UNIVERSITI PUTRA MALAYSIA

IN VITRO EFFECTS OF Phaleria macrocarpa (Boerl.) MAHKOTA DEWA FRUIT AQUEOUS AND METHANOL-CHLOROFORM EXTRACTS ON GLUCOSE UPTAKE AND METABOLISM

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By

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IN VITRO EFFECTS OF *Phaleria macrocarpa* (Boerl.) MAHKOTA DEWA FRUIT AQUEOUS AND METHANOL-CHLOROFORM EXTRACTS ON GLUCOSE UPTAKE AND METABOLISM

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February 2014

Chair: Associate Professor Mohamad Aziz Dollah, PhD

Faculty: Faculty of Medicine and Health Sciences

Diabetes mellitus is the most common metabolic disease worldwide. One of the main pathophysiological defects of diabetes is insulin resistance due to impairment in the insulin-signaling pathway leading to a failure of the insulin stimulated glucose uptake in the target cells. Traditionally, plants such as *Phaleria macrocarpa* have been used to treat diabetes. The mechanism of how this *Phaleria macrocarpa* exert an anti-diabetic effect still obscure and thus a research consisted of five studies was conducted in vitro with the objectives to evaluate the anti-diabetic properties of *Phaleria macrocarpa* aqueous and methanol-chloroform fruit extracts using hepatocytes (HepG2 cells) as a model. In these studies, HepG2 cells were cultured in RPMI media in the presence of *Phaleria macrocarpa* fruit extracts (0, 0.01, 0.1 and 1 mg/mL) or metformin (1 mg/mL) or insulin (100 nM). Metformin and insulin were used as positive control. There are five studies including glucose uptake assay, inhibitor studies using wortmannin (PI3-kinase inhibitor) and genistein (protein tyrosine kinase inhibitor), glycogen synthesis assay and glycogen synthase activity assay. The present studies showed that both *Phaleria macrocarpa* extracts (aqueous and methanol-chloroform) had demonstrated the ability to enhance glucose uptake activity by up to 5-folds ($p<0.05$). That is similarly to the metformin when compared to the control. However, insulin showed the highest in glucose uptake activity (7-folds). The efficacy of aqueous extract was found to be slightly better than the commercial drug, metformin. In inhibitors study, the glucose uptake activity in cell cultures pre-treated with wortmannin or genistein and later with *Phaleria macrocarpa* extracts were significantly reduced ($p<0.05$) suggesting a possible involvement of PI3-kinase pathway and protein tyrosine kinase pathway in *Phaleria macrocarpa*-induced glucose uptake activity. The percentages of

inhibition at all doses of *Phaleria macrocarpa* extracts were comparable with the percentage of inhibition on insulin action. These indicated that the *Phaleria macrocarpa* action was mimicking the action of insulin. Glycogen synthesis was stimulated in HepG2 cells by more than 1-fold after treatment with *Phaleria macrocarpa* extracts. Metformin showed no effect on glycogen synthesis whereas insulin caused a maximum increased in glycogen synthesis activity in two hours. Both aqueous and methanol-chloroform of *Phaleria macrocarpa* fruit extracts significantly increased ($p<0.05$) glycogen synthase activity. The significant changes in enzyme activities were observed at as low as 0.01 mg/mL of *Phaleria macrocarpa* extracts while the maximum effects was observed at 1.0 mg/mL aqueous extract. This was similar to insulin effect and thus *Phaleria macrocarpa* was able to increase glycogen synthase activity at the same rate as insulin. Moreover, metformin also demonstrated significant increase in glycogen synthase activity ($p<0.05$) when compare to control, however its activity was lower than insulin. These studies concluded that *Phaleria macrocarpa* extracts have the ability to increase glucose uptake, glycogen synthesis and glycogen synthase activity similar to insulin action. Thus, *Phaleria macrocarpa* fruit extracts have insulin mimic activity. Therefore, with further investigation including clinical trial, *Phaleria macrocarpa* has a therapeutic potential as an anti-diabetic agents.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan ijazah Sarjana Sains

Kesan Estrak Akues dan Metanol-Kloroform Buah *Phaleria macrocarpa* (Boerl.) Mahkota Dewake Atas Pengambilan dan Metabolisme Glukosa In Vitro

