640 medium	Gibco
Sodium carbonate (Na_2CO_3)	Carlo
Sodium nitrite ($NaNO_2$)	Sigma
Streptavidin peroxidase	Sigma
3,3',5,5'-tetramethylbenzidine (TMB)	Invitrogen
Trypsin (0.25 % EDTA)	Gibco
Tween 20	Bio-rad
Xylene	Carlo

Table 3.2 List of instruments used in the study.

Name	**Source**
96-well plate	Nunc
A15 Analyzer Automatic Clinical Chemistry	Biosystems
Blood autoanalyzer coulter	Beckman Coulter Inc
Centrifuge machine	Hettich
ELISA plate	Nunc
ELISA plate reader	Bio-rad
Glucometer Accu-Chek Performa glucose test strips	Roche Diagnostics
Haemocytometer	Fisher Scientific

Table 3.2 List of instruments used in the study (Continued).

Name	Source
Hot air oven	Memmert
Lyophilizer	Labconco
Microscope (model CX31)	Olympus
Rotary evaporator	Buchi
Rotary microtome	Shandon Finesse
Spectrophotometer	Cecil
Tissue embedding console system	Shandon Finesse
Tissue processor automated	Shandon Finesse

3.2 Plant materials

3.2.1 Collection of plants

D. reticulata was collected from Prachinburi province, Thailand. Plant verification was performed by Dr. Paul J. Grote, School of Biology, Suranaree University of Technology. Its authenticity was identified and confirmed using morphology based on the definition described earlier (Songsak, 1995; Bangkok Herbarium, 2009; Siri Ruckhachati Nature Park, 2010). A voucher specimen (Pharm-Chu-006) was deposited at School of Pharmacology, Suranaree University of Technology. The stems were cut into small pieces and dried at 50 °C in hot air oven. The dried stems were stored at room temperature until required for extraction.

3.2.2 Preparation of the extract

D. reticulata extract was prepared by boiling 100 g of dried plants in 500 ml of distilled water for 10 min. This process was repeated twice. The aqueous extract of

D. reticulata was filtered through cotton gauze and then centrifuged at 2,500 × g for 10 min. Supernatant was collected and concentrated by using a rotary evaporator. The extract was lyophilized into powder by a lyophilizer at -40 °C for three days. The dried extract (yield 16.73%, w/w) was kept at -20 °C until used.

3.3 Phytochemical screening

Phytochemical screening was carried out to identify constituents of *D. reticulata* extract. The extract was screened for anthraquinones, terpenoids, flavonoids, saponins, tannins, and cardiac glycosides. Phytochemical screening was performed based on previous reports (Ayoola et al., 2008; Yadav and Agarwala, 2011).

3.3.1 Test for anthraquinones

D. reticulata extract (0.5 g) was boiled with 10 ml of H_2SO_4 and filtered. Then, 5 ml of chloroform was added to the filtrate and mixed. The chloroform layer was transferred into another test tube and 1 ml of ammonia was added. The resulting solution was observed for color changes. A pink color showed the presence of anthraquinones.

3.3.2 Test for terpenoids (Salkowski test)

D. reticulata extract (0.5 g) was mixed with 2 ml of chloroform. Then 3 ml of H_2S0_4 was added carefully and the tube shaken gently. A reddish brown indicates the presence of terpenoids.

3.3.3 Test for flavonoids (Alkaline reagent test)

D. reticulata extract (0.5 g) was mixed with 2 ml of 2% NaOH. Formation of intense yellow color, which becomes colorless on addition of few drops of dilute acid, indicates the presence of flavonoids.

3.3.4 Test for saponins

D. reticulata extract (0.5 g) was added to 5 ml of distilled water. The mixture was shaken vigorously and observed for stable froth. If persistent froth is produced, it indicates the presence of saponins.

3.3.5 Test for tannins

D. reticulata extract (0.5 g) was boiled with 10 ml distilled water and then filtered. A few drops of 0.1% $FeCl_3$ were added. Observation of brownish green or blue-black coloration indicates the presence of tannins.

3.3.6 Test for cardiac glycosides (Keller-Kilani test)

D. reticulata extract (0.5 g) was mixed with 2 ml of glacial acetic acid containing 1-2 drops of 2% of $FeCl_3$ solution. The mixture was transfer into another test tube containing 2 ml of concentrated H_2SO_4. A brown ring at the interphase indicates the presence of deoxysugar.

The structure of cardiac glycosides includes lactone ring, steroid nucleus and deoxy sugar. Lactone ring was determined by Kedde's test, whereas steroid nucleus was analysed by Liebermann's test and test for deoxy sugar was conducted by Keller-Kilani test. First, deoxy sugar in *D. reticulata* extract was examined. In this study, the result showed the absence of deoxy sugar. Therefore Kedde's test and Liebermann's test were not performed.

3.4 Determination of antioxidant compounds

3.4.1 Determination of total phenolic content

The phenolic compounds of the *D. reticulata* extract was determined by a method previously described (Matthäus, 2002). About 5 mg of *D. reticulata* extract

was dissolved in 1 ml of distilled water. A 100 µl of aliquot was mixed with 2 ml of 2% Na_2CO_3. The mixture was left standing for 2 min at room temperature followed by an addition of 100 µl of Folin-Ciocalteau reagent (diluted with methanol 1:1 v/v). After the incubation for 30 min, absorbance was measured at 750 nm using spectrophotometer. Gallic acid was used for standard curve calibration. Total phenolic content of *D. reticulata* extract was expressed as mg gallic acid equivalents (GAE) per gram extract.

3.4.2 Determination of total flavonoid content

The amount of total flavonoid content was measured according to the method reported by (Liu et al., 2002). In brief, 250 µl of *D. reticulata* extract (5mg/ml) was diluted with 1250 µl of distilled water. 75 µl of a 5% $NaNO_2$ solution was added to the mixture and incubated for 6 min. After the incubation period, 150 µl of a 10% $AlCl_3$ solution was added. The mixture was incubated for further 5 min. Then, 500 µl of 1 M NaOH was added, and the final volume was adjusted to 2500 µl with distilled water. The absorbance was measured at 510 nm and compared to standard catechin. Total flavonoid content of *D. reticulata* extract was expressed as mg catechin per gram extract.

3.5 Determination of antioxidant activity

3.5.1 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) assay

The radical scavenging activity of *D. reticulata* extract against $ABTS^{\bullet+}$ was carried out according to the procedure described previously (Re et al., 1999). Briefly, $ABTS^{\bullet+}$ radical cation was produced by mixing 5 ml of 14 mM ABTS with 5 ml of 4.9 mM potassium persulphate ($K_2S_2O_8$) for 16 h in the dark at room temperature.

Before use, the $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. 50 µl of various concentrations of *D. reticulata* extract was mixed with 950 µl of diluted $ABTS^{\bullet+}$ solution. After 6 min of incubation, the absorbance was read at 734 nm. Butylated hydroxytoluene (BHT) was used as standard. The percentage of radical scavenging activity (% RS) was calculated using the formula given below. The antioxidant activity was expressed as IC_{50} (the concentration required for 50% scavenge for free radical) was determined graphically.

$$ABTS^{\bullet+} \text{ radical scavenging activity (\%)} = [A_0 - A_1] / A_0 \times 100 \quad (1)$$

where A_0 is the absorbance of the control reaction

A_1 is the absorbance of the sample reaction

3.5.2 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The ability of the *D. reticulata* extract to scavenge DPPH free radical was measured according to the procedure described previously (Bor, Chen, and Yen, 2006) with slight modifications. 1 ml of various concentrations of the *D. reticulata* extract was added to 4 ml of DPPH methanolic solution (final concentration of DPPH: 0.2 M). The reaction mixture was shaken and left standing at room temperature for 30 min in the dark and then spectroscopically measured at 517 nm. The percentage of radical scavenging activity (% RS) was calculated using the following formula. The antioxidant activity was expressed as IC_{50} (the concentration required for 50% scavenge for free radical).

$$\text{DPPH radical scavenging activity (\%)} = [A_0 - A_1] / A_0 \times 100$$

where A_0 and A_1 have the same meanings as in the Equation (1).

3.5.3 Ferric reducing antioxidant power (FRAP) assay

The ferric reducing power of the extract was performed according to the procedure described previously (Benzie and Strain, 1996). FRAP reagent consists of

10 mM TPTZ solution (2,4,6-tripyridyl-s-triazine) in 40 mM HCl, 20 mM $FeCl_3$, and 300 mM acetate buffer (pH 3.6), in ratio 1:1:10 (v/v/v), respectively. FRAP reagent was freshly prepared and incubated at 37 °C until used. The reaction was comprised 50 µl of *D. reticulata* extract (5 mg/ml) mixed with 1.5 ml of the FRAP reagent. After 4 min, the absorbance was measured at 593 nm. The reducing potential of the *D. reticulata* extract was determined from a standard curve of $FeSO_4$ and the FRAP value was expressed as µmol Fe^{2+} / mg dried extract.

3.6 Cells and culture conditions

RINm5F, Rattus norvegicus (rat) cell line was obtained from the American Type Culture Collection (ATCC). RINm5F cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% antibiotic-antimycotic solution and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were grown as adherent cells and spread in culture flask. The cells were sub-cultured at 70-80 % confluence. The cells were trypsinized with 0.25% trypsin-EDTA and resuspended in complete culture medium before passage.

3.6.1 *In vitro* cytoprotective study

To investigate cytoprotective effect of *D. reticulata* extract on alloxan-induced RINm5F cell damage, the experiment was carried out according to the method described earlier (Ramkumar et al., 2009) with minor modifications.

3.6.2 Effect of *D. reticulata* extract on the viability of RINm5F cell

The effects of *D. reticulata* extract on the viability of RINm5F cell were first tested to find the range of optimal concentrations in the cytoprotective experiment. RINm5F cells were seeded in 96 well plates at the concentration of 2×10^5 cells/well.

After having been allowed to attach overnight, cells were treated with various concentrations of *D. reticulata* extract at the doses of 0-3500 μg/ml for 24 h. At the end of incubation, cell viability was determined by MTT assay as the followings. After treatment, the medium was removed and then 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added. Cells were then incubated in the dark at 37 °C for an additional 4 h. After the incubation, MTT solution was removed and the formed formazan crystals were dissolved in 50 μl of DMSO. Absorbance was read at 570 nm using a microplate reader. Absorbance of the reaction solution without cells is background. The background was subtracted from the sample value before data analysis. The absorbance of untreated cells was considered as 100 % viability.

3.6.3 Effect of alloxan on the viability of RINm5F cells

In order to find the suitable concentration of alloxan for induction of 50% cell damage, RINm5F cells with density of 2×10^5 cells/well were seeded in 96 well plates and then treated with alloxan at a range of concentrations 0-15 mM (dissolved in 50 mM citrated buffer pH 3.0). After 1 h of incubation, the medium was removed and cells were further incubated for 23 h. At the end of experiment, cell viability was assessed by MTT assay as described in the previous section.

3.6.4 Determination of cell viability on alloxan-induced RINm5F cells damage

In the pre-treatment, RINm5F cells (2×10^5) were incubated with *D. reticulata* extract for 23 h. After the incubation period, the medium was removed. Cells were treated with alloxan at 9 mM (which caused about 50% of cell death) for 1 h. At the end of experiment, cell viability was assessed by MTT assay as described earlier.

In the post-treatment, RINm5F cells (2×10^5) were incubated with alloxan for 1 h. After the incubation, the alloxan medium was removed. After that, cells were

treated with *D. reticulata* extract for 23 h. At the end of experiment, cell viability was assessed by MTT assay.

3.7 Animals

Male and female Wistar rats were used in this study. The rats were obtained from Laboratory Animal, Suranaree University of Technology. Animals were acclimatized for 7 days prior to the experiments. The rats were housed in polypropylene cage, with free access to normal diet and water *ad libitum*. The rats were maintained at room temperature (25 ± 0.5 °C), relative humidity 45-50% and a 12 h light/dark cycle. All procedures in this study were approved and conducted according to guidelines of the Institutional Animal Care and Use Committee, Suranaree University of Technology. All efforts were made to minimize the number of rats used and their suffering.

3.8 Acute toxicity study

Acute oral toxicity was based on the guidelines of the (OECD, 2001). After acclimatization, rats were randomly assigned to each of five groups of 8 rats (4 males and 4 females). The rats were fasted overnight for 14 h and treated as follows: the first group was treated with distilled water and taken as control, while the remaining groups were administered the single doses (250, 500, 1000, and 2000 mg/kg) of the *D. reticulata* extract dissolved in distilled water. The behavior of rats was continuously monitored for 1 h and once daily for 14 days. The following toxicity signs and symptoms were examined: skin, fur, eyes and mucous membranes evaluation, autonomic effects (e.g., salivation), central nervous system effects (tremors and

convulsions), posture, and bizarre behavior. The body weight of the rats was determined on 1st, 7th, and 14th day. At the end of experiment, all animals were anesthetized by CO_2 inhalation and sacrificed by cervical dislocation. Internal organs (heart, lung, liver, kidney, spleen, testes, ovary and uterus) were removed and gross histopathological examinations were performed (Figure 3.1).

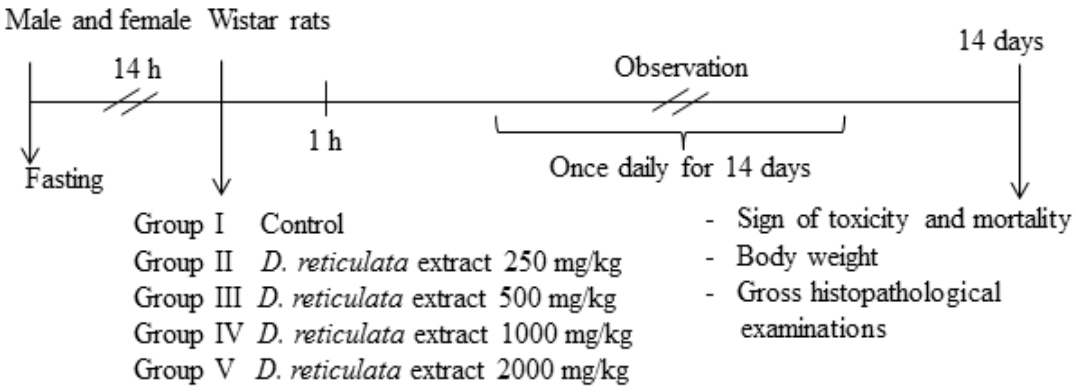

Figure 3.1 Scheme for acute toxicity study of *D. reticulata* extract.

3.9 Sub-chronic toxicity study

Wistar rats of both sexes were randomly assigned into five groups, a control and four treatment groups (n = 8; 4 males and 4 females), the same as in acute toxicity study. All treatment groups were given the extract 250, 500, 1000, and 2000 mg/kg by oral route once a day for 42 days. The body weights were recorded at the end of each week. All animals were observed daily for clinical signs and mortality throughout the treatment period. At the end of treatment, rats were fasted overnight for 14 h. All rats were anesthetized by CO_2 inhalation and blood samples were immediately collected via cardiac puncture for haematological and biochemical analyses. After blood collection, the rats were sacrificed by cervical dislocation. Internal organs were

excised, weighed, and examined macroscopically. The relative organ weight was calculated as (organ weight/body weight) × 100%. Vital organs such as liver and kidney were preserved for histopathological examinations (Figure 3.2).

