ISOLATION AND *IN VITRO* ANTIDIABETIC PROPERTIES OF A PROANTHOCYANIDIN FROM *CINNAMOMUM ZEYLANICUM*

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Dedicated to my beloved parents, brothers and my sister, who had provided me with support spiritually and emotionally throughout this long journey. To my dearest wife; Deny Susanti, her loving contribution has no boundary and kept me going at the difficult times, and my children; Muhammad Ghaisannaufal and Muhammad Luthfirrahman, who had motivated me to complete the study.

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ABSTRACT

Characterization and *in vitro* testing of antihyperglycemic activity of a natural product from the stem bark of *Cinnamomum zeylanicum* have been carried out. Characterization was carried out using HPLC, ultraviolet, infrared, ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy and mass spectrometry. Based on the spectroscopics data, bioactive compound was identified as cinnamtannin B1. Cinnamtannin B1 is a double linked flavan-3-ol trimer known as A-type proanthocyanidin. Its activity was evaluated using cell proliferation, cell differentiation, glucose regulation and phosphorylation of insulin receptor β-subunit in 3T3-L1 cells. Cinnamtannin B1 promoted cell proliferation approximately 2-fold at 48 hours after treatment. Dosage range of cinnamtannin B1 in promoting cell proliferation was 100-150 μg/mL (0.11-0.17 mM). A mixture of 0.1 mM cinnamtannin B1 and 150-200 μg/mL water extract induced differentiation of adipocytes similar to that of the insulin activity. Addition of cinnamtannin B1 into the culture of 3T3-L1 adipocyte increased glucose consumption up to 32%. The mixture of 0.1 mM cinnamtannin B1 and 100 nM insulin stimulated glucose uptake from a basal value by 1.8 and 1.7-fold, respectively. Cinnamtannin B1 and water extract stimulated phosphorylation of insulin receptor β-subunit. There was no phosphorylation of insulin receptor observed in 3T3-L1 preadipocytes. The activity of cinnamtannin B1 in stimulating glucose uptake and phosphorylation were inhibited by wortmannin and cytochalasin B. In contrast, sodium orthovanadate stimulated glucose uptake and phosphorylation. The results demonstrated that activity of cinnamtannin B1 and water extract mimics insulin action. They acted directly on insulin receptor β-subunit by activation of PI3-kinase that stimulates glucose transporter-4 (GLUT-4) translocation. Stimulation of GLUT4 translocation therefore stimulates glucose uptake lead to glucose disposal process in adipocytes. Based on the work that has been carried out, it was suggested that cinnamtannin B1 could be one of the potential lead drug compound in the treatment of type 2 diabetes.

ABSTRAK

Pencirian dan pengujian keaktifan *in vitro* sebagai antihyperglisemia keatas sebatian semula jadi dari kulit kayu manis (*Cinnamomum zeylanicum*) telah dijalankan. Pencirian dijalankan dengan menggunakan HPLC dan spektroskopi ultralembayung, inframerah, ^{1}H dan ^{13}C resonans magnet nukleus dan spektrometri jisim. Berdasarkan data-data spektroskopi yang diperolehi, sebatian bioaktif dikenalpasti sebagai cinnamtannin B1. Cinnamtannin B1 merupakan flavan-3-ol trimer dua rangkaian yang lebih dikenali sebagai proantosianidin jenis-A. Aktiviti cinnamtannin B1 ke atas sel kultur diuji dengan menggunakan metodologi pembiakan sel, pembezaan sel, pengaturan glukosa dan pemfosforilan β-subunit reseptor insulin di dalam tisu 3T3-L1 adiposit. Cinnamtannin B1 meningkatkan pembiakan sel lebih kurang 2 kali ganda setelah 48 jam eksperimen dijalankan. Julat dos cinnamtannin B1 dalam meningkatkan pembiakan sel adalah di antara 100-150 μg/mL (0.11-0.17 mM). Campuran 0.11 mM cinnamtannin B1 dan 150-200 μg/mL ekstrak air didapati menginduksi penukaran sel preadiposit kepada tisu adiposit matang, aktiviti ini serupa dengan aktiviti insulin. Penambahan cinnamtannin B1 ke atas kultur tisu adiposit meningkatkan penggunaan glukosa sebanyak 32%. Campuran 0.1 mM cinnamtannin B1 dan 100 nM insulin merangsangkan penyerapan glukosa sebanyak 1.8 dan 1.7 kali ganda berbanding nilai asas masing-masing. Cinnamtannin B1 dan ekstrak air juga merangsang pemfosforilan β-subunit reseptor insulin. Pemfosforilan reseptor insulin tidak berlaku pada sel 3T3-L1 preadiposit. Perangsangan penyerapan glukosa dan pemfosforilan cinnamtannin B1 disekat oleh wortmannin dan cytochalasin B. Manakala sodium orthovanadate merangsang penyerapan glukosa dan pemfosforilan. Hasil kajian bioaktiviti menunjukan bahawa aktiviti cinnamtannin B1 and ekstrak air mimik kepada aktiviti insulin. Cinnamtannin B1 dan ekstrak air bertindak secara langsung pada reseptor insulin β-subunit dengan mengaktifkan PI3-kinase yang akan merangsang translokasi pengangkut glukosa-4 (GLUT-4). Perangsangan ke atas pengangkut glukosa-4 selanjutnya merangsang pula penyerapan glukosa dan membolehkan pembuangan glukosa oleh tisu adiposit. Kajian yang telah djalankan mendapati bahawa cinnamtannin B1 berpotensi untuk dijadikan sebagai bahan untuk mengubati penyakit diabetes jenis 2.