Oleh

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Penyakit diabetes adalah penyakit metabolik yang tersebar meluas di seluruh dunia.Salah satu punca utama penyakit diabetes adalah disebabkan oleh rintangan hormon insulin yang berpunca dari penurunan dalam penghantaran isyarat insulin yang kemudian membawa kepada kegagalan insulin untuk merangsang pengambilan glukosa oleh sel-sel tubuh. Secara tradisional, *Phaleria macrocarpa* ialah sejenis tumbuhan yang telah digunakan untuk merawat diabetes. Mekanisme bagaimana *Phaleria macrocarpa* boleh memberi kesan anti–diabetik masih tidak diketahui. Oleh yang demikian, lima kajian telah dijalankan secara *in-vitro* menggunakan sel hepatosit (HepG2) sebagai model dengan objektif untuk menilai ciri-ciri anti-diabetik estrak akues dan ekstrak metanol-kloroform buah *Phaleria macrocarpa*. Dalam kajian ini, sel HepG2 dieram dalam media RPMI dan diberi masing-masing estrak buah *Phaleria macrocarpa* (0, 0.01, 0.1 dan 1 mg/mL) atau insulin (100 nM) atau metformin (1 mg/mL).Metformin dan insulin digunakan sebagai kawalan positif. Terdapat lima kajian termasuk kajian pengambilan glukosa, kajian perencat meggunakan wortmannin (perencat PI3-kinase) dan genistein (perencat protein tyrosine kinase), sintesis glikogen dan kajian aktiviti enzim glikogen sintase. Kajian ini menunjukkan bahawa kedua-dua *Phaleria macrocarpa* ekstrak (akues dan metanol-kloroform) mempunyai keupayaan untuk meningkatkan aktiviti pengambilan glukosa sehingga 5 kali ganda ($p<0.05$) dari kawalan. Kesan ini adalah sama seperti kesan metformin. Walau bagaimanapun, insulin menunjukkan aktiviti pengambilan glukosa paling tinggi (7 kali ganda). Keberkesanan ekstrak air didapati sedikit lebih baik daripada ubat komersial iaitu metformin.Dalam kajian kedua dan ketiga, aktiviti pengambilan glukosa dalam sel didik yang diberi wortmannin atau genistein menyebabkan aktiviti pemgambilan glukosa oleh sel didik berkurang dengan ketara ($p<0.05$). Ini menunjukkan terdapat penglibatan PI3-kinase dan tyrosine kinase dalam aktiviti

pengambilanglukosa oleh HepG2 sel. Peratusan perencatan kemasukan glukosa oleh semua dos ekstrak *Phaleria macrocarpa* adalah setara dengan peratusan perencatan terhadap tindakan insulin. Ini menunjukkan bahawa, tindakan *Phaleria macrocarpa* adalah menyamai dengan tindakan insulin.Sintesis glikogen yang dirangsang di dalam sel HepG2 adalah lebih daripada 1 kali ganda selepas diberi ekstrak *Phaleria macrocarpa*. Metformin tidak menunjukkan sebarang kesan ke atas sintesis glikogen manakala insulin menunjukkan kesan maksimum terhadap aktiviti sintesis glikogen dalam masa dua jam. Kedua-dua esktrak akues dan metanol-kloroform *Phaleria macrocarpa* meningkatkan aktiviti enzim glikogen synthase dengan ketara ($p<0.05$). Perubahan ketara aktiviti enzim adalah berkadar dengan dos *Phaleria macrocarpa* ekstrak dan menyamai dengan kesan insulin.Selain itu, metformin juga menunjukkan peningkatan yang ketara dalam aktiviti enzim glikogen synthase ($p<0.05$) apabila dibandingkan dengan kumpulan kawalan, namun aktivitinya adalah lebih rendah daripada insulin.Kajian ini membuktikan bahawa ekstrak *Phaleria macrocarpa* mempunyai keupayaan untuk meningkatkan pengambilan glukosa, sintesis glikogen dan aktiviti enzim glikogen synthase pada sel HepG2 yang menyamai dengan tindakan insulin.Oleh itu, kajian ini dapat membuktikan peranan dan mekanisme tindakan*Phaleria macrocarpa* untuk mengurangkan gula dalam darah. Oleh itu, untuk mengetahui lebih lanjut mengenai potensi *Phaleria macrocarpa* sebagai agen anti-diabetik, adalah dicadangkan membuat siasatan lanjut melibatkan percubaan klinikal.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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DECLARATION

