IN VITRO STUDY ON GLUCOSE UPTAKE AND INSULIN STIMULATING PROPERTIES OF *PLUCHEA INDICA* (L.) LESS.

BY

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A thesis submitted in fulfilment of the requirement for the degree of Master in Pharmaceutical Sciences (Pharmacology)

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ABSTRACT

Insulin resistance and pancreatic β-cells defect are central features of diabetes disorder that may progress to several serious complications. Some of medicinal plants are potential sources for antidiabetic agents. *Pluchea indica* (*beluntas*) is widely distributed in Malaysia and it is believed to have antidiabetic properties. A hypoglycemic effect of *P. indica* in normal rats was reported in the previous study. This research was aimed to study the effects of *P. indica* in glucose and insulin regulation through cell-based *in vitro* model by using 3T3-L1 adipocytes and RIN-5F pancreatic β-cells. *P indica* was extracted using soxhlet apparatus with *n*-hexane, dichloromethane, ethyl acetate and methanol consequtively. The plant also was macerated using water to yield water extract. In cell viability test, the concentration of 0.2 mg/mL was found to be the maximum concentration of *P. indica* extracts in the absence of cytotoxicity. The preadipocytes were induced to differentiate into mature adipocytes prior to assay. The methanol extract at concentration of 0.05 mg/mL increased glucose uptake in adipocytes ($p<0.05$), as indicated by up regulation of adipogenesis-regulator *Pparγ* and insulin-sensitive glucose transporter 4 (*Glut4*) mRNAs. The *n*-hexane and water extracts at concentration of 0.05 mg/mL and 0.1 mg/mL respectively stimulated insulin release in β-cells ($p<0.05$). Moreover, these extracts elevated the transcription level of insulin receptor substrate 2 (*Irs2*) and glucose-transporter *Glut2* in β-cells. Taken together, this *in vitro* study was useful for a screening model of *P. indica* extracts to demonstrate the glucose uptake in adipocytes and insulin secretion activity in β-cells. These findings also suggest that *P. indica* extract deserves further investigation as a potential agent for diabetes management.

ملخص البحث

المقاومة للإنسولين والخلل في الخلايا بيتا البنكرياسية هي مميزات جوهرية في عيوب الداء السكري التي قد تتطور إلى تعقيدات خطيرة. تعتبر بعض النباتات الطبية مصادر محتملة للعوامل المضادة للسكري. نبات *Pluchea indica* (*beluntas*) واسع الانتشار في ماليزيا. ذُكر التأثير الخافض للسكر لـ*P. indica* عند الجرذان في الدراسات السابقة. تهدف هذه الدراسة لدراسة تأثير الـ*P. indica* على الغلوكوز وتنظيم الغلوكوز من خلال نموذج خلوي في الزجاج باستخدام خلايا 3T3-L1 adipocytes و RIN-5F pancreatic β-cells. تم إجراء الاستخلاص المتعاقب للـ *P. indica* للحصول على أربع خلاصات خام اعتماداً على القطبية وهي خلاصات الهكسان، الديكلورومتان، االإيتيل أسيتات، والميتانول. تم تضمين الخلاصة المائية أيضاً في هذه الدراسة. اعتماداً على اختبار حيوية الخلايا، وُجد أن 0.2 mg/mL هو التركيز الأعظمي من خلاصة الـ*P. indica* في غياب السمية الخلوية. تم تحريض طلائع الخلايا الدهنية preadipocytes للتمايز و عملية الصبغ تمت للخلايا البالغة. أظهرت خلاصة المتانول بتركيز 0.05 mg/mL زيادة ملحوظة في قبض الغلوكوز في الخلايا الدهنية ($p<0.05$)، كما هو ملاحظ من خلال زيادة التنظيم للـmRNAs لـ adipogenesis-regulator وناقل الغلوكوز 4 الحساس للإنسولين (*Glut4*). خلاصة الهكسان والخلاصة المائية بتركيز 0.05 mg/mL و 0.1 mg/mL على الترتيب أظهرت تحرير تحريضي للإنسولين في خلايا بيتا ($p<0.05$). إضافة إلى ذلك، هذه الخلاصات رفعت مستوى النسخ لـ*Insulin receptor substrate 2 (Irs2)* وناقل الغلوكوز (*Glut2*) في خلايا بيتا البنكرياسية. بالنظر إلى النتائج معاً، هذه الدراسة ضمن الزجاج مفيدة كنموذج اختباري لخلاصات الـ*P. indica* لتوضيح قبض الغلوكوز في الخلايا الدهنية وفعالية إفراز الإنسولين في خلايا بيتا. هذه النتائج أيضا تقترح أن خلاصة الـ *P. indica* تستحق المزيد من التحري كعامل محتمل للوقاية والعلاج لداء السكري.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it comforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master in Pharmaceutical Sciences (Pharmacology).