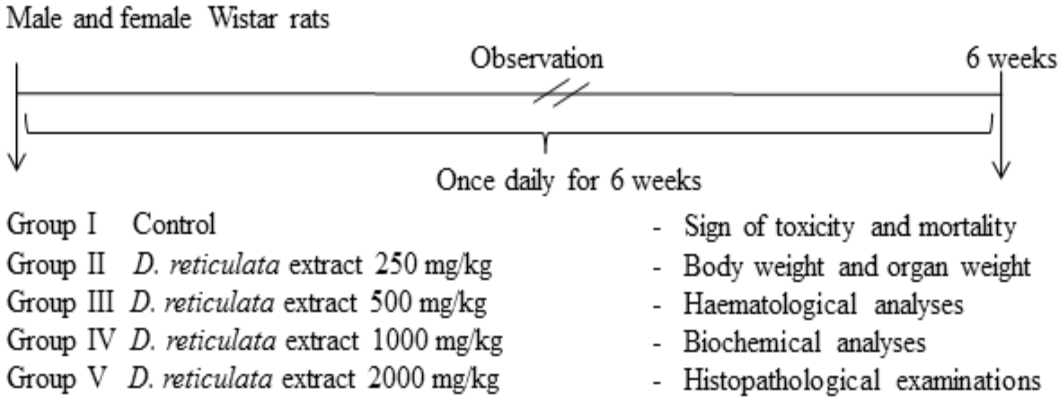

Figure 3.2 Scheme for sub-chronic toxicity study of *D. reticulata* extract.

3.10 Haematological and biochemical evaluations

Haematological analysis was determined using a blood autoanalyzer Coulter (Beckman Coulter Inc., Ireland). The measured haematological parameters included: red blood cell (RBC), white blood cell (WBC), lymphocyte (LYM), monophil (MON), eosinophil (EOS), basophil (BAS), platelet cell (PLT), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC).

For biochemical parameters analysis, blood sample without anticoagulant was centrifuged at 3,000 × g for 5 min to obtain serum. Serum was stored at -20 °C until analysis for biochemical parameters. The serum was analysed by A15 Analyzer Automatic Clinical Chemistry (Biosystems S.A., Spain). Biochemical parameters

assessed included: glucose, total cholesterol, triglyceride, creatinine, aspartate transaminase (AST), and alanine transaminase (ALT).

3.11 Histopathological examinations

Tissue samples of liver and kidney were fixed with 10% neutral buffered formaldehyde. Then, tissues were dehydrated in serial ethanol solution (70%, 95%, and 100%) and acetone. Tissue samples were cleaned with xylene. The tissues were infiltrated with molten paraffin at 65 °C and embedded in paraffin block. Tissues were sectioned at 5 µm thickness. The sections were mounted onto slides using gelatin coating solution. The sections were dried at 56 °C for 45 min in a hot air oven. Tissue sections were deparaffined in xylene. Tissue sections were hydrated with serial ethanol solution (100%, 95%, and 70%). The sections were washed in running tap water, and stained with haematoxylin for 10 min and washed in running tap water. The sections were stained with eosin for 10 min, followed by dehydrated with serial ethanol solution (70%, 95%, and 100%). Finally, the sections were immersed in acetone and xylene and covered with cover slip after drying. The photomicrographs of each tissue section were observed under microscope (Olympus, Japan).

3.12 Investigation of antihyperglycemic activity

3.12.1 Induction of diabetes

Diabetic rats were induced by intraperitoneal injection of alloxan monohydrate in fasted male rats at the dose 150 mg/kg. Alloxan was freshly dissolved in 0.85% (w/v) NaCl. Diabetic rats were orally given 5% glucose solution overnight after alloxan injection to prevent hypoglycemia. On the third day, rats with fasting

blood glucose level higher than 250 mg/dl were considered as diabetic rats and used in the experiment. Animals were divided into 4 groups and each group included 5 rats as follows: Group I (NC), normal control rats received sterile water; Group II (DM), diabetic control rats received sterile water; Group III (DM + Extract), diabetic rats treated with *D. reticulata* extract at 250 mg/kg; and Group IV (DM + Glib), diabetic rats treated with glibenclamide (tablet) 20 mg/kg. The extract and standard drug were administered once daily for 15 days. The freshly prepared solutions were orally administered to animals by gastric intubation with force feeding needle.

3.12.2 Sample collection

Measurements of body weight and blood glucose analyses were done on 5^{th}, 10^{th}, and 15^{th} day of the treatment. Blood samples were collected from tail vein for determination of glucose level using Glucometer Accu-Chek Performa glucose test strips (Roche Diagnostics, USA). At the end of treatment, blood samples were collected via cardiac puncture for biochemical analyses. The animals were then sacrificed by cervical dislocation. Pancreas was removed from the animals and rinsed in 0.85% (w/v) NaCl. Tissue samples were fixed with 10% neutral buffered formaldehyde for histopathological examinations (Figure 3.3).

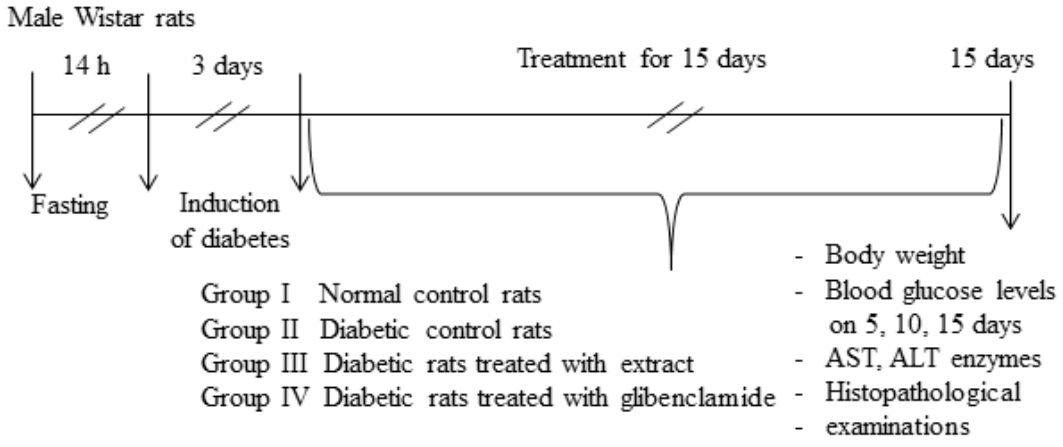

Figure 3.3 Scheme for investigation of antihyperglycemic activity.

3.12.3 Determination of inhibitory effect of *D. reticulata* extract on glucose absorption

Effect on glucose absorption was investigated in rat intestine using method previously described (Krisanapun, Peungvicha, Temsiririrkkul, and Wongkrajang, 2009). Rat jejunum was removed immediately after cervical dislocation and immersed in Kreb-Henseleit solution continuously aerated with carbogen. The jejunum was cut into several pieces and everted with glass rod. Each everted jejunum was tied with a cotton thread at one end. Kreb-Henseleit solution with 140 mg/dl glucose was filled into the everted jejunum, and then the other end of jejunum was tied. Each jejunum sac was incubated in the following solutions: Group I, Kreb-Henseleit with glucose 140 mg/dl; Group II, Kreb-Henseleit with glucose 140 mg/dl mixed with sodium fluoride (NaF) 0.2 M; Group III-V, Kreb-Henseleit with glucose 140 mg/ml mixed with *D. reticulata* extract at 0.25, 0.5, and 2.5 mg/ml respectively. The incubation flasks were shaken at 90 oscillation/min 37 °C for 30 min. After incubation, the sacs were cut and the concentration of glucose in the sac was measured using peroxidase-glucose oxidase (PGO) enzyme commercial kit.

3.12.4 Determination of stimulatory effect on insulin secretion

The insulin secretion assay was performed as a previously described method (Keller et al., 2011). RINm5F cells (2×10^5 cells/well) were seeded in 96 well plates and grown to reach 70-80% confluent state. Culture medium was removed and replaced with Kreb's Ringer buffer. After 60 min of incubation, cells were washed twice with fresh Kreb's Ringer buffer and then incubated with the *D. reticulata* extract at 250 and 500 µg/ml, and glibenclamide 50 µg/ml for 60 min. The extract was diluted with Kreb's Ringer buffer. Glibenclamide was dissolved in DMSO and further diluted with Kreb's Ringer buffer. The final concentration of DMSO (0.5% w/v) did not affect

RINm5F cell viability. The supernatant was collected for measurement of insulin release by ELISA assay.

3.12.4.1 Enzyme-linked immunosorbent assay (ELISA)

The ELISA assay was used to detect insulin release from RINm 5F cells. First, the microtiter plate was coated with 50 µl of anti-insulin antibody (Abcam, USA) in bicarbonate buffer (1:1000). The wells were covered with plastic wrap and incubated at 4 ºC overnight. After the incubation, the solutions were removed and wells were washed with 0.05% Tween 20 in PBS for three times. After blocking with 3% skim milk for 2 h, 50 µl of supernatant from treated RINm5F cells or insulin standard was added in each well. After 2 h of incubation, the solutions were removed and then wells were washed for three times. 50 µl of anti-Insulin+ Proinsulin antibody labeled with biotin (Abcam, USA) at a concentration of 0.5 µg/ml in 3% skim milk were added to microtiter plate. After 2 h of incubation, the detection antibody was removed and washed for three times. Streptavidin peroxidase (50 µl) was used for enzyme conjugation to detect antibody. After washing for three times, the washing solution was removed followed by an incubation of 50 µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate in the dark for 20 min. Finally, the reaction was stopped by adding 1 N HCl. The absorbance was measured at 450 nm. The concentration of insulin was determined from a standard curve of insulin.

3.13 Statistical analysis

Data are expressed as mean ± S.E.M. Comparisons among different groups were performed by analysis of variance (ANOVA) followed by Student-Newman-Keuls test. *P*-values less than 0.05 were set as the level of significance.

CHAPTER IV

RESULTS

4.1 Phytochemical analysis

The phytochemical qualitative analysis revealed the presence of terpenoids, flavonoids, saponins, and tannins but the absence of anthraquinones and cardiac glycosides. The results of preliminary phytochemical screening of *Derris reticulata* extract are summarized in Table 4.1.

Table 4.1 Phytochemical screening of *D. reticulata* extract.

Phytochemical compounds	**Results**
Anthraquinones	-
Terpenoids	+
Flavonoids	+
Saponins	+
Tannins	+
Cardiac glycosides	-

(+) Presence, (-) Absence

4.2 Total phenolic and flavonoid contents

4.2.1 Total phenolic content

Folin-Ciocalteau method was employed to determine the phenolic content of *D. reticulata* extract which was expressed in term of mg GAE/g extract. Total phenolic content of *D. reticulata* extract was 78.84 ± 0.01 mg GAE/g extract.

4.2.2 Total flavonoid content

Total flavonoid content in *D. reticulata* extract was measured using the aluminium chloride colorimetric method and calculated from a calibration curve of catechin standard. Total flavonoid content of *D. reticulata* extract was 54.72 ± 1.81 mg catechin/g extract.

4.3 Antioxidant activity of *D. reticulata* extract

4.3.1 ABTS assay

Antioxidant activities of *D. reticulata* extract determined by ABTS method were shown in Table 4.2. The ABTS radical scavenging activity of the extract and BHT increased with the increasing concentration (Appendix B). The IC_{50} values of *D. reticulata* extract and standard (BHT) were 515.05 ± 0.13 µg/ml and 83.05 ± 0.13 µg/ml, respectively.

4.3.2 DPPH assay

The DPPH radical scavenging activity of *D. reticulata* extract and standard (ascorbic acid) increased in a concentration dependent manner (Appendix B). As shown in Table 4.2, the IC_{50} values of *D. reticulata* extract and ascorbic acid were 239.85 ± 0.13 and 1.23 ± 0.18 µg/ml, respectively.

4.3.3 FRAP assay

The reducing potential of *D. reticulata* extract was calculated by using the $FeSO_4$ standard curve (Appendix A). The result was expressed as μmol Fe^{2+}/mg dried extract. The FRAP value of *D. reticulata* extract was 0.23 ± 0.05 μmol Fe^{2+}/mg dried extract.

Table 4.2 Antioxidant activities of *D. reticulata* extract.

	Methods		
	ABTS (IC_{50}: μg/ml)	**DPPH (IC_{50}: μg/ml)**	**FRAP (μmol Fe^{2+}/mg dried extract)**
D. reticulata extract	515.05 ± 0.13	239.85 ± 0.13	0.23 ± 0.05
Ascorbic acid	-	1.23 ± 0.18	-
Butylated hydroxytoluene (BHT)	83.05 ± 0.13	-	-

Values are expressed as means ± S.E.M. (*n*=3).

4.4 *In vitro* cytoprotective effect of *D. reticulata* extract on alloxan-induced RINm5F cells damage

As shown in Figure 4.1A, after treated with *D. reticulata* extract at concentrations higher than 500 μg/ml for 24 h, the viability of RINm5F cells were significantly decreased compared to control. *D. reticulata* extract at the concentrations of 50-500 μg/ml which did not significantly reduce cell viability were selected for further study. Figure 4.1B shows the results from cytotoxicity study of alloxan. The

half maximal inhibitory concentration (IC_{50}) of alloxan on cell viability was found at 9 mM which was used for studying the cytoprotective effect of the extract.

Both pre and post-treatment with *D. reticulata* extract was found to have significant protective action against alloxan-induced RINm5F cells damage in a dose-dependent fashion as shown in Figures 4.2 and 4.3, respectively.

In the pre-treatment design, it was shown that *Derris reticula* extract at the doses of 50-500 µg/ml increased cell viability from alloxan-induced cell death 50.26 ± 0.30% to 78.51 ± 0.29%. However, the post-treatment was found to produce less cytoprotective effect. With the same range of concentration, post-treatment of the extract enhanced cell viability 52.11 ± 0.23% to 73.25 ± 3.31%.

4.5 Acute toxicity

Single oral administration of *D. reticulata* extract at the doses of 250, 500, 1000, and 2000 mg/kg did not induce abnormal behaviors in rats of both sexes. Normal body weight gains were observed in male and female rats (Table 4.3). There were no animal deaths during 14 days after administration with *D. reticulata* extract. Therefore, the lethal dose of *D. reticulata* extract was higher than 2000 mg/kg. No gross abnormalities of internal organs were observed in the treatment with *D. reticulata* extract.

4.6 Sub-chronic toxicity

4.6.1 Clinical observation and body weight

Sub-chronic administration of *D. reticulata* extract at the doses of 250, 500, 1000, and 2000 mg/kg did not produce clinical signs of toxicity. The body weights of

male and female rats increased throughout the study period in both control and treated groups (Table 4.4.) No lethality was observed during the 42-day period of treatment with *D. reticulata* extract.

