UNIVERSITI PUTRA MALAYSIA

IN VIVO AND IN VITRO EFFECTS OF PHALERIA MACROCARPA (SCHEFF.) BOERL ON LOW DENSITY LIPOPROTEIN AND PCSK9 EXPRESSION

CHONG SOO CHING

FPSK(m) 2011 57

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By

CHONG SOO CHING

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

December 2011

Abstract of thesis presented to the Senate of University Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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Chairman: Associate Professor Mohamad Aziz Dollah, PhD

Faculty: Faculty of Medicine and Health Sciences

Phaleria macrocarpa (*Pm*) fruit is traditionally used to treat high cholesterol level. However its anti-hypercholesterolemic property is still unknown. LDL receptor is a ligand that involves in the cholesterol metabolism by taking in LDL which has high proportion of cholesterol whereas PCSK9 is a protein that mediates the degradation of LDL receptor. This study investigated the effects of *Pm* fruit aqueous extract on weight control and mechanistic basis of its anti-hypercholesterolemic effect in both *in vivo* and *in vitro* conditions. *In vivo* study, 36 male *Sprague dawley* rats were randomized to 6 groups. 5 groups were given 3% (v/v) cholesterol enriched-diet for 52 days to induced to become hypercholesterolemia, followed by *Pm* fruit aqueous extract (0, 20, 30 and 40 mg extract/kg bw) or simvastatin (40 mg/kg) treatment for 84 days. The sixth group was used as a negative control. The effects of *Pm* fruit aqueous extract treatment were determined on the following parameters: 1) body weight, 2) liver weight 3) liver weight-to-body weight ratio 4) blood lipid profiles (TC, TG, HDL and LDL) and 5) expression level of hepatic LDL receptor (160 kDa

and 120 kDa) and PCSK9 proteins. *Pm* fruit aqueous extract significantly ($P<0.05$) reduced body weight gain but tends to reduce liver weight and liver weight-to-body weight ratio. As for the blood lipid profiles, 20 mg extract/ kg bw of *Pm* significantly ($P<0.05$) reduced TC (1.54 mmol/L), TG (0.38 mmol/L), HDL (0.68 mmol/L) and LDL (0.94 mmol/L) whereas 30 mg extract/ kg bw of *Pm* significantly ($P<0.05$) reduced TC (1.55 mmol/L), TG (0.33 mmol/L) and LDL (0.93 mmol/L) as compared to the untreated hypercholesterolemic group [TC (2.4 mmol/L), TG (1.13 mmol/L), HDL (0.94 mmol/L) and LDL (1.51 mmol/L)]. 40 mg extract/ kg bw of *Pm* significantly ($P<0.05$) reduced TC (1.85 mmol/L) and LDL (1.03 mmol/L). On the other hand, 20 mg extract/ kg bw of *Pm* significantly ($P<0.05$) increased LDL receptor and PCSK9 proteins by 1-fold whereas 30 and 40 mg extract/ kg bw of *Pm* had no effect on LDL receptor and PCSK9. Effect of *Pm* fruit aqueous extract in *in vivo* model was then further analyzed in *in vitro* study. *In vitro* study, HepG2 cells were cultured in serum-free RPMI 1640, supplemented with 0.2% BSA with or without LDL (200 μM) and in the presence of *Pm* fruit aqueous extract (0, 0.1, 2, 40 and 1000 μg/ml) or simvastatin (10 μM) for 24 hours. The abundance of both LDL receptor (160 kDa) and PCSK9 proteins and mRNA were then investigated. Similar to the *in vivo* study, *Pm* fruit aqueous extract was found to have increased LDL receptor and PCSK9 proteins by 1-fold in HepG2 cells with significant increment ($P<0.05$) at the concentration of 0.1 μg/ml. Besides that, *Pm* fruit aqueous extract at the concentration of 0.1 μg/ml also significantly ($P<0.05$) increased both LDL receptor and PCSK9 mRNA transcripts, comparable to simvastatin treated group. These study indicated that *Pm* fruit aqueous extract reduces body weight gain, liver weight, liver weight-to-body weight ratio and exhibited anti-hypercholesterolemic

effect by reducing blood lipid profile of hypercholesterolemic rats and upregulating LDL receptor and PCSK9 at both protein and mRNA level.