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DECLARATION

I hereby declare that this thesis is the result of my own investigation, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degree at IIUM or other institutions.

Wastuti Hidayati Suriyah

Signature …… Date ……

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…….. ……...

Signature Date

This work is dedicated to my beloved mother and the memory of my father,

who courageously fought diabetes throughout his life until the very end.

His hard work and patience always inspire to those who know him.

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LIST OF ABBREVIATIONS

2hPG	2 hour Plasma Glucose
A	Adenine
ADA	American Diabetes Association
AHPA	American Herbal Products Association
AIM	Adipogenesis-inducing medium
Akt	Murine thymoma viral oncogene homolog
AMM	Adipogenesis-maintaining medium
AMPK	Adenosine Monophosphate
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
C	Cytosine
C/EBP	CCAAT/enhancer binding protein
Ca_{2+}	Calsium (II)
$CaCl_2$	Calcium Chloride
CCK	Cholecystokinin
CDC	Centers for Disease Control
cDNA	complementary DNA
CHD	Coronary Heart Disease
CODE-2	The Cost of Diabetes in Europe– Type II
cm	centimetre
CVD	Cardiovascular Disease
DCM	Dichloromethane
DEPC	Diethylpyrocarbonate
DEX	Dexamethasone
dH_2O	double Distilled Water
DKA	Diabetic Ketoacidosis
DM	Diabetes Mellitus
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	deoxynucleoside Triphosphate
DPP	Dipeptidyl Peptidase 4
Eds./ed.	Editions/edition
e.g.	(*exempli gratia*); for example
ELlSA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular signal
et al	(*et alia*); and others
EtOH	Ethanol
FBS	Foetal Bovine Serum
FFA	Free Fatty Acid
Fig	Figure
FPG	Fasting Plasma Glucose

g	gram
g	gravity
G	Guanine
GACP	Good Agricultural and Collection Practices
GDM	Gestational Diabetes Mellitus
GIP	Gastric Inhibitory Peptide
GLP-1	Glucagon-Like Peptide 1
GLUT2	Glucose transporter type 2
GLUT4	Glucose transporter type 4
GLUTs	Glucose transporters
Grb	Growth factor receptor-binding protein
GSIS	Glucose-stimulated insulin secretion
h	hour/s
H_2O	Water
HCl	Hydrogen Chloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hex	Hexane
HGO	Hepatic Glucose Output
HGP	Hepatic Glucose Production
IAs	Insulin Analogues
IDDM	Insulin-Dependent Diabetes Mellitus
IFG	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
IRS	Insulin Receptor Substrate
IRS2	Insulin Receptor Substrate2
K_{ATP}	ATP-sensitive potassium channel
KCl	Potassium Chloride
KH_2PO_4	Potassium di-hydrogen phosphate
KRB	Krebs Ringer Bicarbonate
KRPH	Krebs Ringer HEPES
MAP	Mitogen-activated protein
MBq	Megabecquerel
MeOH	Methanol
MetS	Metabolic Syndrome
MgCl2	Magnesium Chloride
$MgSO_4$	Magnesium Sulfate
min	minute
MIX	Methylisobutylxanthine
µL	microlitre
mL	millilitre
mM	millimolar
MMP	Matrix Metalloproteinase
mRNA	messenger Ribonucleic Acid
MTT	3-(4,5-dimethylthiazol-2-y)2,5-diphenyltetrazolium bromide
Na_3VO_4	Natrium Orthovanadate
NaCl	Natrium Chloride
NAFLD	Non Alcoholic Fatty Liver Disease
$NaHCO_3$	Sodium Bicarbonate

NaOH	Sodium Hydroxide
NHMS III	National Health Morbidity Survey III
NIDDM	Non-Insulin-Dependent Diabetes Mellitus
NKHS	Non-Ketotic Hyperosmolar State
nm	nanometer
No.	Number/Numbers
N.T	Not Tested
obj	Objective
OD	Optical Density
ODM	Oral Diabetes Medications
OGTT	Oral Glucose Tolerance Test
PBS	Phosphate Buffer Saline
PCOS	Polycystic Ovary Syndrome
PCR	Polymerase Chain Reaction
PDK	PI-3K–dependent kinase
pH	ATP
PI-3K	Phosphatidylinositol 3-kinase
PIP3	Phosphatidylinositol 3,4,5-phosphate
PKC	Protein Kinase C
PPAR	Peroxisome Proliferator Activated Receptor
PPARγ	Peroxisome Proliferator Activated Receptorγ
RNA	Ribonucleid Acid
RPMI	Roswell Park Memorial Institute medium
RT PCR	Reverse Transcription Polymerase Chain Reaction
RT-qPCR	Real Time Reverse Transcription quantitative PCR
sec	second
SEM	Standard Error of Means
Src	Sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)
SREBP	Sterol Regulatory Element
T	Thymine
TNF-α	Tumor Necrosis Factor-α
TZD	Thiazolidinedione
WHO	World Health Organization

