



UNIVERSITI PUTRA MALAYSIA

**EFFECT OF POLYPHENOL EXTRACT FROM MALAYSIAN COCOA
POWDER ON PLASMA LIPID IN INDUCED RABBIT
HYPERCHOLESTEROLEMIA MODEL**

FAIZUL HELMI BIN ADDNAN

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By

FAIZUL HELMI BIN ADDNAN

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of the Requirement for the Degree of Master of Science**

December 2007





Specially dedicated to,

*mak, ayah,
my family,
and my wife.*

Alg'eee 2007



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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December 2007

Chairman: Associate Professor Amin Ismail, PhD

Faculty: Medicine and Health Sciences

It is estimated that by 2010, cardiovascular disease (CVD) will be the leading cause of death in developing countries. In Malaysia, heart disease and disease of pulmonary circulation is the second major killer, making up 14.31% of total mortality reported in Ministry of Health (MOH), hospitals. Population studies have shown that plant polyphenol is inversely correlated with mortality from cardiovascular disease and numerous dietary flavonoids have been shown to beneficially impact toward atherosclerosis, including lipoprotein oxidation, blood platelet aggregation and vascular reactivity. There were many studied had reported that cocoa flavonoids had shown the similar degree towards being protective against CVD due to: antioxidant, anti-platelet, anti-inflammatory effects, as well as increasing HDL-c, lowering blood pressure and improving endothelial. However, the study on the effect of cocoa polyphenols on atherosclerotic plaque formation is to be documented. The present study was aimed to investigate the protective effects of polyphenol extract (CE) prepared from Malaysian cocoa powder against hypercholesterolemia atherosclerosis. Male New Zealand White



rabbits were fed with the experimental diets for 12 weeks. Plasma total cholesterol (TC) increased up to week 12 for a group fed with diet enriched with 1% of cholesterol. Plasma TC were significantly decreased ($p < 0.01$) from 5 to 12 week when CE was administered at a dosage of 300, 600 and 800 mg/kg body weight/day. Significant increment ($p < 0.01$) and significant decreament ($p < 0.01$) in plasma high density lipoprotein cholesterol (HDL-c) was observed from 0 to 5 week and from 5 to 12 week, respectively for groups administered with CE. Plasma low density lipoprotein cholesterol (LDL-c) was significantly increased ($p < 0.001$) from initial to 5 week, and then decreased ($p < 0.01$) from 5 to 12 week in the group administered with CE, except for the 300 mg CE group. Compared to the standard laboratory diet, cholesterol-enriched diet increased aortic atherosclerosis formation, hepatic superoxide dismutase (SOD), hepatic glutathione peroxidase (GSHPx), hepatic malondialdehyde (MDA), and kidney GSHPx. Administered with 800 mg of CE to rabbits fed with cholesterol-enriched diet showed significant increased ($p < 0.05$) and significant decreased ($p < 0.05$) for activities of hepatic GSHPx and MDA concentration, respectively. Significant decreased ($p < 0.05$) in MDA concentration and significant increased ($p < 0.05$) for activities of hepatic catalase (CAT) was observed in groups of 600 mg CE and 300 mg CE, respectively. Approximately, 50 % area of atherosclerotic lesion in the rabbits fed with cholesterol-enrich diet could be reduced by administering with CE. The effectiveness of CE against hypercholesterolemic atherosclerosis could be due by its effects to lower plasma TC, LDL-c, increase plasma HDL-c and enhances antioxidative defenses against the oxidative stress imposed by hypercholesterolemia.



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

**KESAN EKSTRAK POLIFENOL DARI SERBUK KOKO MALAYSIA KE ATAS
PLASMA LIPID MODEL ARNAB TERARUH HIPERKOLESTEROLEMIA**

Oleh

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Pada tahun 2010, adalah dijangkakan bahawa penyakit kardiovaskular (PKV) akan menjadi penyebab utama kematian di negara-negara membangun. Di Malaysia, penyakit jantung dan penyakit peredaran pulmonari adalah pembunuh kedua yang terbanyak, dengan jumlah mortaliti mencapai 14.31 % seperti yang dilaporkan oleh hospital-hospital Kementerian Kesihatan Malaysia. Kajian-kajian populasi telah menunjukkan polifenol tumbuhan berkadar songsang dengan kadar kematian dari penyakit kardiovaskular dan banyak komponen flavonoids dalam diet telah menunjukkan impak yang baik terhadap aterosklerosis, termasuk pengoksidaan *lipoprotein*, pengumpulan platelet darah dan reaktiviti vaskular. Sehingga kini, banyak kajian telah dilaporkan mengenai kepentingan (faedah) komponen flavonoid koko yang bertindak sebagai pelindung daripada PKV disebabkan oleh: antioksidan, anti-platelet, kesan anti-inflamatori, termasuk juga meningkatkan HDL-c, merendahkan tekanan darah dan memperbaiki saluran endotelial darah. Walaubagaimanapun, kajian mengenai kesan polifenol koko ke atas pembentukan plak aterosklerotik masih belum dibuktikan. Kajian ini bertujuan untuk mengkaji kesan pencegahan ekstrak polifenol (CE) dari serbuk koko Malaysia ke atas aterosklerosis