4.6.2 Haematological and biochemical parameters

Haematological parameters of male and female rats depicted in Tables 4.5 and 4.6 showed that the number of red blood cells and white blood cells of treatment groups were not significantly different from the control group. The number of platelets of all treated female rats significantly increased (Table 4.6) while male rats treated with *D. reticulata* extract at 250 mg/kg had a significant decrease of mean corpuscular haemoglobin (MCH) (Table 4.5). However, this effect was not dose dependent because it was not found in the groups treated with 500, 1000, and 2000 mg/kg of *D. reticulata* extract. There were significant differences ($p < 0.05$) in mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) between the treated and the control groups in male rats. However, it should be noted that all of the parameters in treated groups that were different compared to control are within the normal ranges.

Biochemical parameters profiles of male and female rats are presented in Tables 4.7 and 4.8. Oral administration of *D. reticulata* extract did not cause significant changes in the level of glucose, total cholesterol, triglyceride, creatinine, aspartate transaminase (AST), and alanine transaminase (ALT).

4.6.3 Relative organ weights

Relative organ weights of 42-day treated rats are shown in Tables 4.9 and 4.10. There were no significant changes in the relative organ weight of heart, lung, liver, kidney, spleen, testes, ovaries, and uterus of all animals treated with *D. reticulata* extract at various doses compared to the control group.

4.6.4 Histopathological examinations

There was no macroscopic change of internal organs (i.e., appearance, color, and size) considered to be related to the treatment. The histopathological examinations of liver and kidney revealed no morphological alteration in all treated and control groups. Photomicrographs of liver and kidney are shown in Figures 4.4-4.7.

4.7 Antidiabetic activity *in vivo*

4.7.1 Effects of *D. reticulata* extract on body weight and fasting blood glucose levels

The body weights were found to increase normally in control rats. Diabetic control rats induced by alloxan at the dose of 150 mg/kg had significant weight loss, whereas treatments with *D. reticulata* extract (250 mg/kg) and glibenclamide (20 mg/kg) significantly improved the body weight as shown in Table 4.11. Intraperitoneal injection of alloxan caused elevation of blood glucose however treatments with *D. reticulata* extract and glibenclamide resulted in a reduction of blood glucose levels when compared with diabetic control rats.

4.7.2 Effects of *D. reticulata* extract on biochemical parameters

The levels of liver enzymes (AST and ALT) in normal and experimental rats are shown in Table 4.12. As anticipated, the levels of AST and ALT were markedly increased in alloxan-induced diabetic rats. However, these hepatic enzymes in serum decreased significantly by the treatment with *D. reticulata* extract, similar to the treatment with glibenclamide as positive control.

4.7.3 Protective effect of *D. reticulata* extract on pancreatic islets *in vivo*

Histopathological examinations were used to evaluate the protective effect of *D. reticulata* extract *in vivo*. Areas of Langerhans islets were clearly decreased in

alloxan-induced diabetic control rats (Figure. 4.8B). In addition, the shape of pancreatic islets of this group became irregular when compared to normal rats (Figure. 4.8A). As seen in Figures. 4.8C and 4.8D, the pancreatic islets injury was restored after treated with *D. reticulata* extract and glibenclamide, respectively.

4.8 Antihyperglycemic action *in vitro*

4.8.1 Inhibitory effect of *D. reticulata* extract on glucose absorption

Inhibition of intestinal glucose absorption by *D. reticulata* extract was examined by everted intestinal sacs method. As presented in Table 4.13, glucose concentrations inside the sacs incubated with the extract and NaF (positive control) significantly decreased compared to untreated control ($p < 0.05$). The results showed that *D. reticulata* extract at the doses of 0.25, 0.5, and 2.5 mg/ml suppressed glucose absorption from intestine. The extract exerted this inhibitory effect in a dose-dependent manner.

4.8.2 Effect of *D. reticulata* extract on insulin secretion

RINm5Fcells were used to evaluate the effect of *D. reticulata* extract on insulin secretion *in vitro* and the data were shown in Figure 4.9. The insulin secretagogue glibenclamide significantly increased insulin concentration in the medium from 1.35 ± 0.04 to 2.36 ± 0.15 ng/10^5 cells compared to control. It was found that unlike glibenclamide, *D. reticulata* extract did not stimulate insulin secretion from RINm5F cells. Cells treated with the extract at the doses of 250 and 500 μg/ml did not significantly alter insulin secretion from RINm5F cells.

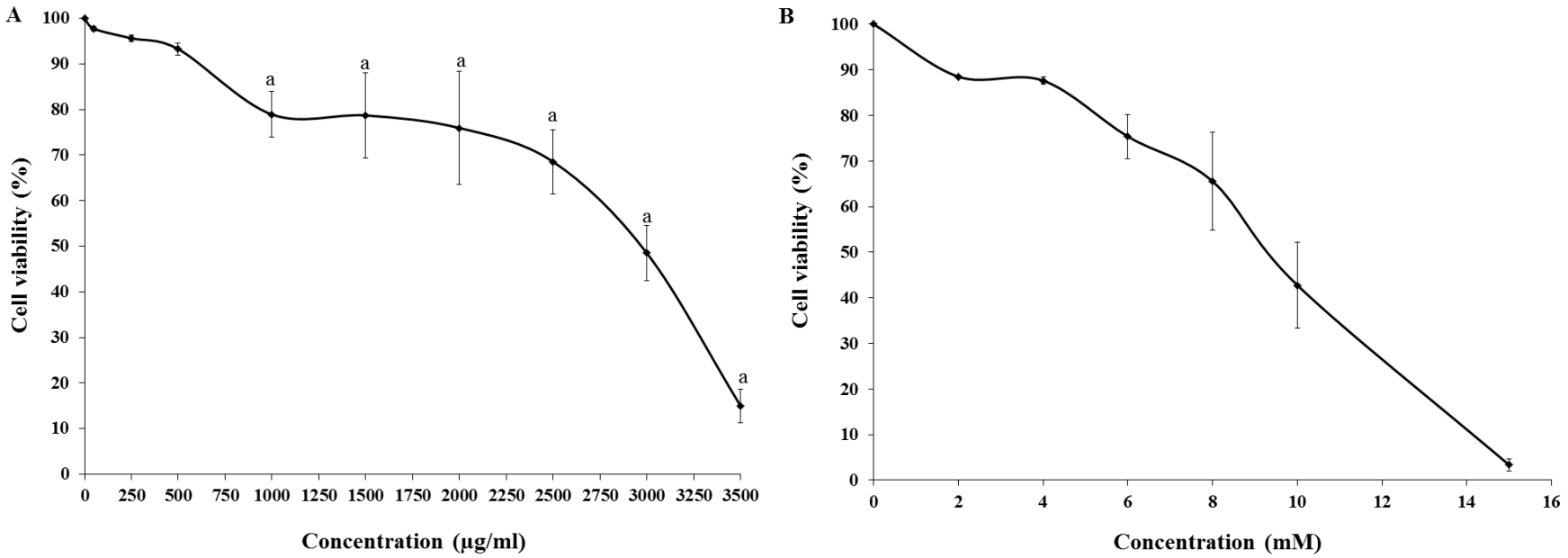

Figure 4.1 Cytotoxicity effect of *D. reticulata* extract and alloxan. Panels A and B are cell viability of RINm5F cells after treated with various concentrations of the *D. reticulata* extract and alloxan, respectively. Values are expressed as mean ± S.E.M. (n = 3).

[a] $p < 0.05$ statistically significant difference from control.

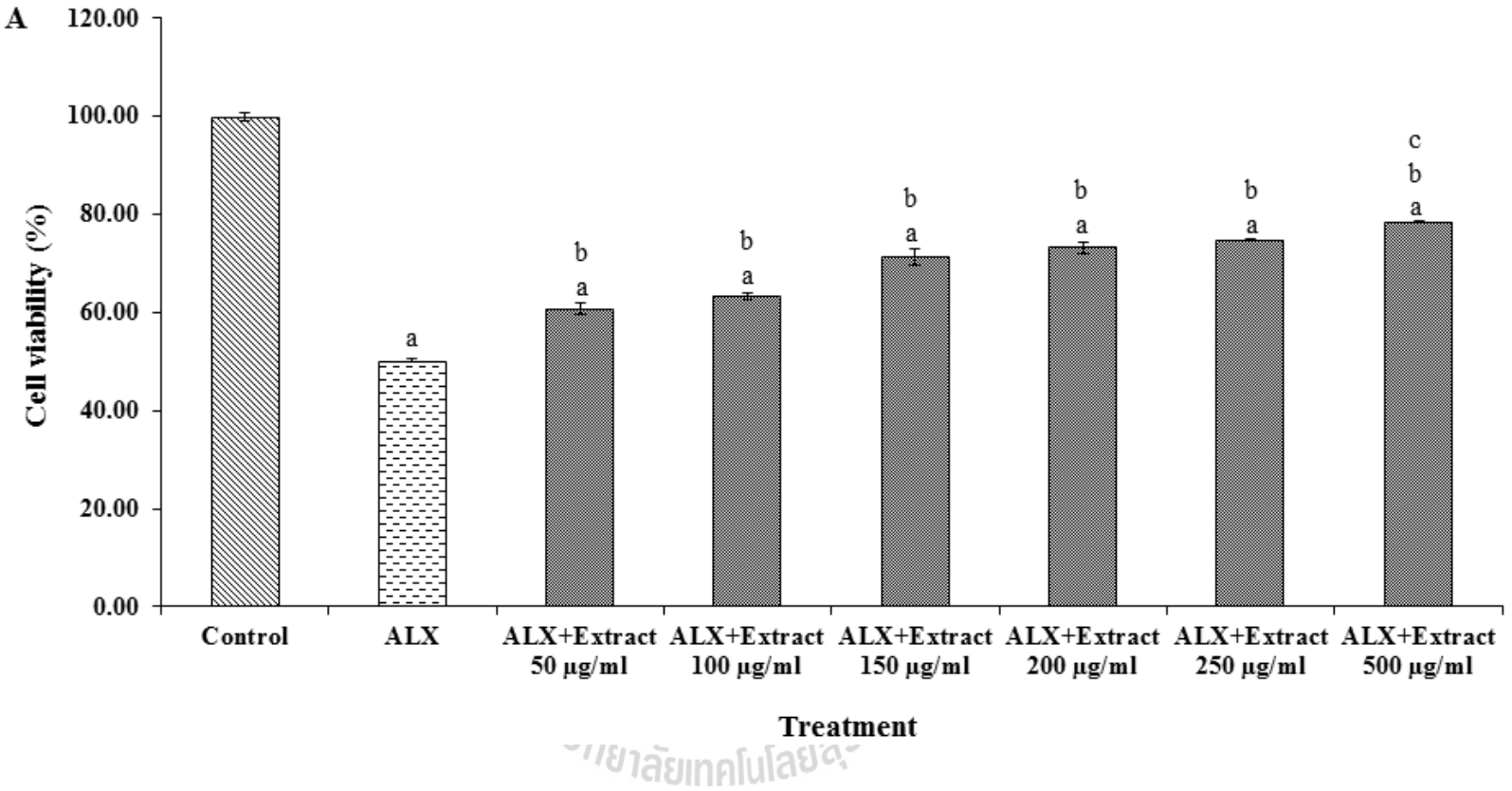

Figure 4.2 Pre-treatment with *D. reticulata* extract on alloxan-induced RINm5F cell damage.

Values are expressed as mean ± S.E.M. (n = 3).

[a] $p < 0.05$ statistically significant difference from control. [b] $p < 0.05$ statistically significant difference from alloxan.

[c] $p < 0.05$ statistically significant difference from the lower doses.

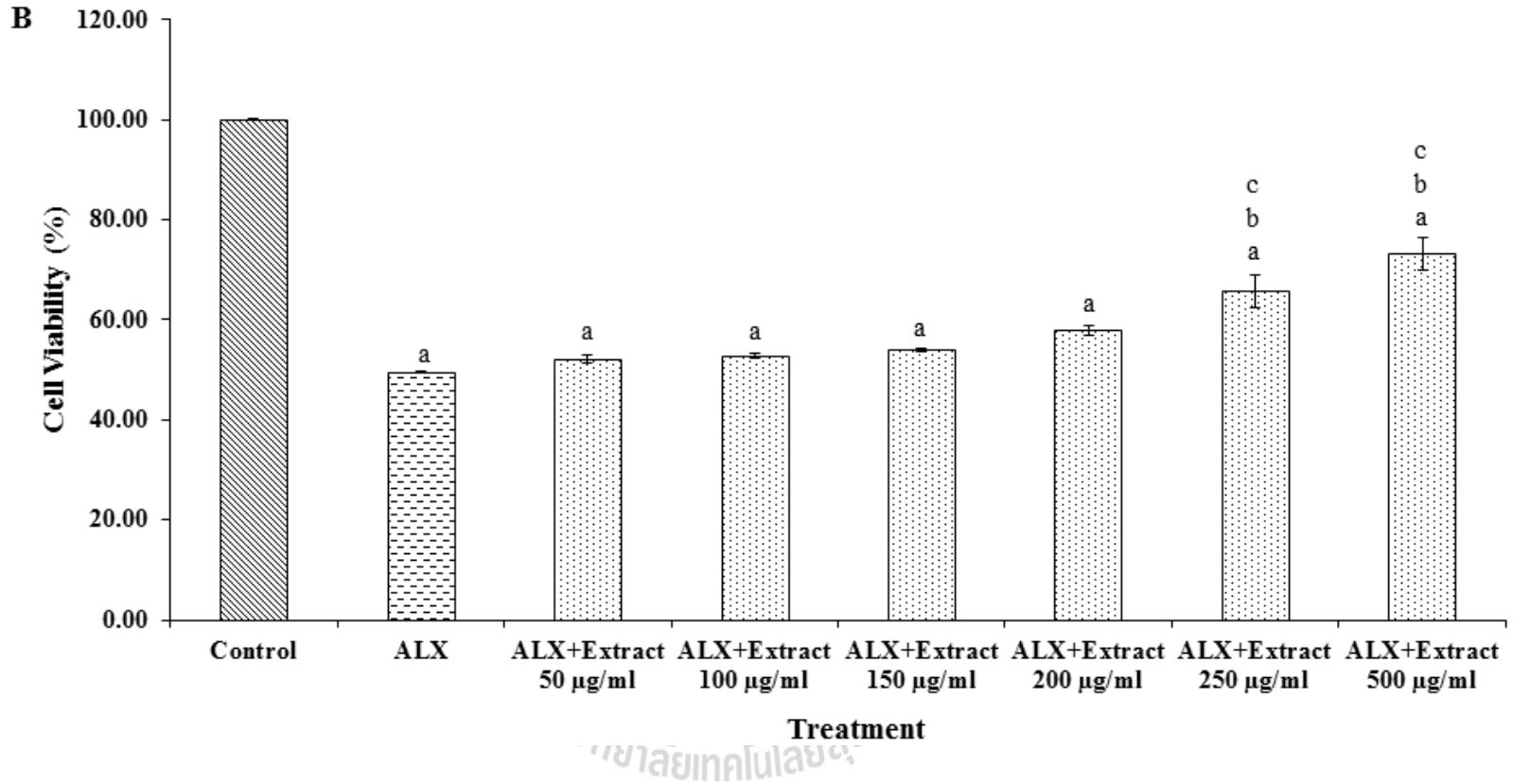

Figure 4.3 Post-treatment with *D. reticulata* extract on alloxan-induced RINm5F cell damage.