LIST OF SYMBOLS

α	alpha
β	beta
γ	gamma
p	the probability of obtaining the result
*	statistical significance denotation
n	sample sizes
^{o}C	degree Celcius
®	registered trademark

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 THE GROWING HEALTH PROBLEM OF DIABETES MELLITUS

Diabetes mellitus (DM) is now being recorded as one of the major threat to human health in the 21st century (Zimmet et al., 2001). DM has an influenced on the quality of life of sufferers and caused a wide range of problems from threatened live sustain to death (Zimmet, 2011). The high rates of morbidity and mortality of DM are resulted from the long-term progression of microvascular and macrovascular complications that caused several organs damage. Thus, people with DM are at a risk in serious health problems such as blindness, renal failure, and/or neuropathy, foot ulcers, amputation, cardio vascular and peripheral vascular diseases (World Health Organization [WHO], 1999). The chronic hyperglycemic condition has been known to accelerate the onset and progression of the DM and its complications (Brownlee, 2001; Reusch, 2003).

1.1.1 Epidemiology, Definition and Classification of Diabetes Mellitus

The worldwide prevalence of patient with DM is projected to rise from 2.8 % in 2000 to 4.4 % by 2030, which is affecting 366 million people. Most of the cases that is 298 million will occur in developing countries. This escalating trend is seen in the age between 45 and 64 years. It happens due to numerous factors such as escalation of population aging, urbanization, the high prevalence of obesity and lack of physical activity (Wild et al., 2004).

In Asia, people with DM present moderate obesity and chronic diabetic complications since juvenile which then leads to a shorter life expectancy compared to people in developed countries. People in Asia also have a highly genetic susceptibility to develop the type 2 DM, which is characterized by β-cell failure and abdominal obesity (Yoon, et al., 2006). In tandem with the rising prevalence of obesity and metabolic syndrome in certain ethnic groups of developing countries such as in South Asians, Hispanics and sub-Saharan Africans, the prevalence of the metabolic syndrome is also rapidly increasing in East Asia and China (Misra and Khurana, 2008). In Malaysia, the highest prevalence according to race is in the Asian Indians (28.8 %), followed by the Malays (24.2 %) and the Chinese (14.8 %) (Tan et al., 2004).

The Malaysian National Health Morbidity Survey III (NHMS III) in 2006 affirmed that the overall prevalence of DM in Malaysia among adults of ≥30 years old had risen from 8.3 % to 14.9 %. The data seemed to be associated with the increase of obesity (Hussein, 2008).

DM is defined as a chronic metabolic disease, which occurs when the level of blood glucose is higher than normal, resulting from dysfunction and/or lack of insulin produced by pancreatic β-cell and leads to distinctive complications (WHO, 1999). The current classification of DM consists of four main types (American Diabetes Association [ADA], 2004). The type 1, previously refers to insulin-dependent diabetes (IDDM), is characterized by absolute insulin deficiency due to β-cell destruction either immune-mediated or idiopathic. More than 90 % of type 1 DM resulting from the autoimmune islet cell destruction (Carver and Abrahamson, 2009). The type 2, a non-insulin-dependent diabetes (NIDDM), is a relative insulin deficiency owing to insulin secretion defect and insulin resistance (Wright, 2003). The third group consists of other less common types of DM that are caused by or associated with certain

specific conditions and/or syndromes. The fourth group considered as diabetes diagnosed during pregnancy which is called gestational diabetes (GDM) (ADA, 2004).

Diagnosis of type 2 DM is based on the fasting plasma glucose (FPG) and/or 2-hour plasma glucose (2hPG) concentration measurement during an oral glucose tolerance test (OGTT). The limits of diabetes level are FPG and 2hPG concentration at 7.0 and 11.1 mmol/L respectively (Drouin et al., 2009). The ADA Expert Committee identified the category of pre-diabetes state include both impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT). The current parameters from ADA revealed that a FPG concentration of 100 to 125 mg/dL (≥5.6 and <7.0 mmol/L) is considered to as IFG and that of more than 126 mg/dL is indicated as diabetes condition. IGT is considered if 2hPG concentration at 140 to 199 mg/dL (≥7.8 and <11.1 mmol/L), meanwhile the concentration > 200 mg/dL is considered as diabetes (ADA, 2004).