hiperkolesterolemia. Arnab jantan putih New Zealand diberi makan dengan diet-diet kajian selama 12 minggu. Jumlah plasma kolesterol (TC) telah meningkat sehingga minggu ke-12 bagi kumpulan yang diberi diet yang diperkaya dengan 1% kolesterol. Plasma TC telah menurun secara signifikan ($p < 0.01$) dari minggu ke-5 hingga minggu ke-12 apabila CE diberi pada dos-dos berbeza iaitu 300, 600 dan 800 mg/ kg berat badan/hari. Kenaikkan dan penurunan yang signifikan ($p < 0.01$) bagi plasma lipoprotein-kolesterol berketumpatan tinggi (HDL-c) telah diperhatikan dari awal minggu kajian hingga minggu ke-5 dan dari minggu ke-5 hingga minggu ke-12, masing-masing bagi kumpulan-kumpulan yang diberi CE. Plasma lipoprotein-kolesterol berketumpatan rendah (LDL-c) telah meningkat secara signifikan ($p < 0.001$) di awal minggu kajian hingga minggu ke-5 dan kemudian menurun ($p < 0.01$) dari minggu ke-5 hingga minggu ke-12, bagi kumpulan yang diberi CE, kecuali kumpulan yang diberi 300 mg CE. Berbanding dengan diet normal, diet diperkaya kolesterol meningkatkan pembentukan aortik aterosklerosis, superoksida dismutase hepatic (SOD), *gluthathione peroxidase* hepatic (GSHPx), malondialdehid hepatic (MDA) dan GSHPx dalam ginjal. Pemberian 800 mg CE kepada arnab yang diberi makan diet diperkaya kolesterol, menunjukkan kenaikan signifikan ($p < 0.05$) dan penurunan signifikan ($p < 0.05$) bagi aktiviti –aktiviti GSHPx hepatic dan kepekatan MDA hepatic, masing-masing. Penurunan signifikan ($p < 0.05$) dalam kepekatan MDA hepatic dan kenaikan signifikan ($p < 0.05$) bagi aktiviti-aktiviti katalase (CAT) hepatic telah diperhatikan dalam kumpulan 600 mg CE dan 300 mg CE, masing-masing. Kira-kira 50 % kawasan lesi aterosklerotik pada arnab yang diberi diet diperkaya kolesterol dapat dikurangkan dengan memberi CE. Keberkesanan CE dalam melawan hiperkolesterolemia arterosklerotik boleh ditunjukkan melalui



kesannya dalam menurunkan plasma TC, LDL-c, dan meningkatkan plasma HDL-c, dan juga meningkatkan pertahanan antioksidatif dalam melawan tekanan oksidatif yang dibebani oleh keadaan hiperkolesterolemia.



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I certify that an Examination Committee has met on 18th December 2007 to conduct the final examination of Faizul Helmi Bin Addnan on his Master of Science thesis entitled “Effect of Polyphenol Extract from Malaysian Cocoa Powder on Plasma Lipid in Induced Rabbit Hypercholesterolemia Model” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the degree of Master of Science.

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

FAIZUL HELMI BIN ADDNAN

Date:



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LIST OF ABBREVIATIONS

300 mg CE	Hypercholesterolemic treated with 300 mg CE
600 mg CE	Hypercholesterolemic treated with 600 mg CE
800 mg CE	Hypercholesterolemic treated with 800 mg CE
AI	Atherogenic Index
CAT	Catalase
CE	Cocoa Polyphenol Extract
CHD	Coronary heart disease
CN	Normal Diet (Negative control) group
CP	Hypercholesterolemic (Positive control) group
CVD	Cardiovascular disease
GSHPx	Glutathione peroxidase
HDL-c	High density lipoprotein-cholesterol
LDL-c	Low density lipoprotein-cholesterol
MOH	Ministry of Health
SOD	Superoxide dismutase
TC	Total Cholesterol
TG	Triglyceride
US FDA	United States, Food and Drug Administration
WHO	World Health Organization



CHAPTER I

INTRODUCTION

Introduction

Coronary heart disease (CHD) has become very common in most industrialized societies and it is occurring increasingly early in life. CHD is also called coronary artery disease and ischemic heart disease. Prevention of CHD could be done by the action predicted from its risk factors.