Declaration by graduate student

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LIST OF ABBREVATIONS

Akt	Protein kinase
ARC	Arcuate nucleus
ATCC	American Type Culture Collection
cAMPK	Cyclic adenosine monophosphate
COX-2	Cyclooxygenase 2
CPLA2	Cytosolic phospholipase A.
DMSO	Dimethylsulfoxide
EFGR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
FDA	Food and Drug Administration
G6P	Glucose-6-phosphate
GLUT	Glucose transporter
GSK 3β	Glycogen synthase kinase-3β
HDL	High density lipoprotein
HepG2	Hepatocellular carcinoma cells
HER2	Human epidermal receptor-2
HGP	Hepatic glucose production
HL-60	Human promyelocytic leukemia cells
IDF	International Diabetes Federation
IGF	Insulin growth factor
IR	Insulin receptor
IRS 1/2	Insulin receptor substrate 1/2
IRTK	Insulin receptor tyrosine kinase
KIF3	Kinesins superfamily-3
LC-MS	Liquid Chromatography/Mass Spectrometry
LDL	Low density lipo protein
LKB1	Liver kinase B1
mRNA	Messenger ribonucleic acid
MTT	Methylthiazol Tetrazolium Assay
NEFAs	Non-esterified fatty acids
NKC	Natural killer cells

PBS	Phosphate buffered saline
PCSK9	Proprotein convertase subtilisin kexin 9
PDK1	Phosphoinositide-dependent kinase-1
PEPCK	Phosphoenolpyruvate carboxykinase
PET	Positron Emission Tomography
PI3-kinase	Phosphatidylinositol-3-kinase
PIP3	Phosphatidylinositol (3,4,5)- trisphosphate
PKB	Protein-kinase B
PKC	Protein-kinase C
PTK	Protein tyrosine kinase
SDS	Sodium dodecyl sulfate
STAT3	Signal transducers and activators of transcription 3
UDP	Uridine di-phosphate
UTP	Uridine tri-phosphate
VEGF-C	Vascular endothelial growth factor C

LIST OF ANNOTATIONS

>	More than
<	Less than
cm^2	Centimeter square
dpm	Disintegration per minute
g	gram
mg/mL	Miligram per mililiter
mL	Mililiter
nM	Nanomolar
^oC	Degree celcius
S.E.M	Standard error of mean
µCi/mL	Micro curie per mililiter
µL	Microliter

CHAPTER 1

INTRODUCTION

1.0 Overview

Diabetes is a complex and multi-factorial disease mainly caused by insulin deficit that leads to abnormalities in carbohydrate, fat and protein metabolism on target tissues (American Diabetes Association, 2010). Diabetes is considered the most prevalent disorder in the world. In 2011, there were already 336 million patients with diabetes mellitus worldwide. This value is expected to increase up to 552 million cases by the year of 2030 (International Diabetes Federation, 2011). Malaysia is very likely heading towards epidemic proportions based on the result in Malaysian National HealthMorbidity Survey III 2006 reported by Letchuman et al. (2010), which showed that Malaysia has alreadyreached the projected prevalence for year 2025. The number of diabetic patients increased from 8.3% in 1996 to 11.6 % in 2006.

Diabetes mellitus is a common metabolic disorder of the endocrine system. It is described by high blood glucose (sugar). Glucose is the main source of energy for the cells and tissues especially brain. Both adults and children require an intake of 130 grams of dietary glycemic carbohydrates per day to cover the glucose requirement of the brain (EFSA Panel on Dietetic Products Nutrition and Allergies, 2010). In a normal healthy people, blood glucose is strictly maintained by a close ranged, around 3.9 to 6.1 mmol/L (Philip, 2007). However, in diabetic patients, this range cannot be controlled by the body and always causes extra glucose circulating in the blood.

Insulin is the hormone that responsible in maintaining blood glucose level by stimulating phosphorylation and utilization of glucose. Liver is the primary site of insulin action. After a meal, insulin stimulates glucose uptake and store surplus glucose in the form of glycogen in the liver. Insulin initiates the glucose uptake into the cells by activating the insulin receptor (IR). It is then phosphorylates and recruits different substrate adaptors such as the insulin receptor substrates family (IRS)including IRS-1 and IRS-2.This initiatesthe phosphatidylinositol-3-kinase (PI3-kinase) pathwayandconducts the modulation of other proteins necessary for the metabolic effects of insulin (Klover and Mooney, 2004). PI3-kinase is known to play an important role in glucose transporter translocation (Lizcano and Alessi 2002). The inhibition of catalytic subunit of PI3-kinase, p110 with inhibitors for example wortmannin, can totallyblock most actions of insulin (Okada et al., 1994). Previous studies postulate that defect in glucose transporter translocation and impaired in insulin signaling cascade have been identified in patients with type 2 diabetes (Leng et al., 2004).

