UNIVERSITI PUTRA MALAYSIA

EFFECTS OF ETHANOLIC EXTRACT OF COCOA ON BLOOD GLUCOSEAND LIPID PROFILE IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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RUZAIDI AZLI BIN MOHD MOKHTAR

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

April 2005

This Thesis is specially dedicated to my beloved:

Father
Mohd Mokhtar

Mother
Siti Ramalah

& Family

For the unconditional patient, love and support.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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April 2005

Chairman: **Amin Ismail, PhD**

Faculty: **Medicine and Health Sciences**

This study aims to investigate the hypoglycaemic and hypocholesterolaemic properties of Malaysian cocoa (*Theobroma cacao*) polyphenols extract *in-vivo* and *in-vitro*. Cocoa extract (contained 190 - 286 mg total polyphenol per g of extract) was prepared from fermented and roasted (140 °C, 20 min) beans by extracting with 80% ethanol in the ratio of 1 to 10. The total phenolic content was estimated according to the Folin-Ciocalteu reagent method. The eluted individual polyphenol was monitored by using a normal-phase HPLC. Monomer is the predominant polyphenols present in cocoa extract (CE) followed by dimer and tetramer. To study the effect of CE on plasma glucose levels and lipid profiles in normal and diabetic rats, two different batches of animal (*in-vivo*) studies were performed. In the first batch, rats were given free excess to diet containing CE in the form of powder, while in the second batch, rats were force-fed with CE suspended in normal saline daily. The CE was given in three dosages (100, 200 and 300 mg per kg body weight) to both batches for a period of 4 weeks. The result showed that 100 mg/kg and 300

mg/kg CE significantly reduced ($p < 0.05$) the plasma glucose levels in the diabetic rats of both the first and second batch of studies. In the first batch, supplementation of 100 mg/kg and 300 mg/kg CE had significantly reduced ($p < 0.05$) the level of total cholesterol in diabetic rats. In addition, 100, 200 and 300 mg/kg CE diets had significantly lowered ($p < 0.05$) the total triglycerides. Interestingly, this study found that plasma HDL-cholesterol had increased significantly ($p < 0.05$) in diabetic rats fed with 200 mg/kg CE, while the LDL-cholesterol had decreased significantly ($p < 0.05$) in group treated with 100 mg/kg CE. In the second batch, plasma cholesterol, HDL-cholesterol and LDL-cholesterol levels showed no siginificant difference in both normal and diabetic rats. Meanwhile, there was a significant decrease ($p < 0.05$) in plasma triglyceride level in diabetic rats. In another study, rats were pretreated with CE to investigate the protective effect of CE against streptozotocin diabetogenic action. In 200 mg/kg CE pretreated rats, there was a 163% increase in plasma glucose levels, compared with a 226% increase in diabetic control rats. There were no protective effects on plasma lipid profiles in CE pretreated rats. Results also exhibited CE could normalize the body weight loss caused by STZ. BRIN-BD11 cell lines (*in-vitro*) were used to evaluate the effect of CE on insulin secretion. This *in-vitro* study demonstrated that CE at a concentration of 0.1 mg/ml significantly increase ($p < 0.05$) insulin secretion compared to control. In conclusion, the study shows that Malaysian cocoa polyphenol extract may possess potential hypoglycaemic and hypochlosterolaemic properties. Further studies are needed to elucidate the exact mechanism by which polyphenols present in CE can lower the plasma glucose levels and improved lipid profiles in diabetic rats, and stimulate insulin secretion in BRIN-BD11 cell lines.