CHD means a partial or complete blockage of one or more of the coronary arteries which supply blood to the muscular walls of the heart (the myocardium). It arises initially because of the development of fatty deposits in the arterial wall (atherosclerosis), which may affect arteries in the body, and can impair the blood supply. Fatality can occur when the blood supply to the heart is blocked. At the initial stage, the restriction of blood supply to the heart may appear as chest pain (angina), when the demand on the heart increases. Later, a complete blockage may occur as a result of a blood clot at the site of the atherosclerotic lesions (Frayn, 1996). This condition is popularly known as heart attack. Consequences of this event will lead to death of myocardial tissue (heart tissue) and interruption of the heart function (Randall and Romaine, 2005).



There are two main events in the pathogenesis of atherosclerosis. Firstly, the lipid infiltration events and secondly continue on damage of the arterial wall. The lipid event will have fatty substances from the blood circulation, filtrate into the arterial wall. This event explains the high degree of atherosclerosis with fatty infiltrations in the arterial wall in the presence of hypercholesterolemia. According to Ross (1999), high plasma cholesterol concentrations, in particular those of low density lipoprotein-cholesterol (LDL-c) are one of the foremost risk factors for atherosclerosis. Research on the etiological factors bearing on heart disease, show that a key reaction is the oxidation of LDL-c (Zock and Katan, 1998; Fuller and Jialal, 1994; Steinberg et al. 1996, Cox and Cohen, 1996; Ishikawa et al. 1997). The process of atherogenesis has been considered due to the accumulation of lipids within the artery wall. The high level of this lipoprotein, its transportation to the arterial walls, and its subsequent oxidative modification, expose the activation and dysfunction of endothelial cell. Along with these changes, production of chemotactic factors attracts circulating monocytes and T-lymphocytes to the activated endothelium with a subsequent transmigration into the arterial intima. Clotting of platelets at the site of injury is supposed to play an important role and indeed patients with CHD, are found to have platelets that aggregate more easily than those in normal individuals. Each little place of damage can thus causes platelet adhesion and later bleeding in the wall (Libby et al. 2002).

Impact of high plasma cholesterol on CHD risk was acquired more slowly than other risk factors i.e., cigarette smoking and elevated blood pressure. There is evidence that a link between the elevations of plasma cholesterol concentrations and the development of



CHD (Bbriener and Hetnecke, 1996). The driving force for this evidence was the cholesterol hypotheses that postulate a rising plasma cholesterol concentration enhances the risk of CHD whereas a falling level reduces the risk (Stoclet et al. 2004).

The hypothesis that elevated plasma cholesterol causes atherosclerosis came from studies in laboratory animals. For example, the feeding of excess cholesterol to rabbits produced marked hypercholesterolemia and caused deposition of cholesterol in arteries (Mohamedain and Fred, 2000; Prasad, 1997). These depositions closely resemble human atherosclerosis. It provides a strong base of support for the concept that elevated serum cholesterol, particularly when the cholesterol is carried in low-density lipoproteins (LDL-c) and remnant lipoproteins-is highly atherogenic.

In addition, the cholesterol hypothesis comes from epidemiological investigations, where prospective and cross-sectional studies have examined the correlation between serum cholesterol concentrations and incidence and prevalence of CHD. A notable example includes the Seven Countries Study and the Framingham Heart Study (Dawber and Kannel, 1966)

Furthermore, another cholesterol hypothesis comes from genetic disorders of lipoprotein metabolism. These disorders are due to genetic forms of elevated serum cholesterol (namely, familial hypercholesterolemia) and familial defective apolipoprotein B-100. Apolipoprotein B-100 serves as a ligand site for the uptake of LDL-cholesterol by apolipoprotein B/E receptor (LDL receptor) (Frayn, 1996). When these disorders are



present, coronary atherosclerosis develops rapidly, leading to early onset of CHD. Genetic forms of hypercholesterolemia can produce premature CHD even in the absence of other risk factors. The occurrence of premature CHD in persons with genetic hypercholesterolemia provides the strongest evidence that a high level of serum cholesterol is a direct cause of atherosclerosis in humans. Atherosclerosis event not only develop as a result of high plasma cholesterol concentration, but also from the disturbances of glucose metabolism.