1.1.2 Complications of Diabetes Mellitus and Metabolic Aspect Related to Insulin Resistance

DM complications are the leading causes of morbidity and mortality of patients and require costly treatment (Umpierrez et al., 2002). In the United States, the estimation of total direct and indirect diabetes costs for 25.8 million people with diabetes reached $174 billion in 2007 (Centers for Disease Control [CDC], 2011). According to 'The Cost of Diabetes in Europe – Type II (CODE-2) study', the total medical cost for patients with both microvascular and macrovascular complications was increased up to 250 % compared to those with no complications (Williams et al., 2002).

Diabetic ketoacidosis (DKA) and non-ketotic hyperosmolar state (NKHS) are two types of acute diabetes complications that correlated to absolute or relative insulin

deficiency (Tripathi and Srivastava, 2006). The extreme hyperglycemic emergencies stages can cause lethargy and coma, with 2–5 % and 15 % of the mortality rates for DKA and NKHS respectively (Umpierrez et al., 2002).

Long-term hyperglycemia leads to the damage of several tissues of the body and produces chronic complications, such as retinopathy, neuropathy and nephropathy in microvascular disorders. The macrovascular problems include atherosclerosis, coronary artery, peripheral vascular and cerebrovascular diseases. Other chronic complications of nonvascular are gastroporesis, sexual dysfunction and skin changes (Tripathi and Srivastava, 2006).

Insulin resistance occurs in the majority of people with the metabolic syndrome (MetS). Reaven (1988) noted that the MetS or syndrome X refers to cluster of various metabolic risk factors that include abdominal obesity, hypertension, glucose intolerance or hyperglycemia and dyslipidemia. Dandona et al. (2005) postulated that the insulin resistance was induced by proinflammatory state of obesity through cytokine TNF-α factor and promoted clinical and biochemical manifestations of the MetS.

Insulin resistance is an underlying factor in this syndrome and it is also a characteristic feature of most of type 2 diabetic obese patients. In addition to that it is correlated with the increase risk of metabolic and cardiovascular cluster of disorders, such as coronary heart disease (CHD) and cardiovascular disease (CVD) (Kassi et al., 2011; Levesque and Lamarche, 2008). However, Insulin resistance syndrome or that associated with hyperinsulinemia are independent risk factors for CVD (Grundy et al., 2004). It is pathophysiologically occurs in insulin-resistant or hyperinsulinemic persons which do not develop to type 2 DM (Figure.1.1) (Reaven, 2005).

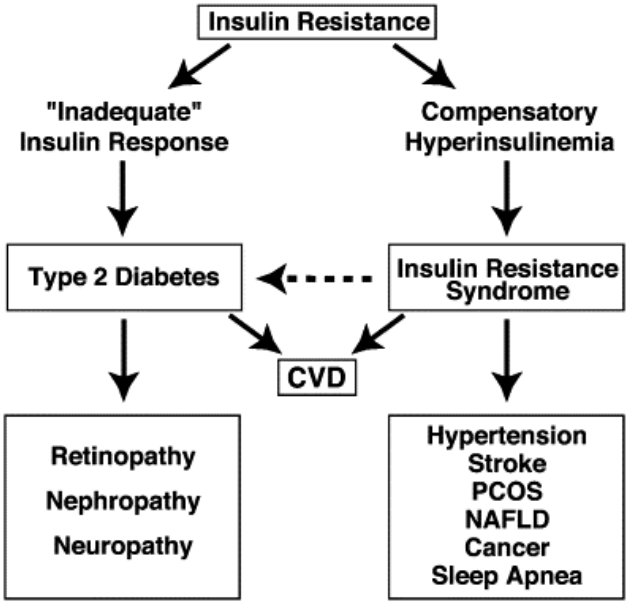

Figure 1.1: Clinical syndromes associated with insulin resistance. Insulin resistance leads to type 2 diabetes and other syndromes that linked to the compensatory hyperinsulinemia. The long-term of hyperglycemia produced microangiopathic complications. PCOS, polycystic ovary syndrome; NAFLD, non-alcoholic fatty liver disease; CVD, cardiovascular disease. (Retrieved from Reaven, 2005).

1.1.3 Interactions between Insulin Resistance and Insulin Secretion in The Development of Glucose Intolerance

The progressive failure of the pancreatic β-cell to compensate insulin resistance is the common pathway responsible for the development of type 2 DM (Kahn, 1998). The hypersecretion of insulin compensatory results in the expansion of β-cell mass and increase the expression of β-cell glucose metabolism enzymes (Cavaghan et al., 2000). In insulin resistance, β-cell may be exhausted due to over secreted insulin to maintain euglycemia. The β-cell failure will results in increase plasma free fatty acid (FFA) concentration, due to insufficient of insulin suppression on the plasma FFA. The elevation of plasma FFA concentration leads to increase hepatic glucose production (Reaven, 1988).