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

KESAN EKSTRAK ETANOLIK KOKO KEATAS GLUKOSA DARAH DAN PROFAIL LIPID TIKUS DIABETES DIARUH STREPTOZOTOCIN

Oleh

RUZAIDI AZLI BIN MOHD MOKHTAR

April 2005

Pengerusi: **Amin Ismail, PhD**

Fakulti: **Perubatan dan Sains Kesihatan**

Kajian ini bertujuan untuk mengkaji ciri-ciri hipoglisemik dan hipokolesterolemik ekstrak polifenol koko Malaysia (*Theobroma cacao*) secara *in-vivo* dan *in-vitro*. Ekstrak koko (mengandungi 190 – 286 mg polifenol per g ekstrak) disediakan daripada biji koko yang telah difermentasi dan dipanggang (140 °C, 20 min) dengan mengekstrak menggunakan 80% etanol berdasarkan nisbah 1 kepada 10. Pengiraan jumlah kandungan polifenol menggunakan kaedah *Folin-Ciocalteu*. Polifenol individu dianalisis dengan menggunakan fasa normal HPLC. Monomer adalah polifenol yang paling banyak terdapat di dalam CE diikuti oleh dimer dan tetramer. Untuk mengkaji kesan CE ke atas paras plasma glukosa dan profil lipid tikus normal dan diabetik, dua kumpulan kajian haiwan (*in-vivo*) telah dijalankan. Dalam kajian kumpulan pertama, tikus tersebut bebas untuk mengambil makanan dalam bentuk serbuk yang mengandungi CE, sementara dalam kumpulan kedua, tikus diberi CE yang dilarutkan di dalam salin normal secara oral (suapan paksa). CE diberi dalam tiga dos (100, 200 dan 300 mg per kg berat badan), dan diberi kepada kedua-dua

kumpulan selama 4 minggu. Keputusan menunjukkan 100 mg/kg dan 300 mg/kg CE menurunkan paras plasma glukosa secara signifikan ($p < 0.05$) di dalam kedua-dua kumpulan. Di dalam kajian kumpulan pertama, pemberian 100 mg/kg dan 300 mg/kg CE menurunkan paras plasma kolesterol secara signifikan ($p < 0.05$) di dalam tikus diabetik. Tambahan pula, 100, 200 dan 300 mg/kg diet CE menurunkan kandungan trigliserida secara signifikan ($p < 0.05$). Kajian ini juga mendapati plasma HDL-kolesterol meningkat secara signifikan ($p < 0.05$) di dalam tikus diabetik yang diberi 200 mg/kg CE, sementara LDL-kolesterol menurun secara signifikan di dalam tikus diberi 200 mg/kg CE. Di dalam kajian kumpulan kedua, paras plasma kolesterol, HDL-kolesterol dan LDL-kolesterol tidak menunjukkan perubahan yang signifikan di dalam tikus normal dan diabetik. Sementara itu, terdapat penurunan signifikan ($p < 0.05$) di dalam paras plasma trigliserida tikus diabetik. Untuk mengkaji kesan perlindungan CE melawan tindakan diabetogenik STZ, tikus diberi CE terlebih dahulu sebelum suntikan STZ. Tikus yang terlebih dahulu diberi 200 mg/kg CE, didapati terdapat peningkatan 163% paras plasma glukosa, berbanding dengan peningkatan 226% di dalam tikus kontrol diabetik. Tiada kesan perlindungan ke atas plasma profil lipid di dalam tikus yang diberi CE terlebih dahulu. Keputusan juga mendapati CE berupaya mengnormalkan kehilangan berat badan disebabkan oleh STZ. BRIN-BD11 sel (*in-vitro*) digunakan untuk menilai kesan CE ke atas rembesan insulin. Di dalam kajian ini menunjukkan CE pada kepekatan 0.1 mg/ml meningkatkan rembesan insulin secara signifikan ($p < 0.05$) berbanding kontrol. Secara kesimpulan, kajian ini menunjukkan ekstrak polifenol koko Malaysia kemungkinan mempunyai potensi ciri-ciri hipoglisemik dan hipokolesterolemik. Kajian selanjutnya diperlukan untuk menerangkan mekanisme keupayaan polifenol

daripada CE dalam menurunkan paras plasma glukosa dan memperbaiki profil lipid di dalam tikus diabetik, dan merangsang rembesan insulin di dalam BRIN-BD11 sel.