Phenolic substances, especially polyphenol containing several hydroxyl groups directly associated with a cyclic benzene ring, are widely distributed in plants. Polyphenol can be defined as part of a broader group of compounds that have antioxidant activity (Hertog et al. 1993; Frankel et al. 1993). Growing epidemiological evidences suggest the existence of a negative correlation between consumption of polyphenol-rich foods or beverages such as fruits, vegetables, red wine, grape juice and tea and the incidence of CHD (Nakachi et al. 2000; Renaud and de Lorgeril, 1992; Saint-Leger et al. 1979; Sasazuki et al. 2000; Knekt et al. 1996; Hertog et al. 1993; Hollman et al. 1996). By virtue of their chemical nature, polyphenols can acts as antioxidants and may help to maintain the body's natural defences against a variety of diseases associated with oxidative stress, such as cancers, cardiovascular disease and inflammation. These polyphenol acts as hydrogen donating antioxidants and also as a chelators of metal ions, preventing metal catalysed formation of initiating radical species (Salah et al. 1995). Catechins (example of polyphenol compound) serve as powerful antioxidants for eliminating superoxide anion radicals (Bearden et al. 2000).



Research indicates that antioxidant may help the body cells resist damage by free radicals, which are formed by numerous processes (Jacob and Burri, 1996). A protective role of cocoa polyphenols against atherosclerosis has been suggested by several studies (Osakabe et al. 2001; Kondo et al. 1996). The effect is due to the antioxidant activity of polyphenols that can inhibit low density lipoprotein (LDL-c) from oxidation and inhibit platelet aggregations (Rein et al. 2000) that is central to the formation of atherosclerotic plaque. It is clear that the prevention or decrease of the key oxidation reactions could be an important means of lowering the risk of coronary heart disease. In the field of a lower risk of coronary heart disease, it has been shown, through studies in humans as well as animal models, that the intake or use of foods containing antioxidants is beneficial.

Polyphenol compounds in cocoa are quite similar compared to green tea, black tea and wine. Tea, wine and cocoa are rich with flavanols, proanthocyanidin and anthocyanidin. Sanbongi et al. (1997) and Kondo et al. (1996) reported cocoa polyphenols contain potential antioxidant properties mainly from (-)-epicatechin, (+)-catechin, clovamide and quercetin *in vitro* and *in vivo*. Osakabe et al. (1998) showed cocoa polyphenols had reduced significantly lipid oxidation.

Cocoa is also known as *Theobroma cacao*, means 'food of the God' and it was first discovered in the ancient civilization of the Olmecs, Mayas and Aztecs in South America and Mexico. The Aztecs considered the beverage as a royal drink. Cocoa beans were thought to have magical powers by the Mayas and carefully used in rituals, religious ceremonies and healing. From sixteenth to early twentieth century, a manuscript

produced in Europe and Spain revealed more than 100 medicinal uses for cocoa or cocoa products. Three consistent roles can be attributed to this manuscript such as to treat emaciated patients to gain weight, to stimulate nervous system of apathetic, exhausted or feeble patients and to improve digestion and elimination (Dillinger et al. 2000).

A growing number of evidence suggest that numerous pathobiological conditions are associated with an increased production of reactive oxygen species (ROS) and other radicals, the most prominent of which are superoxide and hydroxyl radicals and oxidants like H₂O₂ and peroxynitrite (Harrison and Ohara, 1995; Kojda and Harrison, 1999; Griending et al. 2000; Ferdinandy and Schulz, 2001). Several factors have been identified to promote significant effect towards cardiovascular health, including low density lipoprotein oxidation and platelet aggregation and blood vessel function (Ross and Epstein, 1993). Studies done *in vitro* and *in vivo* reveal that polyphenols may play a positive role in regulating these factors. Polyphenolic substances derived from tea and grape seed showed effectiveness in prevention of atherosclerosis in hypercholesterolemic rabbits. These studies suggested that the anti-atherosclerotic effect of this compound is due to inhibition of low density lipoprotein oxidation (Tjiburg et al. 1997; Yamakoshi et al. 1999)

Lipid oxidation mainly low density lipoprotein (LDL-c) particles have an important role in the development of atherosclerosis (Kondo et al. 1996). The development of atherosclerosis is also dependent on the nitrite oxide syntheses from endothelium-dependent relaxation system. Nitric oxide (NO), an endogenous vasodilator, has many