Values are expressed as mean ± S.E.M. (n = 3).

[a] $p < 0.05$ statistically significant difference from control. [b] $p < 0.05$ statistically significant difference from alloxan.

[c] $p < 0.05$ statistically significant difference from the lower doses.

Table 4.3 Body weight gain in rats treated with different doses of *D. reticulata* extract in acute toxicity study.

Treatment (mg/kg)	Body weight (g)		
	Day 1	Day 7	Day 14
Male			
0	220.00 ± 9.13	237.50 ± 8.54	260.00 ±14.72
250	220.00 ± 2.50	230.00 ± 4.08	247.50 ± 2.50
500	222.50 ±11.75	235.00 ±10.21	255.00 ±13.98
1000	232.50 ± 7.50	262.50 ±10.31	270.00 ±10.80
2000	227.50 ± 7.50	260.00 ± 9.31	270.00 ±12.91
Female			
0	180.00 ± 5.77	192.50 ± 7.50	202.50 ± 6.29
250	187.50 ± 4.79	200.00 ± 7.07	202.50 ± 8.54
500	177.50 ± 2.50	192.50 ± 7.50	192.50 ± 7.50
1000	187.50 ± 7.50	200.00 ± 8.16	210.00 ± 7.07
2000	190.00 ± 5.77	202.50 ± 2.50	210.00 ± 4.08

Values are expressed as mean ± S.E.M. (n = 4/group).

There was no significant difference among control and treatment groups ($p > 0.05$).

Table 4.4 Body weight gain in rats treated with different doses of *D. reticulata* extract in sub-chronic toxicity study.

Treatment (mg/kg)	**Body weight (g)**						
	Day 1	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**Day 35**	**Day 42**
Male							
0	220.00 ± 9.13	227.50 ± 12.5	275.00 ± 12.58	280.00 ± 14.72	302.50 ± 14.93	325.00 ± 15.54	347.50 ± 16.00
250	202.50 ± 4.79	227.50 ± 7.50	237.50 ± 8.53	280.00 ± 9.12	287.50 ± 11.08	282.50 ± 17.01	285.00 ± 15.54
500	217.50 ± 4.79	237.50 ± 4.78	250.00 ± 4.08	282.50 ± 6.29	297.50 ± 7.50	327.50 ± 13.15	325.00 ± 14.43
1000	207.50 ± 17.02	227.50 ± 17.01	242.50 ± 18.87	275.00 ± 18.48	300.00 ± 22.73	302.50 ± 22.86	305.00 ± 23.27
2000	205.00 ± 12.58	220.00 ± 7.07	232.50 ± 9.46	267.50 ± 11.81	282.50 ± 14.36	300.00 ± 21.21	302.50 ± 21.36
Female							
0	175.00 ± 2.89	190.00 ± 5.77	207.50 ± 4.78	212.50 ± 6.29	227.50 ± 4.78	237.50 ± 10.38	242.50 ± 7.50
250	175.00 ± 5.00	180.00 ± 4.08	192.50 ± 4.79	217.50 ± 4.79	222.50 ± 2.50	235.00 ± 5.00	235.00 ± 5.00
500	175.00 ± 5.00	182.50 ± 4.78	192.50 ± 4.79	212.50 ± 4.79	222.50 ± 4.79	227.50 ± 6.29	230.00 ± 7.07
1000	170.00 ± 9.13	182.50 ± 8.53	187.50 ± 7.50	207.50 ± 6.29	215.00 ± 5.00	227.50 ± 6.29	232.50 ± 8.53
2000	167.50 ± 4.79	177.50 ± 6.29	180.00 ± 5.77	202.50 ± 10.30	212.50 ± 10.31	227.50 ± 13.76	230.00 ± 14.72

Values are expressed as mean ± S.E.M. (n = 4/group).

There was no significant difference among control and treatment groups ($p > 0.05$).

Table 4.5 Haematological parameters of male rats administered with *D. reticulata* extract for 42 days.

Parameters	Control	Treatment			
		250 mg/kg	500 mg/kg	1000 mg/kg	2000 mg/kg
Male					
RBC ($\times 10^6$/µl)	9.15 ± 0.21	9.55 ± 0.11	8.56 ± 0.24[b]	8.82 ± 0.28	8.80 ± 0.16
HGB (g/dl)	17.75 ± 0.25	17.50 ± 0.29	16.50 ± 0.50	17.25 ± 0.63	16.75 ± 0.25
HCT (%)	52.50 ± 1.56	50.25 ± 0.63	47.00 ± 1.68	48.00 ± 2.12	47.00 ± 0.91
WBC ($\times 10^3$/µl)	1.48 ± 0.28	5.58 ± 1.84	4.50 ± 1.43	3.80 ± 0.38	2.15 ± 0.70
LYM (%)	83.50 ± 2.96	75.50 ± 5.72	85.00 ± 2.48	85.50 ± 1.66	82.50 ± 5.12
MON (%)	3.25 ± 2.02	3.25 ± 1.11	2.75 ± 1.44	2.75 ± 0.85	2.00 ± 1.00
EOS (%)	0.25 ± 0.25	0.50 ± 0.29	0.75 ± 0.25	0.50 ± 0.29	0.75 ± 0.75
BAS (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
PLT (10^3/µl)	731.3 ± 45.3	863.0 ± 44.5	638.8 ± 35.7	667.8 ± 21.4	798.8 ± 43.5
MCV (fl)	57.10 ± 0.58	52.63 ± 0.37[a]	54.90 ± 0.38[a b]	54.30 ± 0.86[a]	53.45 ± 0.43[a]
MCH (pg)	19.25 ± 0.24	18.35 ± 0.10[a]	19.40 ± 0.14[b]	19.18 ± 0.18[b]	19.03 ± 0.11[b]
MCHC (g/dl)	33.65 ± 0.30	34.88 ± 0.17[a]	35.38 ± 0.29[a]	35.28 ± 0.26[a]	35.63 ± 0.36[a]

Values are expressed as mean ± S.E.M. (n = 4/group).

[a] $p < 0.05$ statistically significant difference from control.

[b] $p < 0.05$ statistically significant difference from 250 mg/kg group.

Table 4.6 Haematological parameters of female rats administered with *D. reticulata* extract for 42 days.

Parameters	Control	Treatment			
		250 mg/kg	500 mg/kg	1000 mg/kg	2000 mg/kg
Female					
RBC ($\times 10^6$/µl)	8.10 ± 0.17	7.94 ± 0.32	8.21 ± 0.19	8.12 ± 0.15	8.40 ± 0.16
HGB (g/dl)	16.00 ± 0.00	15.50 ± 0.65	16.25 ± 0.25	16.00 ± 0.00	16.75 ± 0.25
HCT (%)	45.50 ± 0.65	43.25 ± 2.10	44.00 ± 0.71	44.50 ± 0.87	45.00 ± 0.82
WBC ($\times 10^3$/µl)	1.33 ± 0.25	5.18 ± 2.26	1.08 ± 0.40	1.35 ± 0.35	1.58 ± 0.11
LYM (%)	86.25 ± 0.95	87.50 ± 3.40	76.25 ± 4.63	82.25 ± 4.84	88.50 ± 1.04
MON (%)	2.00 ± 0.41	3.50 ± 1.56	2.00 ± 0.71	1.75 ± 1.11	0.75 ± 0.48
EOS (%)	2.25 ± 0.85	1.00 ± 1.00	5.50 ± 4.27	3.50 ± 2.22	1.50 ± 0.29
BAS (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
PLT (10^3/µl)	618.5 ± 43.1	811.8 ± 34.8[a]	807.3 ± 49.0[a]	818.8 ± 43.3[a]	752.8 ± 45.2[a]
MCV (fl)	56.00 ± 0.58	54.43 ± 0.80	53.78 ± 0.60	54.80 ± 0.46	53.63 ± 0.29
MCH (pg)	19.75 ± 0.40	19.68 ± 0.20	19.63 ± 0.34	19.78 ± 0.14	19.60 ± 0.06
MCHC (g/dl)	35.28 ± 0.55	36.10 ± 0.27	36.50 ± 0.27	36.08 ± 0.27	36.55 ± 0.25

Values are expressed as mean ± S.E.M. (n = 4/group).

[a] $p < 0.05$ statistically significant difference from control.

Table 4.7 Biochemical parameters of male rats administered with *D. reticulata* extract for 42 days.

Parameters	Control	Treatment			
		250 mg/kg	500 mg/kg	1000 mg/kg	2000 mg/kg
Male					
Glucose (mg/dl)	125.8 ± 10.4	114.3 ± 10.7	135.8 ± 5.1	114.0 ± 2.0	118.5 ± 4.3
Total cholesterol (mg/dl)	61.50 ± 2.60	52.75 ± 3.50	61.50 ± 4.52	44.50 ± 2.84	50.75 ± 5.82
Triglyceride (mg/dl)	80.75 ± 18.44	85.50 ± 6.61	81.75 ± 13.83	76.75 ± 6.16	93.75 ± 23.02
Creatinine (mg/dl)	0.78 ± 0.09	0.65 ± 0.03	0.69 ± 0.04	0.68 ± 0.01	0.67 ± 0.04
AST (U/l)	96.25 ± 7.39	96.00 ± 6.49	99.00 ± 9.46	95.00 ± 10.98	98.00 ± 8.37
ALT (U/l)	32.75 ± 4.27	28.25 ± 3.64	33.25 ± 2.93	25.50 ± 2.72	29.25 ± 3.09

Values are expressed as mean ± S.E.M. (n = 4/group).

There was no significant difference among control and treatment groups ($p > 0.05$).

Table 4.8 Biochemical parameters of female rats administered with *D. reticulata* extract for 42 days.

Parameters	Control	Treatment			
		250 mg/kg	500 mg/kg	1000 mg/kg	2000 mg/kg
Female					
Glucose (mg/dl)	101.0 ± 8.2	91.00 ± 2.1	104.5 ± 7.0	101.8 ± 7.0	83.75 ± 4.4
Total cholesterol (mg/dl)	53.00 ± 2.80	45.50 ± 3.20	45.75 ± 4.64	44.25 ± 1.49	42.25 ± 5.47
Triglyceride (mg/dl)	40.50 ± 10.21	47.25 ± 3.50	52.25 ± 14.63	35.75 ± 2.87	47.75 ± 7.12
Creatinine (mg/dl)	0.74 ± 0.06	0.83 ± 0.06	0.73 ± 0.04	0.74 ± 0.07	0.76 ± 0.02
AST (U/l)	93.75 ± 6.21	86.00 ± 5.87	88.00 ± 7.36	75.00 ± 5.45	86.00 ± 4.06
ALT (U/l)	24.25 ± 2.50	35.25 ± 13.33	23.75 ± 1.55	19.25 ± 0.48	23.00 ± 1.47

Values are expressed as mean ± S.E.M. (n = 4/group).

There was no significant difference among control and treatment groups ($p > 0.05$).

Table 4.9 Relative organ weights of male rats administered with *D. reticulata* extract for 42 days.

Organs	Control	Treatment			
		250 mg/kg	500 mg/kg	1000 mg/kg	2000 mg/kg
Male					
Heart	0.26 ± 0.00	0.29 ± 0.01	0.27 ± 0.01	0.29 ± 0.01	0.28 ± 0.02
Lung	0.43 ± 0.04	0.42 ± 0.01	0.46 ± 0.07	0.50 ± 0.03	0.44 ± 0.02
Liver	2.62 ± 0.10	2.84 ± 0.02	2.77 ± 0.12	2.75 ± 0.11	2.63 ± 0.04
Left kidney	0.27 ± 0.01	0.31 ± 0.02	0.28 ± 0.02	0.28 ± 0.01	0.26 ± 0.01
Right kidney	0.28 ± 0.01	0.31 ± 0.01	0.30 ± 0.02	0.29 ± 0.01	0.28 ± 0.02
Spleen	0.17 ± 0.01	0.17 ± 0.02	0.16 ± 0.01	0.18 ± 0.01	0.16 ± 0.02
Left testes	0.46 ± 0.01	0.53 ± 0.03	0.51 ± 0.01	0.54 ± 0.03	0.50 ± 0.04
Right testes	0.46 ± 0.01	0.52 ± 0.02	0.54 ± 0.02	0.53 ± 0.02	0.49 ± 0.03

Values are expressed as mean ± S.E.M. (n = 4/group).

There was no significant difference among control and treatment groups ($p > 0.05$).

Table 4.10 Relative organ weights of female rats administered with *D. reticulata* extract for 42 days.

Organs	Control	Treatment			
		250 mg/kg	500 mg/kg	1000 mg/kg	2000 mg/kg
Female					
Heart	0.31 ± 0.01	0.28 ± 0.01	0.30 ± 0.01	0.28 ± 0.00	0.29 ± 0.01
Lung	0.44 ± 0.02	0.47 ± 0.04	0.57 ± 0.08	0.40 ± 0.03	0.55 ± 0.06
Liver	2.41 ± 0.09	2.56 ± 0.04	2.60 ± 0.05	2.46 ± 0.04	2.38 ± 0.08
Left kidney	0.27 ± 0.01	0.29 ± 0.01	0.29 ± 0.01	0.27 ± 0.01	0.28 ± 0.01
Right kidney	0.29 ± 0.01	0.31 ± 0.01	0.31 ± 0.00	0.28 ± 0.01	0.29 ± 0.02
Spleen	0.24 ± 0.01	0.19 ± 0.02	0.22 ± 0.01	0.22 ± 0.02	0.20 ± 0.01
Left ovary	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
Right ovary	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
Uterus	0.13 ± 0.02	0.11 ± 0.02	0.11 ± 0.02	0.15 ± 0.02	0.10 ± 0.01

Values are expressed as mean ± S.E.M. (n = 4/group).

There was no significant difference among control and treatment groups ($p > 0.05$).