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δ	-	Chemical shift
ε	-	Molar extinction
^{1}H	-	Proton
J	-	Coupling constant in Hz
λ	-	Wavelength
Mr	-	Molecular weight
v	-	Wavenumber

LIST OF ABBREVIATIONS

Ac_2O	-	anhydride acetic acid
br	-	broad
^{13}C	-	carbon
$CaCl_2$	-	calcium chloride
C/EBP	-	C/AAT Enhancer Binding Protein
CD_3OD	-	deuterium methanol
COSY	-	Correlated Spectroscopy
d	-	doublet
dd	-	doublet of doublet
DMEM	-	Dulbecco's Modified Eagle Medium
ELISA	-	Enzyme-linked Immunosorbent Assay
EtOAc	-	ethyl acetate
FAB-MS	-	Fast Atom Bombardment-Mass Spectrometry
FBS	-	foetal bovine serum
$FeCl_3$	-	ferric chloride
GLUT-4	-	glucose transporter-4
HEPES	-	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HPLC	-	High Performance Liquid Chromatography
HCOOH	-	formic acid
HOAc	-	acetic acid
Hz	-	Hertz
IDDM	-	Insulin-Dependent Diabetes Mellitus
IGF-I	-	Insulin-Like Growth Factor I
IgG	-	immunoglobulin G
IR	-	infrared
IR	-	insulin receptor

IRS	-	insulin receptor substrate
IUPAC	-	International Union of Pure and Applied Chemistry
KBr	-	kalium bromide
KCl	-	kalium chloride
kDa	-	kiloDalton
KRPH	-	Krebs Ringer Phosphate Hepes
L	-	Liter
LDL	-	low-density lipoprotein
lit	-	literature
m	-	multiplet
M	-	molar
MAPK	-	mitogen-activated protein kinase
max	-	maximum
MeOH	-	methanol
$MgSO_4$	-	magnesium sulphate
mL	-	milliliter
µg	-	microgram
MHz	-	megaHertz
mM	-	millimolar
m.p	-	melting point
MTT	-	3- (4, 5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide
Na_2HPO_4	-	disodium hydrogen phosphate
Na_3VO_4	-	sodium orthovanadate
NIDDM	-	Non-Insulin-Dependent Diabetes Mellitus
nM	-	nanomolar
NMR	-	Nuclear Magnetic Resonance
PBS	-	phosphate buffered saline
PI3-K	-	phosphatidylinositol 3-kinase
PPARγ	-	peroxisome proliferator-activated receptor gamma
rel. int.	-	relative intensity
R_f	-	retention factor
SDS-PAGE	-	sodium dodecylsulfate-polyacrylamide gel
TLC	-	thin layer chromatography

UV	-	ultraviolet
w/v	-	weight per volume

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CHAPTER 1

INTRODUCTION

1.1 General

Type 2 diabetes (non-insulin-dependent diabetes mellitus) is a chronic metabolic disease that results from defects in insulin secretion and insulin receptor kinase. Investigation of novel small active molecule that can potentiate insulin action or having a similar action as insulin is important in the treatment of diabetes. World ethnobotanical information on medicinal plants reports almost 800 plants used in the treatment of diabetes mellitus. However, only a small number of them have been studied thoroughly (Alarcon-Aguilar *et al*., 1998).