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LIST OF ABBREVIATIONS

ADA	: American Diabetes Association
b.p	: boiling point
CE	: cocoa extract
CV	: coefficient of variance
CVD	: cardiovascular disease
g	: gram
g	: gravity (relative centrifugal force)
HDL	: high density lipoprotein
HPLC	: High Performance Liquid Chromatography
hr	: hour
I.D.	: internal diameter
kDa	: kilodalton
kg	: kilogram
KRB	: Krebs-Ringer bicarbonate
l.	: liter
LDL	: low density lipoprotein
M	: molarity
mg	: milligram
ml	: milliliter
min	: minute
MOH	: Ministry of Health, Malaysia
ng	: nanogram
nm	: nanometer
PBS	: phosphate buffer-saline
STZ	: streptozotocin
v/v	: volume/volume
WHO	: World Health Organization
w/v	: weight/volume
µl	: microliter
µm	: micrometer

CHAPTER I

INTRODUCTION

Diabetes mellitus is a serious and costly disease which is becoming increasingly common, especially in developing countries. It is a disease with major long-term implications, not only on the health and well-being of the affected individuals, but also on the costs incurred by the government. For example in Canada, the annual estimated cost of treating patients with diabetes mellitus is 9 billion dollars, while in the United States it is estimated to be near 132 billion dollars in 2002 in medical expenditures and lost productivity (Dawson *et al.*, 2002; ADA, 2003). There are no available statistics on the cost of treating diabetic patients in Malaysia, but WHO (2002) estimated that direct health care costs of diabetic patients range from 2.5% to 15% of total annual budget, depending on local diabetes prevalence and the effectiveness of the treatment available.

In the latest WHO estimation (WHO, 2004), there are over 171 million people worldwide who are afflicted with diabetes mellitus. Diabetic individuals can suffer from ketoacidosis, a serious acute complication, as well as chronic complications that affect essentially every organ system in the body, among them cardiovascular diseases, stroke, blindness, kidney failure, neurological dysfunction, necrosis and gangrene.

The discovery of insulin in 1921 revolutionized diabetes treatment and greatly reduced the acute complication of diabetes mellitus. As diabetics began to live longer, however, the chronic complications have taken over as the principal cause of morbidity and mortality. Advances in our understanding of the pathophysiology of diabetes in the past several decades have produced significant improvements in therapy. There are two main types of diabetes mellitus: type 1, which was previously known as insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes mellitus, and is caused by absolute insulin deficiency; and type 2 or non-insulin dependent diabetes mellitus (NIDDM) or maturity-onset diabetes mellitus, which occurs mainly in adulthood, is often associated with obesity, and results from a combination of insulin resistance and β-cell dysfunction, leading to relative insulin deficiency (Kahn, 2003).

Although diabetes mellitus is a non-communicable disease, it is considered one of the five leading causes of death in the world. Recently, the search for appropriate hypoglycaemic agents has focused on plants used in traditional medicine, partly because of leads provided by traditional medicine to natural products that may be better treatments than currently used drugs (Lu *et al.,* 2002; Jang *et al.,* 2003; Kar *et al.,* 2003). Drug such as sulphonylureas, lead to higher risk of hypoglycaemia, and metformin brings a higher risk of lactic acidosis (Shenfield, 2001). Due to the side effects of these drugs, many researches have been conducting studies on natural products derived from plants with potential antidiabetic activities (Kamtchouing *et al.*, 1998; Jayakar and Suresh, 2003; Ladeji *et al.*, 2003; Maghrani *et al.*, 2003).

Besides the traditional medicinal plants, cocoa beans were thought to have fearsome magical powers by the Mayas and were carefully used by priests in rituals, religious ceremonies and healings. The Mayas used cocoa medicinally as a treatment for fever, coughs and to help dispel even discomfort during pregnancy. After the 16th century conquest of Central America by Spain, Cortes introduced cocoa to Europe, where it was typically viewed as a healthy and nutritious beverage (Dillinger *et al.*, 2000). Therefore, to evaluate the hypoglycaemic and hypocholesterolaemic effect of cocoa beans, this study was designed to test its effectiveness in reducing hyperglycaemia and hypercholesterolaemia based on *in-vivo* (animal) and *in-vitro* studies.