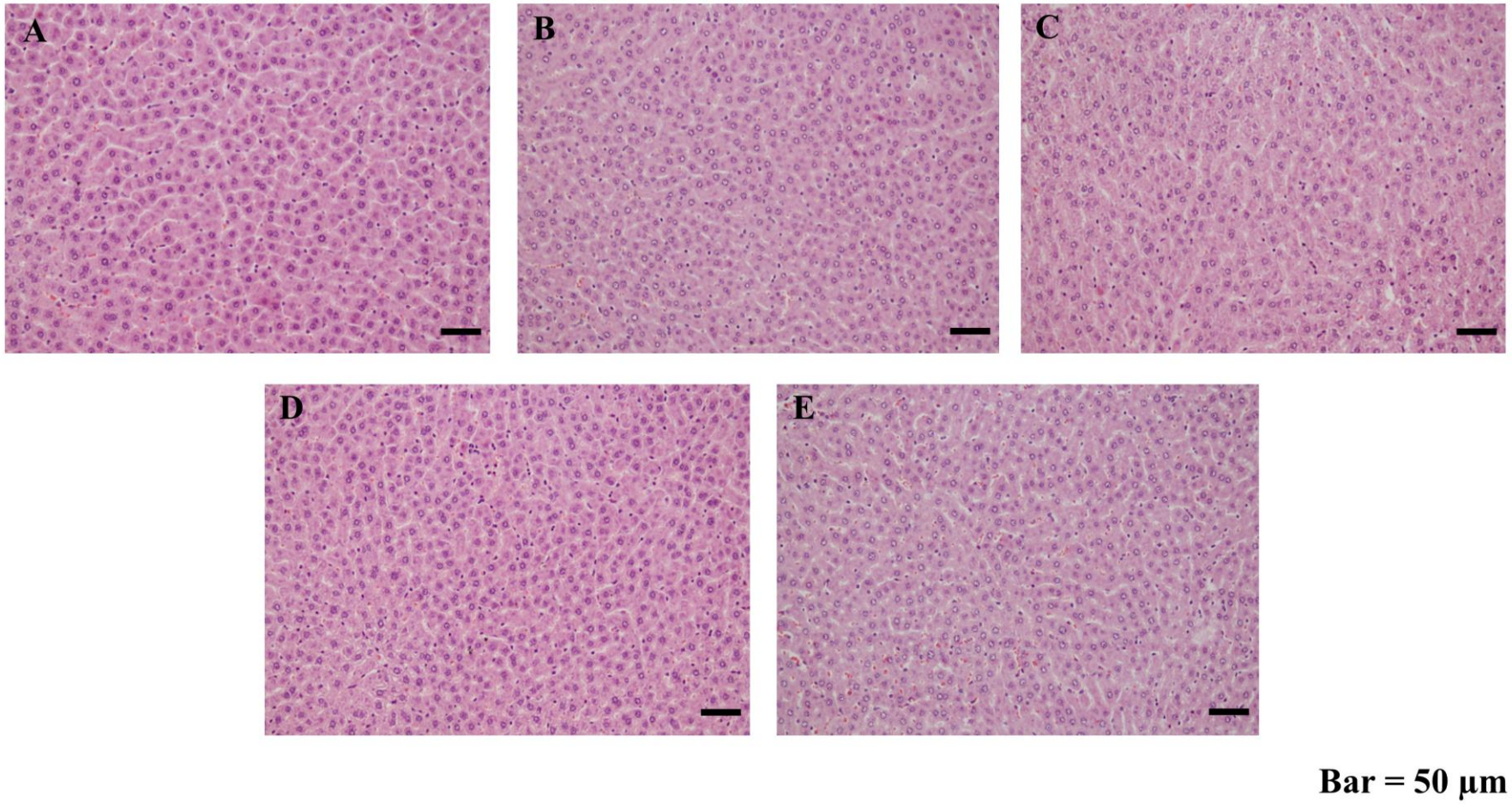

Figure 4.4 Photomicrographs of liver of male rats after administered with the aqueous extract of *D. reticulata* extract.

A: Control; B - E: Treatment at 250, 500, 1000, and 2000 mg/kg, respectively. Hematoxylin and eosin staining (200×).

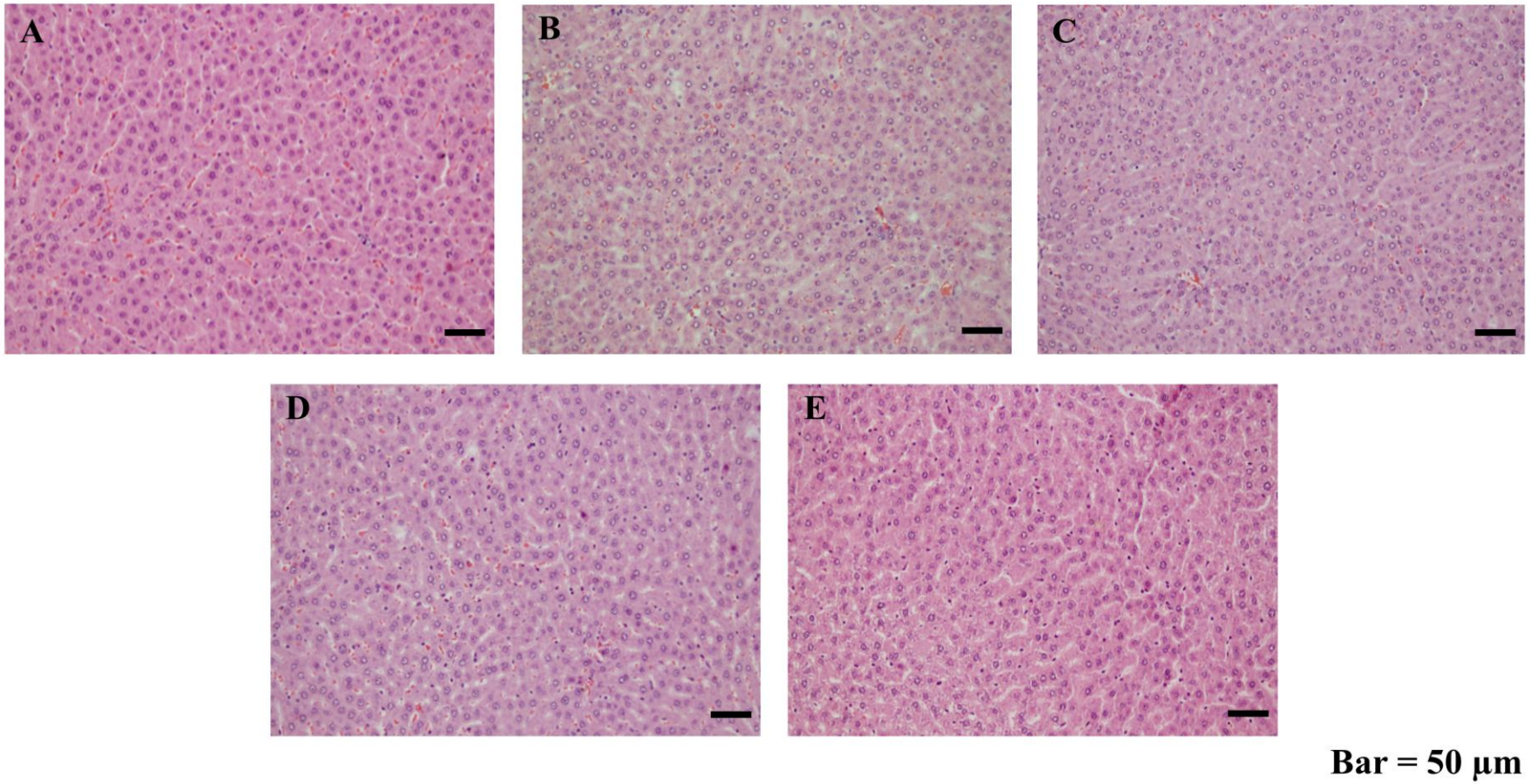

Figure 4.5 Photomicrographs of liver of female rats after administered with the aqueous extract of *D. reticulata* extract.

A: Control; B - E: Treatment at 250, 500, 1000, and 2000 mg/kg, respectively. Hematoxylin and eosin staining (200×).

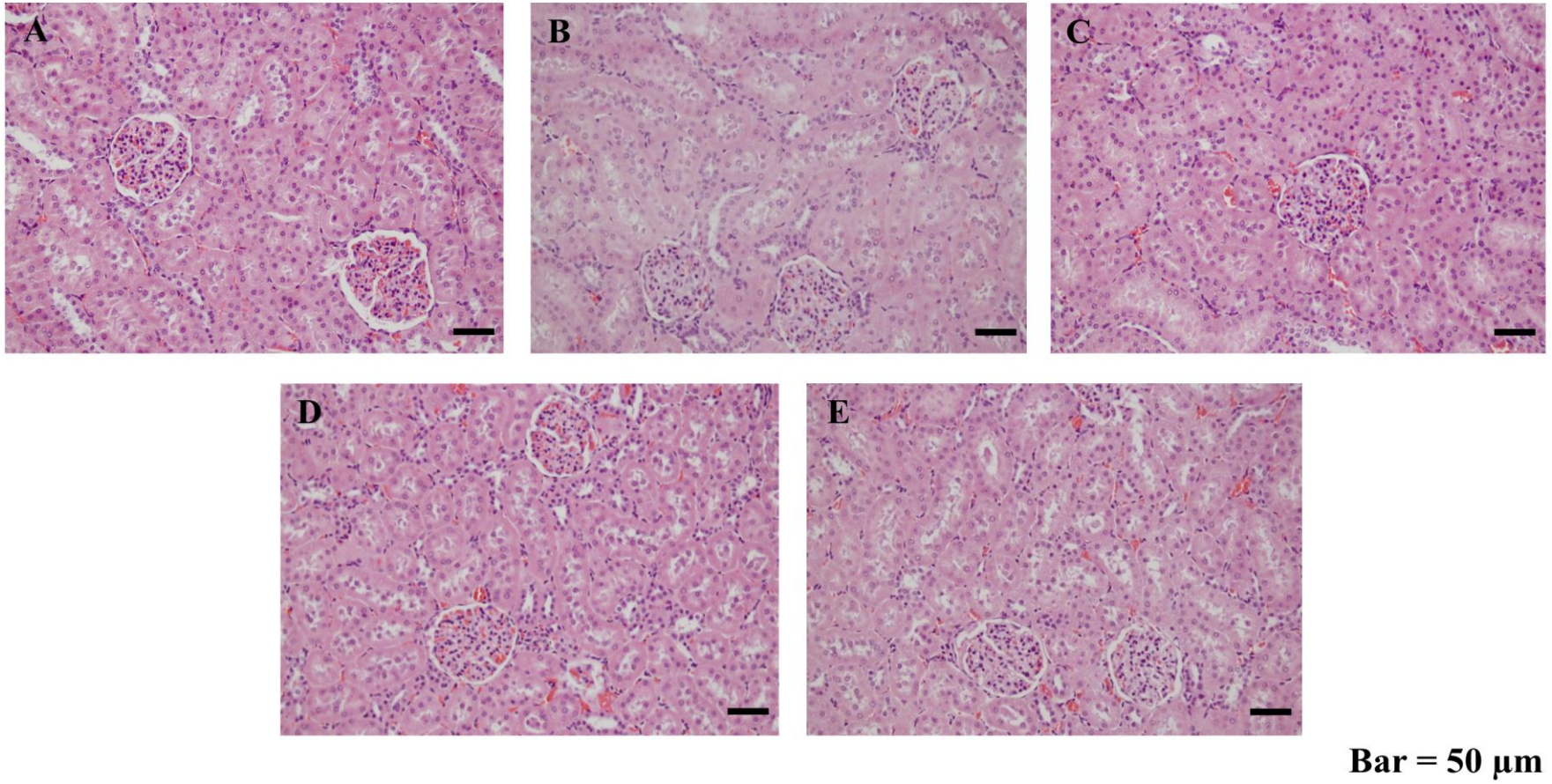

Figure 4.6 Photomicrographs of kidney of male rats after administered with the aqueous extract of *D. reticulata* extract.

A: Control; B - E: Treatment at 250, 500, 1000, and 2000 mg/kg, respectively. Hematoxylin and eosin staining (200×).

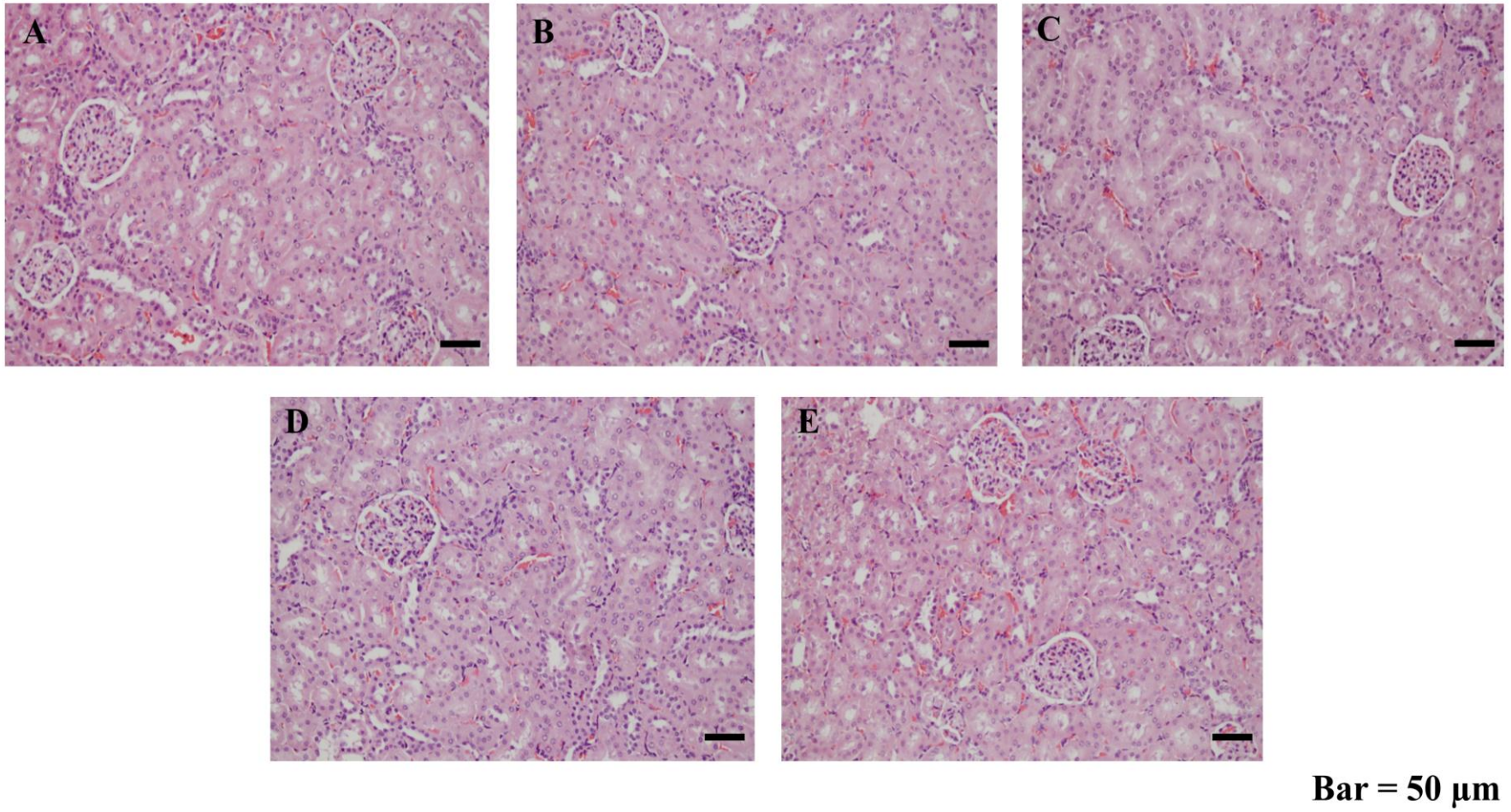

Figure 4.7 Photomicrographs of kidney of female rats after administered with the aqueous extract of *D. reticulata* extract.