Cell line provides a continuous source of large numbers of cells necessary for study of proliferation and differentiation. The 3T3-L1 cell line is selected for this study because it plays an important role in lipid storage and glucose homeostasis. 3T3-L1 adipocytes have been used extensively to study the regulation of such as glucose transporters, cell proliferation and insulin signaling. During differentiation, 3T3-L1 cells experience a 20-fold increase in the number of insulin receptors and acquire the ability to utilize glucose in response to insulin (Frost and Lane, 1985). The most frequently employed adipocytes cell lines are 3T3-F442A and 3T3-L1. They were clonally isolated from Swiss 3T3 cells derived from disaggregated 17-to19-day mouse embryos (Green and Kehinde, 1975 and 1976).

1.2 Plants in Type 2 Diabetes Treatment

The plant extracts and its product play an important role in treating many symptoms. Pioneering studies on the active constituents of *Podophyllum peltatum* followed by the discovery and development of the antileukemic agents, vinblastine and vincristine from *Catharantus roseus* provided convincing evidence that plants could be sources of novel and potential chemotherapeutic agents (Baker *et al*., 1995).

Imparl-Radosevich *et al*. (1998), Jarvill-Taylor *et al*. (2001), Anderson *et al*. (2004) and Pszczola (2001) have introduced method to evaluate plants compound for antihyperglycemia activity. The plant used is cinnamon and suggested to contain a novel phenolic polymer. The compound stimulated phosphorylation insulin receptor and enhance glucose uptake in 3T3-L1 adipocytes. Khan *et al*. (2003) reported the effect of *Cinnamomum cassia* on the diabetes patients. The results of their study demonstrate that intake of 1, 3, or 6 g of cinnamon per-day reduces serum glucose, triglyceride, LDL cholesterol, and total cholesterol in people with type 2 diabetes. They suggested that the inclusion of cinnamon in the diet of people with type 2 diabetes reduces the risk factors associated with diabetes and cardiovascular diseases.

There are several bioactive plant extracts that have been studied for antidiabetic agent. A bioactive compound from Chinese plant *Lithospermum erythrorhizon* stimulates glucose uptake in 3T3-L1 adipocytes (Kamei *et al*., 2002). An extract from *Lagerstroemia speciosa* has insulin like glucose uptake stimulatory effect (Liu *et al*., 2001). In addition, an antidiabetic fungal metabolite from culture broth of *Pseudomassaria* sp. was discovered as an insulin agonist and showed to be highly effective in animal models diabetes (Qureshi *et al*., 2000).

1.2.1 Insulin-mimetic Compounds

The cellular response to insulin is mediated through the insulin receptor (IR), which is a tetrameric protein consisting of two identical extracellular α-subunits that bind insulin as well as two identical transmembrane β-subunits that have intracellular

tyrosine kinase activity (White, 1997; White *et al*., 1985 and 1994). Insulin resistance, an important feature of type 2 diabetes, is manifested as attenuated insulin receptor (IR) signaling in response to insulin binding. A drug that promotes the initiation of IR signaling by enhancing IR autophosphorylation should, therefore, be useful for treating type 2 diabetes.

In order to discover a non peptide, small active compound that exhibited insulin-mimetic activity, Salituro *et al*. (2001) screened over 50,000 samples of natural extracts for their ability to mimic insulin activity. They recently discovered a small non-peptidyl molecule (L-783,281) from a fungal (*Pseudomassaria*) extract (Zhang *et al*., 1999; Ding *et al*., 2002; Qureshi *et al*., 2000). Purification of the active compound revealed that demethylasterriquinone B1 (known as L-783,281) structurally belong to quinone-like structure of natural product. L-783,281 seems to bind directly to the intracellular β-subunit of the insulin receptor containing the insulin receptor tyrosine kinase activity. Binding leads to a conformational change resulting in activation of the kinase and induction of the insulin signaling cascade downstream of the receptor at micromolar concentrations. L-783,281 leads to phosphorylation of a number of proteins of the insulin signaling pathway including the β-subunit of the insulin receptor, the insulin receptor substrate-1 and the Akt-kinase (or protein kinase B). In addition, it stimulates phosphoinositol 3-kinase. L-783,281 was also shown to increase glucose uptake in primary adipocytes and in soleus muscle.