Malaysian is one of the main cocoa-based products producer in the world and the biggest in Asia. However, our local cocoa-based markets are more prefer other cocoa beans (West African and Ghanian beans) due to some weaknesses in Malaysian beans quality (low cocoa aroma, astringent and bitter taste). One of the factors causing this is believed to be due to the high amount of the polyphenol substances. Recently, polyphenols have become intense focus of research interest because of their antioxidant capacity and possible beneficial health effects. Thus, this study uses the superiority of Malaysian beans in order to evaluate its potential beneficial health effects especially in diabetes mellitus.

Historically, animal models have been used to screen out extracts, pure compounds or drugs, and obtain information to help in understanding health disorders and to test

the safety of these materials before putting them on the market. Scientists from around the world usually use animal model to study the way the disease progresses, and factors that are important to the disease process. Animal models are also used to study the treatment of diseases before it can be applied to humans. Usually, after animal study is performed, it will be followed up with additional laboratory studies using cell culture (*in-vitro* model) for result confirmation.

Streptozotocin (STZ)-induced hyperglycaemia in rats have been described as a useful experimental model to study the activity of antidiabetic agents with or without insulin (LeDoux *et al.*, 1986). A range of STZ doses provide a wider range compared to alloxan, as an inducer of hyperglycaemia. The frequently-used single intravenous or intraperitoneal dose in adult rats to induce diabetes mellitus type 1 is between 40 and 60 mg/kg body weight (Pepato *et al.*, 2001; McAnuff *et al.*, 2002), but higher doses are also used (Ladeji *et al.*, 2003). In this study, two different batches of animal fed with cocoa extract (CE) via two different techniques were used: (1) CE mixed with purified diet, powder form and (2) forced-fed CE suspended in liquid solution. The aim of the experiment was to study the hypoglycaemic and hypocholesterolaemic effect of Malaysian cocoa extract. The rationale of using two different feeding techniques is to ensure the results derived from the second batch (force-feeding) supported the findings of the first batch (free excess to diet containing CE). In addition, for the first batch study, actual amount of food intake cannot be measured due to a lot of food powder being spilled. Furthermore, the food intake varied from one rat to another and because of that, it is difficult to measure the

exact polyphenol intake of the rats. Therefore, in order to make sure the exact doses of CE were taken by the rats, force-feeding using intubation needle (second batch) was designed to improve the effectiveness of this first batch design.

The search for a safer and more effective compound in protecting the β-cells from inflammatory destruction by STZ is still being done. Several compounds such as metallothionein, nicotinamide, glucose and (-)epicatechin have been reported to inhibit the diabetonic action of streptozotocin or alloxan (Kamtchouing *et al.*, 1998; Yang and Cherian, 1994). Khalid (2002) found that palm Vitee (palm oil Vitamin E) has a protective property against the toxic inflammation caused by single dose STZ administration. Thus, this study was also designed to evaluate the protective action of CE against the destruction of insulin-producing β-cells of the pancreas in STZ-induced diabetic rats.

To understand the possible mechanisms by which CE improves hyperglycaemia, the effect of CE on insulin secretion by insulin-secreting cells was investigated. Insulin-secreting cell lines have provided useful systems for the study of pancreatic β-cell function. The BRIN-BD11 cell line is a clonal glucose responsive insulin-secreting cell line which is responsive to a range of pharmacological modulators of insulin secretion. Most of the available cell lines exhibit glucose insensitivity or moderate responsiveness to sub-physiological concentrations of glucose (Newgard, 1994). Thus, this study utilizes this cell line superiority to examine the effects of CE upon insulin secretion alone without the presence of glucose.