A: Control; B - E: Treatment at 250, 500, 1000, and 2000 mg/kg, respectively. Hematoxylin and eosin staining (200×).

Table 4.11 Effects of *D. reticulata* extract on body weights and fasting blood glucose levels.

Treatment	Body weight (g)		Blood glucose levels (mg/dl)			
	Initial	Final	0 day	5 day	10 day	15 day
NC	234.00 ± 5.70	274.00 ± 10.37	82.80 ± 2.79	79.00 ± 3.48	82.80 ± 1.51	81.20 ± 2.27
DM	230.00 ± 3.53	190.00 ± 10.00[a]	335.80 ± 3.13	346.20 ± 3.41[a]	355.40 ± 4.08[a]	406.60 ± 12.90[a]
DM+Extract	230.00 ± 9.35	222.00 ± 12.45[a, b]	332.60 ± 12.94	275.20 ± 14.53[a, b]	240.40 ± 10.01[a, b]	172.80 ± 6.57[a, b]
DM+Glib	225.00 ± 7.50	230.00 ± 6.12[a, b]	336.00 ± 7.17	263.00 ± 18.07[a, b]	220.80 ± 17.84[a, b]	126.20 ± 5.95[a, b]

Values are expressed as mean ± S.E.M. (n = 5/group).

NC, normal control rats received sterile water; DM, diabetic control rats received sterile water; DM + Extract, diabetic rats treated with the extract 250 mg/kg; DM + Glib, diabetic rats treated with glibenclamide 20 mg/kg.

[a] $p < 0.05$; statistically significant difference from normal control.

[b] $p < 0.05$; statistically significant difference from diabetic control.

Table 4.12 Effects of *D. reticulata* extract on biochemical parameters.

Treatment	**Liver enzymes**	
	AST (U/l)	**ALT (U//l)**
NC	75.00 ± 1.77	28.60 ± 1.48
DM	309.00 ± 73.20[a]	65.60 ± 24.45
DM + Extract	119.40 ± 4.08[a, b]	37.60 ± 4.62[a, b]
DM + Glib	122.80 ± 5.04[a, b]	37.40 ± 5.04[a, b]

Values are expressed as mean ± S.E.M. (n = 5/group).NC, normal control rats received sterile water; DM, diabetic control rats received sterile water; DM + Extract (250 mg/kg), diabetic rats treated with the extract; DM + Glib, diabetic rats treated with glibenclamide (20 mg/kg).

[a] $p < 0.05$; statistically significant difference from normal control.

[b] $p < 0.05$; statistically significant difference from diabetic control.

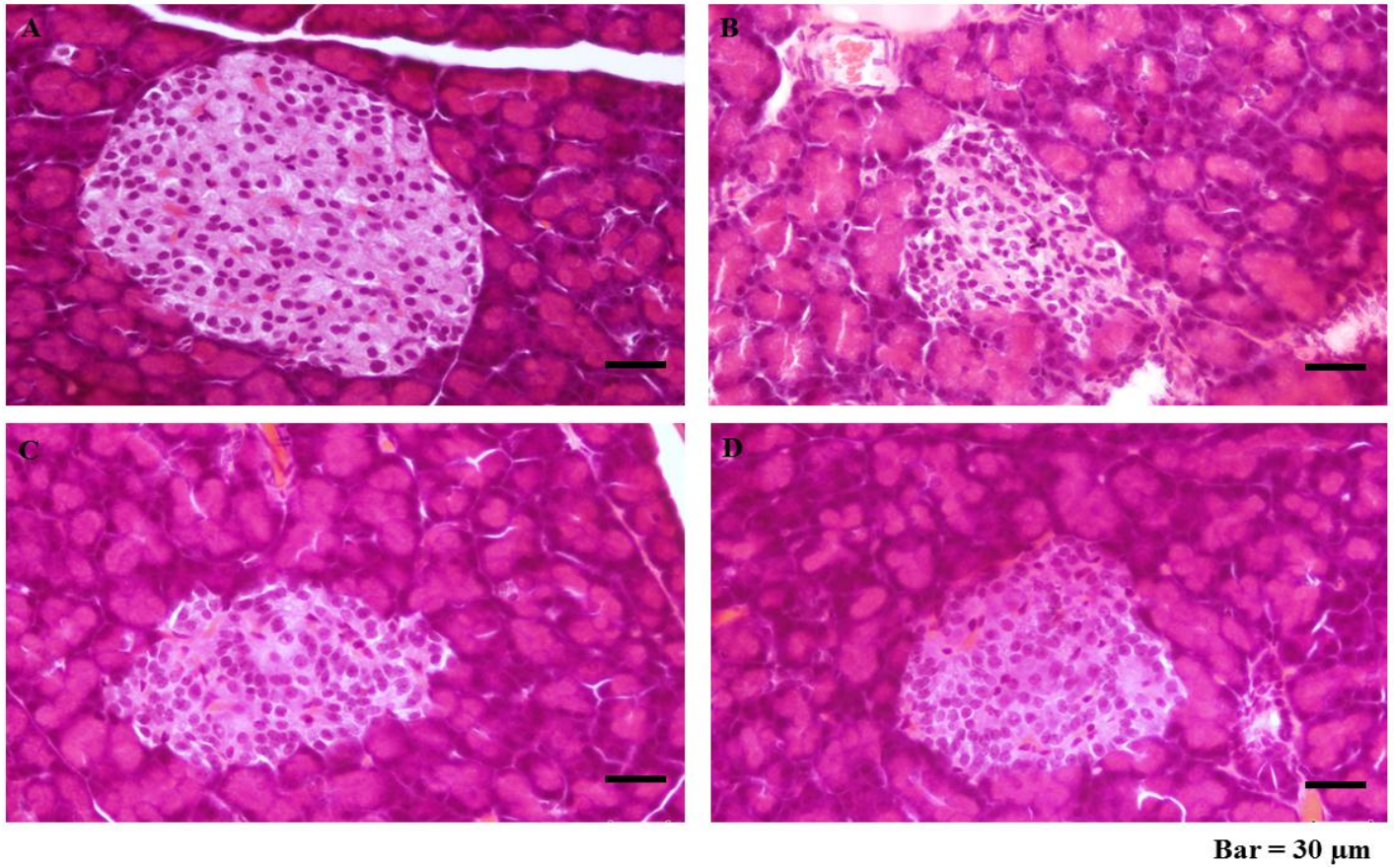

Figure 4.8 Photomicrographs of pancreatic islets of normal and diabetic rats. Hematoxylin and eosin staining (400×); Panel A: normal control rats, Panel B: diabetic control rats receiving steriled water, Panel C: diabetic rats treated with the extract (250 mg/kg) and Panel D: diabetic rats treated with glibenclamide (20 mg/kg).

Table 4.13 Inhibitory effect of *D. reticulata* extract on glucose absorption.

Treatment	**Glucose concentration inside the sacs (mg/dl)**	**% Inhibition**
Control	306.84 ± 4.89	0.00 ± 0.00
Extract 0.25 mg/ml	283.47 ± 6.84[a]	7.65 ± 0.88 [a]
Extract 0.5 mg/ml	269.65 ± 3.73[a, b]	12.1 ± 1.01[a, b]
Extract 2.5 mg/ml	225.63 ± 7.11[a, b]	26.4 ± 2.26 [a, b]
NaF 0.2 M	188.02 ± 2.38[a]	38.7 ± 0.71[a]

Values are expressed as mean ± S.E.M (n = 5).

[a] $p < 0.05$ statistically significant difference from control.

[b] $p < 0.05$ statistically significant difference from the lower doses.

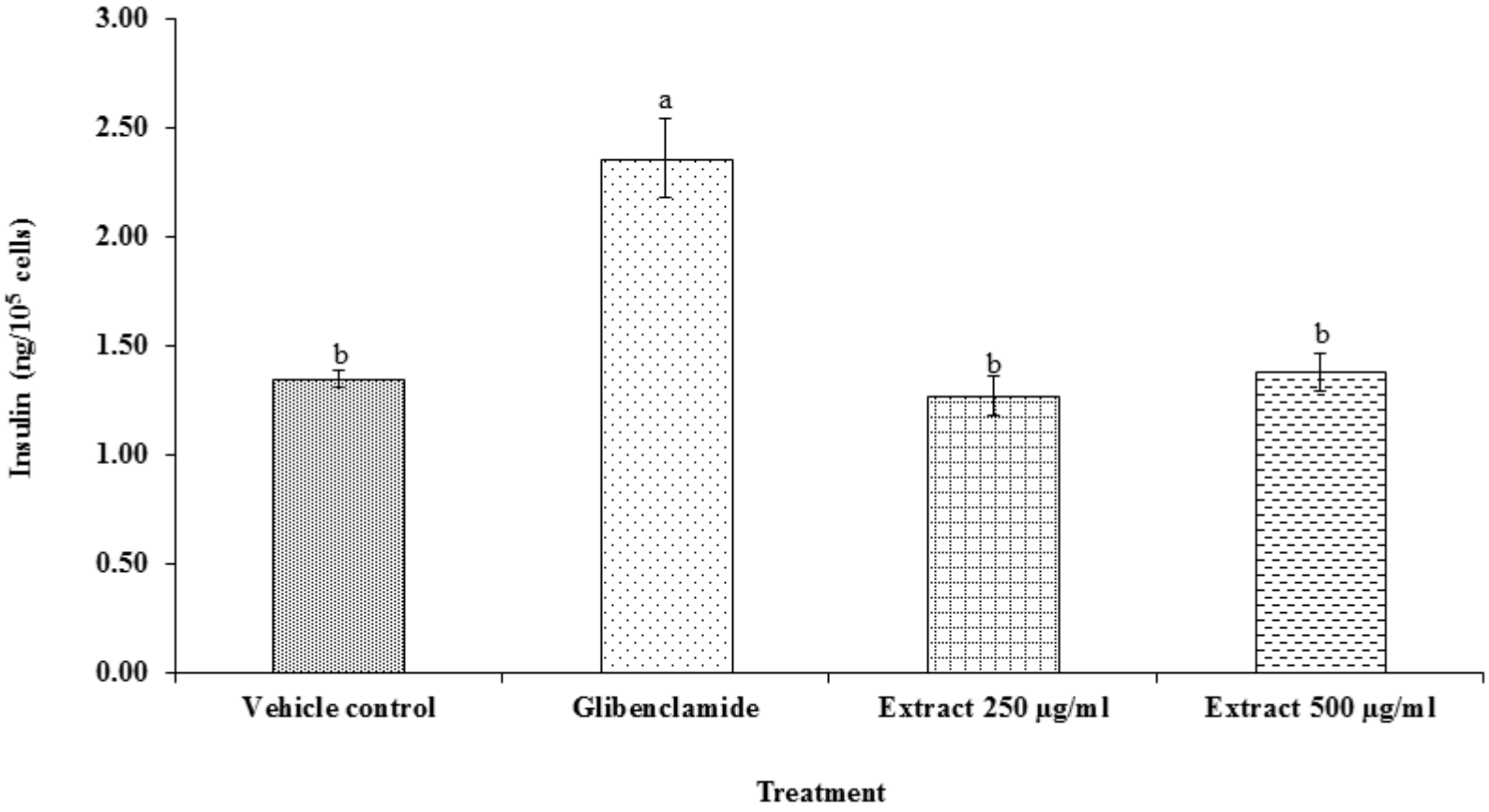

Figure 4.9 Effect of *D. reticulata* extract on insulin secretion. Values are expressed as mean ± S.E.M. (n = 3).

[a] $p < 0.05$ statistically significant difference from control. [b] $p < 0.05$ statistically significant difference from glibenclamide.

CHAPTER V

DISCUSSIONS AND CONCLUSIONS

Phenolic compounds are secondary metabolites of plants that are widely distributed throughout the plant kingdom. Plants need phenolic compounds for pigmentation, growth, reproduction, resistance to pathogens and for many other functions (Lattanzio, Lattanzio, and Cardinali, 2006). Phenolics are gaining attention as being implication for the health benefits, due to their antioxidant activities (Imeh and Khokhar, 2002; Parr and Bolwell, 2000). It is widely accepted that plants are useful sources of remedies for many diseases. Phenolic compounds in plants have exhibited health protective effects in many ailments such as diabetes and hypertension (Randhir, Kwon, and Shetty, 2008). The antioxidant activity of plant materials is well correlated with phenolic compounds (Velioglu, Mazza, Gao, and Oomah, 1998). Folin-Ciocalteu method has been used to determine phenolic content, based on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes, forming blue complexes, which are determined spectroscopically at 750 nm (Magalhães, Segundo, Reis, and Lima, 2008). In this study, total phenolic content of *Derris reticulata* extract was 78.84 ± 0.01 mg GAE/g extract. Flavonoids are the largest group of phenolic compound. Several studies have been reported that flavonoids possess antioxidant activity (Quezada, Asencio, Del Valle, Aguilera, and Gómez, 2004). The hydroxyl groups in flavonoids are responsible for free radical scavenging activities (Kiranmai, Kumar, and Ibrahim, 2011). Total flavonoid content

in *D. reticulata* was measured using the aluminium chloride colorimetric method. In this method, the aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. It also forms acid labile complexes with the orthodihydroxyl groups in the A- or B-ring of flavonoids (Chang, Yang, Wen, and Chern, 2002). The results showed that the value of the total flavonoid content of *D. reticulata* extract was 54.72 ± 1.81 mg catechin/g extract.

Currently, antioxidant activity of plants appears to be a topic of interest. It has been demonstrated that *in vitro* analytical methods are reliable determination of antioxidant activity of biological sample. However, it has been recommended that assays based on hydrogen atom transfer and electron transfer reaction together should be used to provide a better view of antioxidant activity than a single method (Sacchetti et al., 2005). Accordingly, in this study, three analysis methods were performed to determine and confirm antioxidant activity of *D. reticulata* extract.

ABTS radical cation scavenging assay measures the antioxidant activity in both of lipophilic and hydrophilic substances. A sample possessing ABTS free radical scavenging activity indicates that its mechanism of action is as a hydrogen donor and terminates the oxidation process by converting free radicals to more stable product (Tachakittirungrod, Okonogi, and Chowwanapoonpohn, 2007). The absorbance is detected at 734 nm because the interference from other absorbing components and from sample turbidity is minimized (Magalhães et al., 2008).

DPPH radical scavenging assay is one of the most extensively used antioxidant assay for plant sample (Ali et al., 2008). DPPH is a stable free radical of purple color. The purple color of DPPH solution fades to yellow color when the antioxidant is present in the sample that can donate hydrogen atom. Antioxidants react with DPPH and

convert it to 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine, which produces a decrease in absorbance at 517 nm (Tepe et al., 2011).