Manchem *et al*. (2001) found a chemical called as TLK16,998. This compound activated the tyrosine kinase domain of the IR β-subunit at concentrations of 1 μmol/l or less but had no effect on insulin binding to the IR α-subunit even at much higher concentrations. TLK16,998 alone had no effect on IR signaling in mouse 3T3-L1 adipocytes but, at concentrations as low as 3.2 μmol/l, enhanced the effects of insulin on the phosphorylation of the IR β-subunit and IR substrate-1, and on the amount of phosphatidylinositol 3-kinase that coimmunoprecipitated with IR substrate-1.

1.3 Diabetes Mellitus

According to International Diabetes Federation, currently more than 194 million people with diabetes worldwide and the epidemiological estimates that by 2025 there will be 333 million diabetes sufferers. It will be almost twice as many sufferers as today, and has become a serious public health problem, particularly in developed countries. This will be predominantly individuals with type 2 diabetes (Vessby, 2000; Seidell, 2000; Kim *et al*., 2001; Barrett, 2004).

Type 2 diabetes mellitus is an increasingly common disorder of carbohydrate and lipid metabolism (Nadler and Attie, 2001). Two important characteristics of this disease are insulin resistance, the failure of peripheral tissues; including liver, muscle, and adipose tissue, to respond to physiologic doses of insulin, and failure of pancreatic β-cells to properly secrete insulin in response to elevated blood glucose levels. Obesity is a significant risk factor for the development of type 2 diabetes mellitus. An extremely lean and lipoatrophic models have revealed a similar predisposition to developing diabetes. Although it may seem paradoxical that both increased adiposity and severely reduced fat mass cause diabetes, a common pathophysiologic process in fat may be responsible for the predisposition to develop hyperglycemia in both conditions (Kim *et al*., 2001; Nadler and Attie, 2001).

Broadhurst (1997) proposed the major causative factors for non-insulin dependent-diabetes mellitus (NIDDM) involving obesity and overfatness; carbohydrate and fat over nutrition; lack of polyunsaturated fatty acids (PUFA) in plasma membranes and unbalanced triglyceride intake; chromium deficiency; and lack of soluble fiber and relevant beneficial phytochemicals.

NIDDM is a complex disease that is currently thought to be influenced by more than a single gene or environmental factor. Although the relative contribution of genetic and environmental factors to the development of NIDDM differs among individuals, patients generally have two common metabolic abnormalities: insulin resistance and defects in glucose-stimulated insulin secretion, which lead to disease state (Fig.1.1).

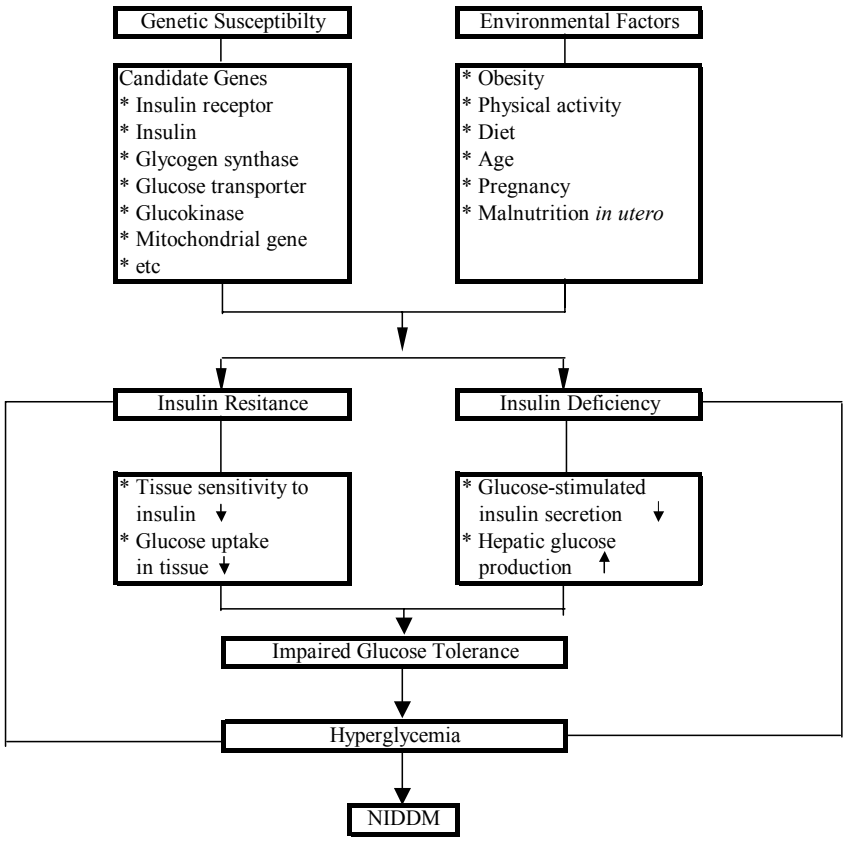

Figure 1.1 Schematic diagram of progressive pathogenesis of NIDDM (Jun *et al.*, 1999).