By looking at the prevalence mentioned earlier, many researchers interested to investigate new anti-diabetic drugs that can treat hyperglycemia better than the commercially available drugs due to its clinical side effects such as gastrointestinal side effect and increase cardiovascular risk(Hoffmann et al.,

2003; Nissen and Wolski, 2007). Therefore, many diabetic patients are also recommended to receive complementary and alternative medicine therapies.

Herbal medicines have been practiced many years ago and research showed their value in treatment and prevention of various kinds of diseases. Recently, much attention has been paid to the discovery of natural products that may be advantageous in reducing the risks of diabetes. Normally, these natural products are usually has less toxicity, with little side effects than over the counter drugs (Jung et al., 2006). Diabetes is known can be controlled using natural products and therefore, the discovery and development of novel drugs for diabetic mellitus based on naturalproducts are very important (Dham et al., 2006). The available literature shows that there are more than 400 plant species possess anti-hyperglycemic activity (Rai, 1995).

Phaleria macrocarpa typically known as Crown God originated from Papua, Indonesia has been used traditionally to treat diabetic mellitus and other diseases such as rheumatic, kidney, gout, heart, hypertension and eczema (Lisdawati, 2002). *Phaleria macrocarpa* fruit is rich in alkaloids, saponins and flavoids while the leaves contain mangiferin, and saponins compound, which are classified into triterpenoid and steroid saponins, based on the structure of aglycon (Zhang et al., 2006; Saufi et al., 2008). Both of these compounds are reported to have anti-inflammatory and cytotoxic effects (Gotama et al., 1999). The isolated chemical constituents of *Phaleria macrocarpa* fruits extract include Icariside C3, magniferin, and gallic acid (Oshimi et al., 2008). It is found that mangiferin has a wide range of therapeutic effects including anti-diabetic, anti-HIV, anti-cancer, immunomodulatory and anti-oxidant activities (Yoshimi et al., 2001). *Phaleria macrocarpa* is one of the herbs that has been claimed traditionally to reduce high blood glucose levels.Sri Sugiwati et al., (2006) demonstrated that an oral administration of *Phaleria macrocarpa*leaves extract exhibited hypoglycemic effect and its ability to act as α-glucosidase inhibitors. Study in our laboratory has successful screened this plant on itsanti-diabetic properties. After 28 days of administration with *Phaleria macrocarpa*fruit aqueous extract, blood glucose level of diabetes-induced rats found to reduce from 25 mmol/L to5 mmol/L (Raudhah and Dollah, 2008).In addition, it was reported that the bioassay-activity guided of anti-diabetic study of the methanol extract of*Phaleria macrocarpa*fruit showed that the flavonoids sub-fraction, which contains 22% of magniferin, has the highest anti-diabetic activity on diabetic rats (Rabyah et al., 2012). However, the mechanism of action of which *Phaleria macrocarpa* reduces the blood glucose still remains to be elucidated.

Hence, the present study focuses on finding the possible mechanism of actions of *Phaleria macrocarpa* extracts on glucose metabolism by determining in-vitro the glucose uptake, glycogen synthesis and glycogen synthase enzyme activity. In this study, the possible mechanism of action of *Phaleria macrocarpa* extracts on glucose uptake was also identified using specific inhibitors.

1.1 Objectives

1.1.1 General Objective

To investigate the ability of *Phaleria macrocarpa*aqueous and methanol-chloroform fruits extracts to exert glucose metabolism effect in HepG2 cells by measuring the glucose uptake activity and its possible mechanism using inhibitors (wortmannin and genistein), glycogen synthesis and glycogen synthase enzyme activity.

1.1.2 Specific Objectives

i. To study on the effect of *Phaleria macrocarpa* fruits extracts on glucose uptake by measuring the uptake activity of 2-Deoxy D-[1-2,^{3}H (N)] glucose in HepG2 cells.

ii. To determine on the effect of *Phaleria macrocarpa* fruits extracts on glucose uptake in HepG2 cells following incubation with wortmannin (PI3-kinase inhibitor).

iii. To determine on the effect of *Phaleria macrocarpa* fruits extracts on glucose uptake in HepG2 cells following incubation with genistein (protein tyrosine kinase inhibitor).

iv. To measure on the effect of *Phaleria macrocarpa*fruits extracts on glycogen synthesis by measuring the activity of D-[^{14}C]glucose in HepG2 cells.

v. To study on the effect of *Phaleria macrocarpa* fruits extract on glycogen synthase by measuring the activity of [^{3}H]-UDP glucose in HepG2 cells.

1.2 Hypothesis

Phaleria macrocarpa extracts stimulate glucose uptake, glycogen synthesis and glycogen synthase activity in HepG2 cells and mimic insulin.

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