Ferric reducing antioxidant power (FRAP) assay was used for determination of antioxidant with reducing capacity. The FRAP assay measures the reduction of a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex to the blue color of ferrous which can be detected at 593 nm (Benzie and Strain, 1996). The antioxidant compound is an electron donor and it terminates the oxidation chain reaction by reducing the oxidized intermediates into stable form (Tachakittirungrod et al., 2007).

As shown in appendix B, the antioxidant activity of *D. reticulata* extract determined by radical scavenging assay was increased in dose dependent manner. The maximal percentage of ABTS radical scavenging activity of the extract (93.71%) was found at 2400 µg/ml, whereas that of DPPH scavenging activity (88.36 %) was observed at 1200 µg/ml. In addition, the FRAP value of the extract was 0.23 ± 0.05 µmol Fe^{2+}/mg dried extract.

The current study was designed to explore the *in vitro* cytoprotective potential of *D. reticulata*. Pre- and post-treatments of *D. reticulata* extract provided a significant protection effect from alloxan-induced RINm5F cells damage. Cytoprotective effect may be due to the free radical scavenging activity of the *D. reticulata* extract. Thereafter, *in vivo* antihyperglycemic effect of *D. reticulata* was examined using alloxan induced-diabetic rats. A number of chemicals or plant extracts have been shown to possess potent and clinically relevant activities both *in vitro* and *in vivo*, however some of them cannot be used as therapeutic agents due to unacceptable toxicity. To assure the potential of this extract for clinical use, toxicity studies were conducted. In the acute toxicity study, single dose oral administration of *D. reticulata* extract at 250, 500, 1000, and 2000 mg/kg to male and female rats did not produce any toxic

signs and adverse effects on gross histopathology of rats. All rats treated with *D. reticulata* extract survived throughout the 14 days observation period. The results indicated that LD_{50} of the extract was greater than 2000 mg/kg. Thus, the aqueous extract of *D. reticulata* can be classified as category 5 (low or no toxicity) in accordance with Globally Harmonized Classification System of OECD.

Because diabetes mellitus is a chronic metabolic disease which requires long term treatment, safety assessment on a sub-chronic basis is necessary for clinical research in the future. Therefore, the present study was also designed to evaluate sub-chronic toxicity of *D. reticulata* extract in rats for 42 days. Changes in body weight have been used as an indicator of adverse effects of drugs and chemicals (Hilaly, Israili, and Lyoussi, 2004; Mukinda and Eagles, 2010). *D. reticulata* extract did not affect body weight of the treated animal compared to control. This suggested that oral administration of *D. reticulata* extract had no adverse effect on the normal growth of rats. Animal behaviors were also examined. The observed behavioral signs of toxicity include skin, fur, eyes, and mucous membranes evaluation, autonomic effects (e.g., salivation), central nervous system effects (tremors and convulsions), posture and bizarre behavior. Sub-chronic administration of *D. reticulata* extract did not produce these signs of toxicity.

Organ weight changes have long been accepted as a sensitive indicator of chemically induced changes to organs (Michael et al., 2007). The result showed that *D. reticulata* extract did not induce any changes in relative organ weight. Blood parameter analysis is relevant to risk evaluation of exogenous compounds including plant extracts and drugs. The changes in the haematological system show a high predictive value for human toxicity, when the data are extrapolated from animal studies (Olson et al., 2000). *D. reticulata* extract produced changes in some haematological

parameters. In female rats, there was significant increase in the platelets cells. Haematological examination also revealed significant differences in mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) between the treated and the control group in male rats but the extract significantly decreased MCH of male rats in the treated group at the dose of 250 mg/kg. Nevertheless, the results were still within the normal reference range for Wistar rats (Appendix C). These effects are not considered to have toxicological significance. MCV is a measure of the average red blood cells (RBC) size and mean corpuscular haemoglobin (MCH) provides the average weight of hemoglobin in the red blood cell, whereas MCHC measures the average concentration of haemoglobin in red blood cells. These parameters are useful as the RBC indices for diagnosis of anaemia (Priyadharshini, 2013). Furthermore, in the sub-chronic toxicity study with *D. reticulata* extract, no biochemical parameter change was observed in all animals. AST is an enzyme that is present in high concentration in the cytoplasm and mitochondria of different tissues, including liver, heart, skeletal muscle, kidney, and brain. ALT is a cytoplasmic enzyme that is found limited to hepatocyte (Al-Habori, Al-Aghbari, Al-Mamary, and Baker, 2002). AST and ALT levels, combined with histopathological examinations have been widely used to indicate liver damage (Ramaiah, 2007). In the present study, the levels of AST and ALT of treated rats showed no significant differences from control. These results indicated that *D. reticulata* extract did not cause damage to the liver. In addition, no significant difference in blood creatinine level was observed, indicating that *D. reticulata* extract did not alter kidney function of rats. Creatinine is a catabolism product of creatine which is made by the liver and transported to muscles. Creatinine is usually produced at a fairly constant rate and is mainly eliminated from blood by kidneys. When kidney is injured, blood creatinine level is increased (Maphosa, Masika,

and Moyo, 2010). Moreover, histopathological observation on liver and kidney of both control and treatment groups revealed that sub-chronic oral administration of *D. reticulata* extract did not cause any alteration of organ morphology. These corroborated the results from biochemical examinations that *D. reticulata* extract did not cause toxic effects to the liver and kidneys. The results suggested that the aqueous extract of *D. reticulata* did not cause mortality or produce any remarkable haematological, biochemical and histopathological adverse effects both in acute and sub-chronic toxicity studies in rats. These results have demonstrated that there is a wide margin of safety of the extract and further support use of this plant as alternative medicine.

The antihyperglycemic effect of *D. reticulata* extract was investigated using alloxan-induced diabetic rats. There is a wide variability in the dose of alloxan required to produce diabetic state. Different doses of alloxan produce varying intensities of hyperglycemia (Rathi, Grover, and Vats, 2002; Szkudelski, 2001). It is generally accepted that severe diabetic is of type-I diabetes or insulin dependent diabetes mellitus (IDDM) and mild diabetic is of type-II diabetes or non-insulin dependent diabetes mellitus (NIDDM) (Maiti, Das, and Ghosh, 2005). Rats with fasting blood glucose level between 150 and 250 mg/dl are considered as mild diabetic and severe diabetic rats have fasting blood glucose level more than 250 mg/dl (Gupta, Sharma, Bansal, and Prabhu, 2009). Alloxan of which structure mimics the glucose structure enters selectively through GLUT2 glucose transporter of the β-cell membrane and destroys the pancreatic β-cells, leading to a decrease of insulin secretion. The cytotoxic action of alloxan is mediated by reactive oxygen species (ROS) in a cyclic redox reaction and hydroxyl radicals produced are responsible for the death of β-cells (Lenzen, 2008; Szkudelski, 2001). The insulin reduction causes poor glucose utilization in

experimental animals, resulting in an increase of catabolism, protein depletion, muscle wasting and loss of body weights (Kasetti et al., 2010; Shabeer et al., 2009). The *D. reticulata* extract significantly increased the body weights of diabetic rats compared to the diabetic control group. In accordance with the results on body weights, diabetic rats treated with *D. reticulata* extract showed significant decreases in blood glucose level similar to glibenclamide-treated group. It has been suggested that an increase in the body weight of diabetic treated rats might be due to an enhancement in glycemic control and increased synthesis of structural protein (Eliza, Daisy, Ignacimuthu, and Duraipandiyan, 2009).

In diabetic control rats, the microscopic photograph from pancreatic hematoxylin/eosin staining demonstrated a destruction of the pancreatic islet cells, such as shrinkage and irregular shape. The islet cells of diabetic rats treated with *D. reticulata* extract and glibenclamide showed less degeneration. The degenerative changes caused by alloxan in islet cells were restored during the treatment of glibenclamide similar to a study reported by Venkatesh and colleagues (Venkatesh, Reddy, Reddy, Mullangi, and Lakshman, 2010). The effect of regeneration islet cells by *D. reticulata* extract might be due to its antioxidant activity by reducing oxidant status of the diabetic rats (Premanath, Lakshmidevi, Jayashree, and Suresh, 2012).

Liver is the vital organ of metabolism, detoxification, storage and excretion of xenobiotics and their metabolites. Biomarker enzymes such as AST, ALT were used for evaluation of liver function (Ohaeri, 2001). The increase in levels of hepatic enzymes, such as AST and ALT, has been established in diabetic rats induced by toxic chemicals, including alloxan and streptozotocin (Eliza et al., 2009; Ju et al., 2008). In this study, alloxan-induced increase of AST and ALT were mitigated in rats administrated with

D. reticulata extract compared to diabetic control group. This result also suggested that the extract may possess hepatoprotective activity.

An impact of postprandial hyperglycemia risk on diabetic complication progress has been reported (Ceriello et al., 2002). The reduction of postprandial hyperglycemia has been approached by suppression of carbohydrate absorption from gastrointestinal tract (Si et al., 2010). The effect of *D. reticulata* extract on glucose absorption was examined using everted sac of jejunum. This method was used for describing active transferences of substances across the membrane into the everted sacs (Wilson and Wiseman, 1954). Sodium fluoride acts as an inhibitor for glycolytic pathway by inhibiting glycolytic enzyme enolase. Inhibition of this enzyme reduces pyruvate and ATP. The later is necessary in the process of intestinal glucose absorption (Sanford, Smyth, and Wathing, 1965; Gumińska and Sterkowicz, 1976). The data obtained showed that incubation with *D. reticulata* extract at the doses of 0.25, 0.5, and 2.5 mg/ml as well as sodium fluoride inhibited glucose absorption from small intestine. The result suggested that this action possibly contribute to the mechanisms underlying its anti-hyperglycemic effect. Moreover, this result implied that the extract could be useful for the control of postprandial hyperglycemia.

RINm5F cells have been used for studying the effect of insulin secretagogues. In this study, the insulin concentrations of the cell culture supernatant were determined by enzyme-linked immunosorbent assay (ELISA). Glibenclamide, an insulin secretagogue, was selected as positive control. Glibenclamide stimulates insulin secretion by blocking ATP-sensitive potassium channels of the β-cell membrane, thereby causing depolarization calcium influx, and rise in cytoplasmic calcium concentration (Finkel et al., 2009). RINm5F cells treated with glibenclamide showed significant increase in insulin secretion. In contrast, the result showed that *D. reticulata*

extract did not possess a stimulatory effect on insulin release. The data suggested that the extract may have some advantages over glibenclamide or other insulin secretagogues by causing a fewer clinical events of hypoglycemia.

The use of aqueous extract has attracted attention because of this form of plant can be consumed in a daily basis as decoction (Skotti, Anastasaki, Kanellou, Polissiou, and Tarantilis, 2014). The phytochemical analysis of the *D. reticula* extract revealed some constituents which could have potential antidiabetic property as shown in some other herbs. For example, it has been reported that *Solanum torvum* Swartz extract containing phenolic compounds (rutin, caffeic acid, gallic acid, and catechin) exhibits hypoglycemic activity and are known for their ability of β-cells regeneration (Gandhi, Ignacimuthu, and Paulraj, 2011). Flavonoids and triterpenoids, two major types of the compounds that were found in *Potentilla discolor* extract have protective effects on β-cells in diabetic rats (Zhang et al., 2010). Saponins from *Bryonia laciniosa* extract administered to diabetic rats significantly reduced the levels of blood glucose and restored the function of liver enzymes and lipid profiles (Patel, Santani, Shah, and Patel, 2012). Another research has reported that tannins produced glucose lowering effects. In addition, tannins showed free radical scavenging activity and increased the levels of antioxidant enzymes such as superoxide dismutase and catalase (Velayutham, Sankaradoss, and Ahamed, 2012). The disadvantage of this study is the lack of identification of the active compounds which are responsible for the antidiabetic activity.

In conclusion, the present study has demonstrated that *D. reticulata* extract exerts antihyperglycemic activity in diabetic rats presumably via cytoprotective effect on pancreatic cells and inhibition of intestinal glucose absorption with relatively wide margin of safety. However, further experiments are still needed for the isolation,

identification and pharmacological evaluation of the active compounds in *D. reticulata* extract.

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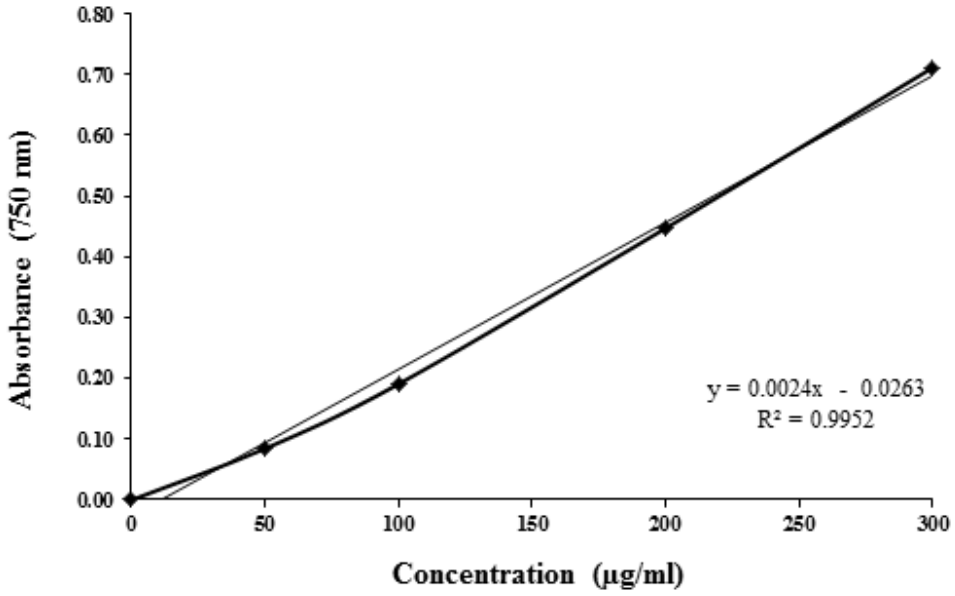
APPENDICES