Figure 1.2 shows that glucose affects insulin release by acting in multiple ionic and metabolic mechanisms. β cells are sensitive to the concentration of glucose. If β cells are exposed to elevated glucose levels for more than 15 min, they become primed so that their response to glucose becomes greater than their initial response. Glucose inhibits ATP-sensitive K^+ channels (possibly by its stimulation of ATP production), which causes membrane depolarization. Depolarization activates Ca^{2+} channels resulting in Ca^{2+} entry and an increase in cytosolic Ca^{2+}. Extracellular Ca^{2+} production and energy production are required for stimulation of insulin secretion. During glucose stimulation of healthy β cells, normal insulin secretion takes place. However, in defective β cells, impaired insulin secretion may cause delayed insulin secretion. The insulin receptor substrate-1 (IRS-1) molecule is

thought to transmit the intracellular signal from the insulin receptor. The binding of insulin to the insulin receptor leads to activation of insulin receptor kinase through autophosphorylation of the insulin receptor (β-subunit). Insulin receptor kinase is essential for insulin action. In type II diabetes, the insulin receptor kinase activity appears to be lower in target tissue due to a decreased number of insulin receptors and a reduction in intrinsic insulin receptor kinase activity per-receptor.

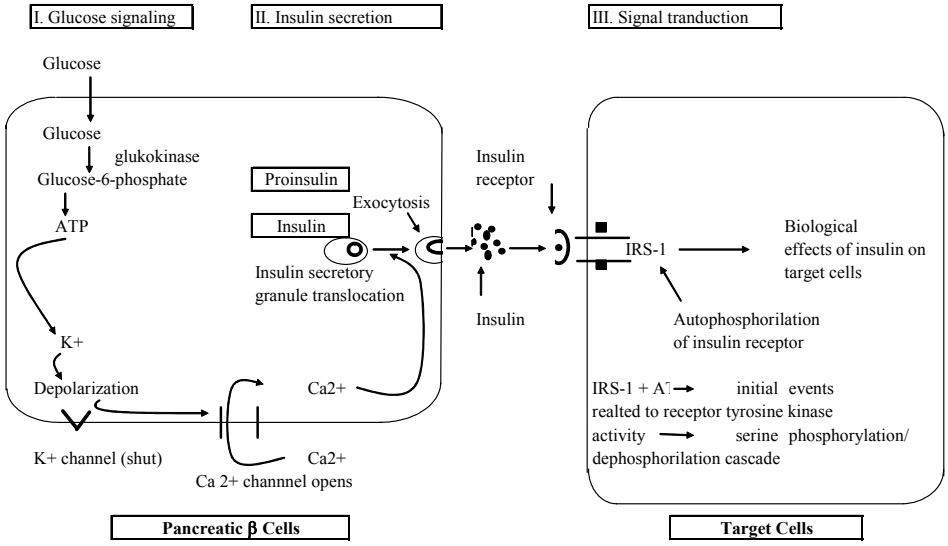

Figure 1.2 Schematic diagram of metabolic functions in β cells, insulin secretion in β cells, and insulin action in target tissues (Jun *et al.*, 1999).

1.4 Culture Model for Hypoglycemic Activity

Mammalian cell cultures are continuously drawing major research effort. A great deal of progress has recently been made in cellular physiology, especially in factors adversely affecting cell growth and viability. There are many advantageous in using cell culture for assay. They provide a continuous supply of homogenous cellular material for biochemical experiments as well as for practical use in medical and public health work. The cells *in vitro* can be manipulated advantageously in

many ways, unlike with cells *in vivo* (Jacoby *et al*., 1979). The screening of new compounds including plant extracts for antidiabetic effects have been investigated by the researchers. The recommended method for the study antidiabetic effect of plant extracts is by *in vitro* or *in vivo* (Verspohl, 2002).

1.5 Objective of the Study

The objective of the study was to evaluate insulin mimetic activity of *Cinnamomum zeylanicum* on 3T3-L1 adipocyte. To achieve the objective, two major research scopes were carried out:

1. Isolation and characterization of active compound from *Cinnamomum zeylanicum*.
2. Cell-based *in vitro* assay for the insulin mimetic activity of the active compound on 3T3-L1 adipocytes.