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

IN VIVO DAN *IN VITRO* KESAN BUAH *PHALERIA MACROCARPA* (SCHEFF.) BOERL PADA LIPOPROTEIN BERKETUMPATAN RENDAH DAN PCSK9

Oleh

CHONG SOO CHING

Disember 2011

Pengerusi: Profesor Madya Mohamad Aziz Dollah, PhD

Fakulti: Fakulti Perubatan dan Sains Kesihatan

Buah *Phaleria macrocarpa* (*Pm*) adalah ubat tradisional untuk anti-hiperkolesterol tetapi mekanisme tindakan anti-hiperkolesterolemianya masih tidak diketahui. Reseptor LDL membantu pengambilan LDL oleh sel hepar untuk didegradasi manakala PCSK9 mengdegradasi reseptor LDL. Kajian *in vivo* dan *in vitro* telah dijalankan untuk menentukan mekanisme anti-hiperkolesterolemia akues ekstrak buah *Pm*. 36 ekor *Sprague dawley* tikus jantan dibahagikan kepada 6 kumpulan dimana 5 kumpulan di beri makanan yang mengandungi tambahan 3% kolesterol selama 52 hari untuk diaruh menjadi hiperkolesterolemia. 5 kumpulan tikus itu kemudian diberi akues ekstrak buah *Pm* (0, 20, 30 dan 40 mg extrak/kg) atau simvastatin secara oral selama 84 hari. Kesan akues ekstrak buah *Pm* kemudian ditentukan dengan mengukur beberapa parameter seperti berikut: 1) berat badan, 2) berat hepar, 3) nisbah berat hepar kepada berat badan, 4) profil lipid (TC, TG, HDL LDL) dan 5) konsentrasi reseptor LDL (160 kDa dan 120 kDa) dan PCSK9 hepar. Akues ekstrak buah *Pm* menurunkan berat badan tikus ($P<0.05$), berat hepar dan

nisbah berat hepar kepada berat badan. Selain daripada itu, 20 mg extrak/ kg buah *Pm* menurunkan ($P<0.05$) profil lipid TC tikus hiperkolesterolemia kepada 1.54 mmol/L, TG kepada 0.38 mmol/L, HDL kepada 0.68 mmol/L dan LDL kepada 0.94 mmol/L berbanding dengan tikus hiperkolesterolemik yang tidak diberi extrak buah *Pm* [TC (2.4 mmol/L), TG (1.13 mmol/L), HDL (0.94 mmol/L) and LDL (1.51 mmol/L)]. 30 mg extrak/ kg buah *Pm* pula menurunkan ($P<0.05$) profil lipid TC tikus hiperkolesterolemia kepada 1.55 mmol/L, TG kepada 0.33 mmol/L and LDL kepada 0.93 mmol/L manakala 40 mg extrak/ kg buah *Pm* pula menurunkan ($P<0.05$) profil lipid TC tikus hiperkolesterolemia kepada 1.85 mmol/L dan LDL kepada1.03 mmol/L. Selain it, 20 mg extrak/ kg buah *Pm* meningkatkan paras reseptor LDL (160 kDa dan 120 kDa) dan PCSK9 ($P<0.05$) sebanyak satu kali ganda manakala 30 mg extrak/ kg dan 40 mg extrak/kg buah *Pm* pula tidak mempunyai apa-apa kesan terhadap reseptor LDL dan PCSK9. Kesan buah *Pm* akuaes ekstrak terhadap reseptor LDL dan PCSK9 di *in vivo* kemudian dikaji dalam eksperimen *in vitro* dengan mengunakan Hepatocellularcarcinoma (HepG2) sel. HepG2 sel diinkubasi dalam RPMI yang ditambahkan dengan 0.2% sera albumin lembu, LDL dan akues ekstrak buah *Pm* (0, 0.1, 2, 40 dan 1000 µg/ml) atau simvastatin (10 µM) selama 24 jam. Paras reseptor LDL (160kDa) dan PCSK9 protein dan mRNA kemudian ditentukan. Akues ekstrak buah *Pm* meningkatkan kepekatan protein LDL receptor (160 kDa) dan PCSK9 ($P< 0.05$) di HepG2 sel sebanyak satu kali ganda walaupun pada dos serendah 0.1 µg/ml. Selain itu, akues ekstrak buah *Pm* pada dos 0.1 µg/ml juga meningkatkan paras reseptor LDL (160 kDa) dan PCSK9 mRNA ($P<0.05$), standing dengan sel yang diinkubasi dengan simvastatin. Kesimpulannya, akues ekstrak buah *Pm* menurunkan berat badan, berat hepar dan nisbah berat hepar kepada berat badan. Selain daripada itu, akues ekstrak buah *Pm* juga menunjukkan kesan anti-

hiperkolesterolemia dengan menurunkan paras profil lipid tikus hiperkolesterolemia dan meningkatkan paras reseptor LDL dan PCSK9 pada tahap protein dan mRNA.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my appreciation and gratitude to my project supervisor, Assoc. Prof. Dr. Mohamad Aziz Dollah, for his help, advice, suggestion and encouragement throughout my postgraduate study. Besides that, I would also like to extend my gratitude to my co-supervisor, Assoc. Prof. Dr. Chong Pei Pei and Dr. Maha Abdullah for their guidance, advice and support throughout this experimental project. Without them, this experiment will not be a successful one.

My deepest appreciation also goes to all the academic and non-academic stuff of Faculty Medicine and Health Sciences for their technical support and assistant towards the completion of this project.

In addition, I would also like to express my appreciation to Biomedical Lab and Immunology lab's students, Ms Shaneeta, Mr. Peishan and Ms. Tham Chau ling for their constant advice and support throughout the experimental period.