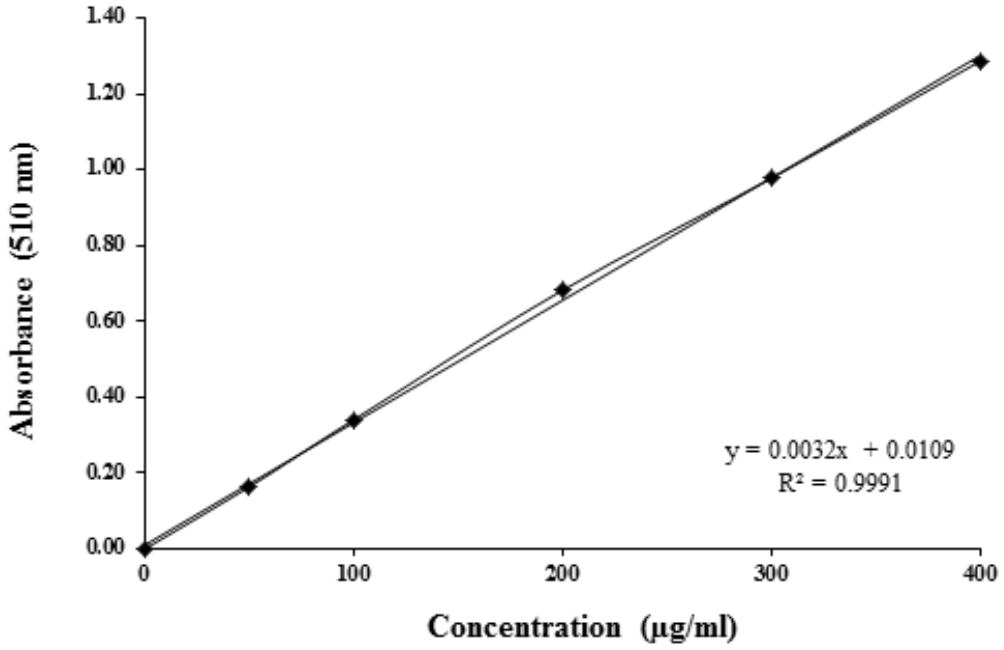
APPENDIX A

STANDARD CURVE

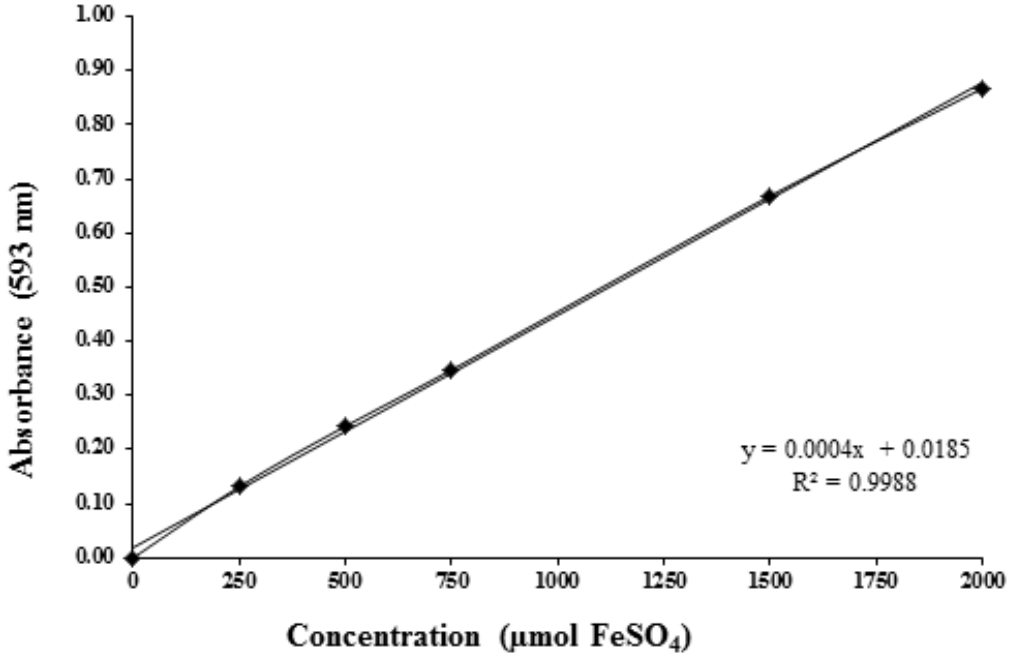
A.1 Standard gallic acid



A.2 Standard catechin

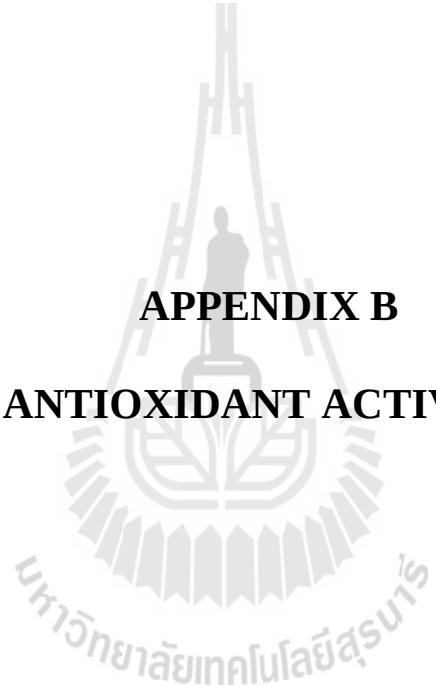


A.3 Standard $FeSO_4$

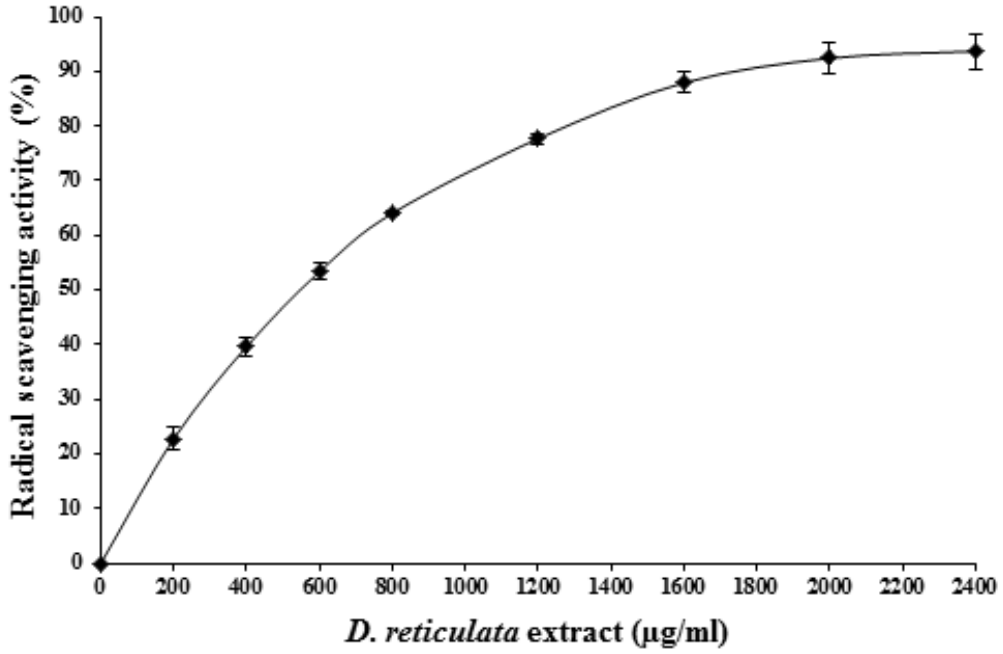


APPENDIX B

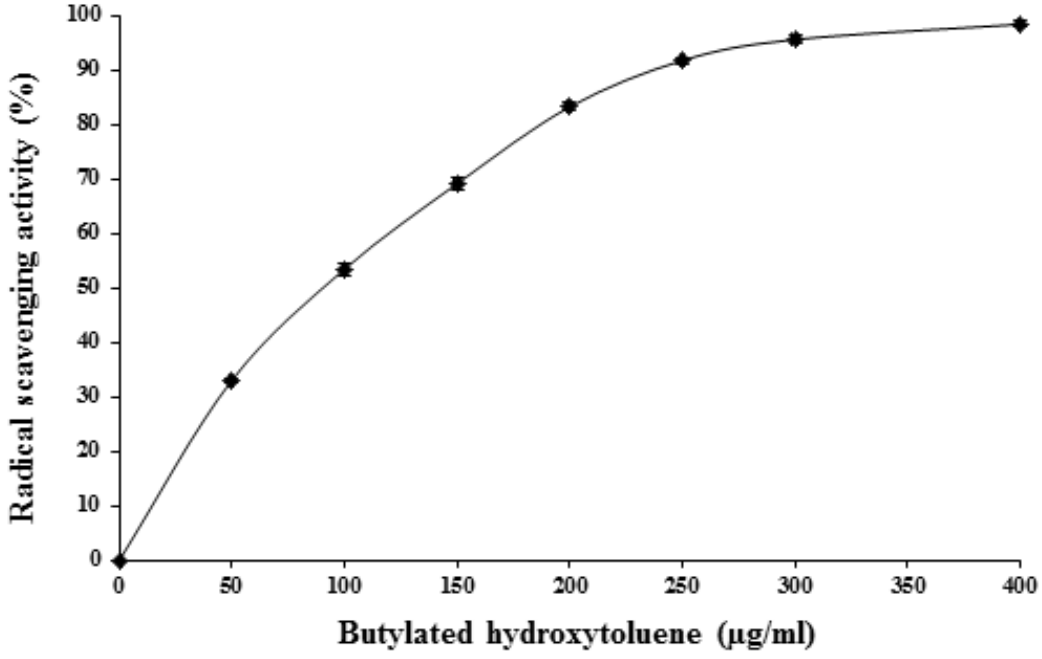
ANTIOXIDANT ACTIVITY



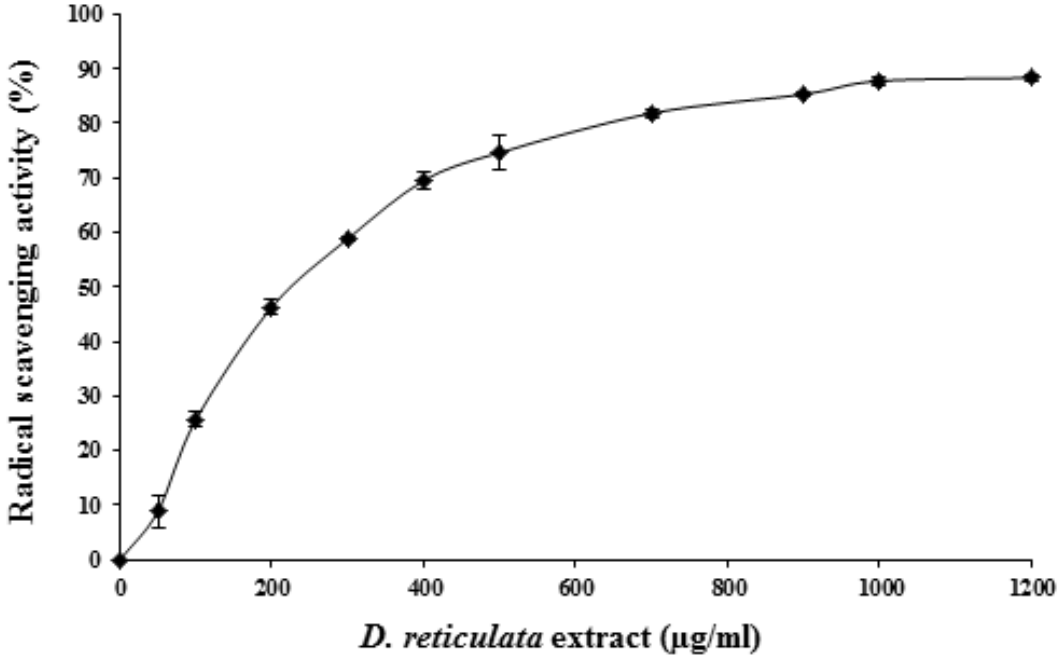
B.1 ABTS assay



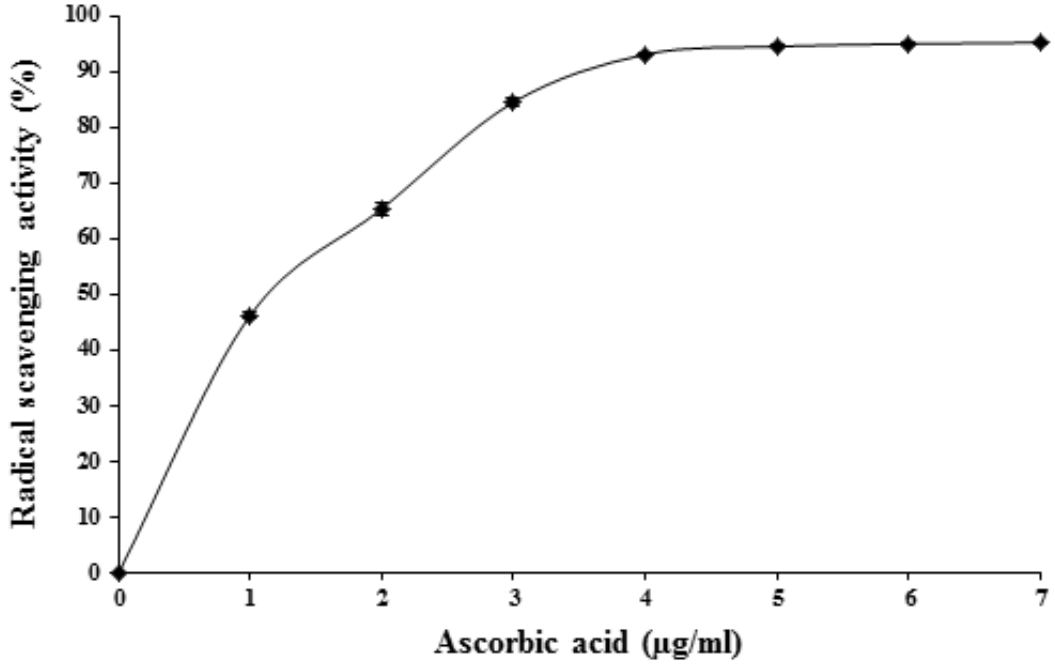
B.2 ABTS assay



B.3 DPPH assay



B.4 DPPH assay



APPENDIX C

REFERENCE RANGE OF

HAEMATOLOGICAL PARAMETERS

Table C1 Reference range of haematological parameters.

Parameters (Units)	Reference range	
	Male Wistar rat	**Female Wistar rat**
RBC ($\times 10^6$/µl)	7.6 - 9.0	7.3 - 8.5
HGB (g/dl)	15.5 - 17.1	15.0 - 16.2
HCT (%)	41.7 - 46.3	39.4 - 43.2
WBC ($\times 10^3$/µl)	4.6 - 8.6	1.9 - 6.4
LYM (%)	69.0 - 93.0	66.0 - 93.0
MON (%)	0.0 - 1.0	0.0 - 1.0
EOS (%)	0.0 - 5.0	0.0 - 3.0
BAS (%)	-	-
PLT ($\times 10^3$/µl)	780 - 975	665 - 1083
MCV (fl)	49.9 - 54.7	49.3 - 55.1
MCH (pg)	18.4 - 20.6	18.7 - 20.8
MCHC (g/dl)	36.2 - 37.9	36.6 - 39.0

Table C2 Reference range of biochemical parameters.

Parameters (Units)	Reference range	
	Male Wistar rat	**Female Wistar rat**
Glucose (mg/dl)	100.10 - 173.30	73.3 - 128.7
Total cholesterol (mg/dl)	49 - 76	44 - 82
Triglyceride (mg/dl)	83 - 223	43 - 101
Creatinine (mg/dl)	0.57 - 0.69	0.62 - 0.75
AST (U/l)	64 - 120	89 - 190
ALT (U/l)	25 - 52	20 - 39

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