Last but not least, I would also like to thank my family members for their support, encouragement and glorious care all this time.

I certify that a Thesis Examination Committee has met on 15th December 2011 to conduct the final examination of Chong Soo Ching on her thesis entitled "*In Vivo* and *In Vitro* Effects of *Phaleria macrocarpa* (Scheff.) Boerl on Low Density Lipoprotein and PCSK9 Expression" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1988. The Committee recommends that the student be awarded the degree of Master of Science.

Members of the Thesis Examination Committee were as follows:

Sabrina Sukardi, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Zuraini Ahmad, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Cheah Yoke Kqueen, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Siti Balkis Budin, PhD
Associate Professor
Faculty of Health Sciences
Universiti Kebangsaan Malaysia
Malaysia
(External Examiner)

SEOW HENG FONG, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 2nd March 2012

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:

Mohamad Aziz Dollah, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Chong Pei Pei, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Maha Abdullah, PhD
Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 19 MAR 2012

DECLARATION

I declare that the thesis is 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.

CHONG SOO CHING
Date:15th December 2011

TABLES OF CONTENTS

LIST OF TABLES

LIST OF FIGURE

LIST OF ABBREVIATIONS

ACAT	acyl-CoA:cholesterol acyltransferase
ACUC	Animal Care and Use Committee
Apo	apolipoprotein
ATP	adenosine triphosphate
BMI	body mass index
BSA	bovine serum albumin
bw	by weight
cCEH	Cytosolic cholesteryl ester bydrolase
CEH	cholesteryl ester bydrolase
Cho	3% cholesterol enriched diet
CVD	cardiovascular disease
DEPC	diethyl dicarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
ECL	Enchanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	epidermal growth-factor
ERK	extracellular signal regulated kinase
FA gel	formaldehyde agarose gel
FBS	fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HDL	high density lipoprotein

HepG2 cell	Heptocellularcarcinoma cell
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HSDA	sodium N-(2-hydroxyl-3-sulfupropyl)-3,5-dimetoxyaniline
IDL	intermediate density lipoprotein
ISO	isoproterenol
LCAT	lecithin-cholesterol acyltransferase
LDL	low density lipoprotein
LPL	lipoprotein lipase
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NTC	non-template control
OD	optical density
PBS	phosphate buffer saline
PCSK9	proprotein convertase subtilisin / kexin type 9
PCR	polymerase chain reaction
PEG-cholesterol esterase	polyethylene glycol- cholesterol esterase
Pm	*Phaleria macrocarpa*
PVDF	polyvinyl difluoride
RPMI 1640	Roswell Park Memorial Institute
RT-PCR	reverse transcriptase- polymerase chain reaction
SCAP	SREBP cleavage-activating protein

SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SR-B1	scavenger receptor B class 1
SREBP	Sterol regulatory element binding proteins
SREBP-2	Sterol regulatory element binding proteins-2
TAE	Tris-Acetate-EDTA
TBST	Tris-Buffered Saline Tween-20
TC	total cholesterol
TG	triglyceride
VLDL	very low density lipoprotein
WHO	World Health Organization

LIST OF ANNOTATIONS

A	ampere
bp	base pair
g	gravity
kDa	kilo dalton
kg	kilogram
mg	milligram
mg/dl	milligram per deciliter
mg extract/ kg bw	milligram extract per kilogram by weight
mg/kg	milligram per kilogram
mg/ml	milligram per milliliter
ml	milliliter
nm	nanometer
rpm	revolutions per minute
U	units
µg	microgram
µg/ml	microgram per milliliter
µl	microliter
µM	micromolar
V	Volts
%	percent
^{o}C	degree celcius
±	plus and/ or minus

>	more than

CHAPTER 1

INTRODUCTION

Cholesterol, a waxy, fat-like substance is an essential metabolite that enables the normal physiological functions of the body in all animals including human. Cholesterol serves as a precursor for the synthesis of steroid hormones such as vitamin D, glucocorticoids, mineral corticoids and sex hormones. Apart from that, cholesterol is also the precursor for the synthesis of bile acids that are responsible for fat absorption in the small intestine into the circulatory system via the lymphatic system. Cholesterol is insoluble in the blood. Thus, it is transported in the blood through the water-soluble carrier molecules known as lipoprotein. There are five types of lipoprotein which can be categorized depending on their density, namely chylomicron, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). Through the lipoprotein carrier, cholesterol is transported to other cells in the body such as steriodogenic tissues, muscles and adipocytes (Grummer & Carroll, 1988). Apart from dietary cholesterol, cells in the body synthesize their own cholesterol through 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase) activity which is regulated by sterol regulatory element binding protein-2 (SREBP-2) (Ahmad, 1994; Basile-Borgia & Abel, 1996; Burger, Gimpl, & Fahrenholz, 2000).

There are two types of cholesterol: bad cholesterol and good cholesterol. HDL is good cholesterol as HDL transports excess cholesterol from cells to liver where as LDL is considered bad cholesterol as it transports cholesterol from liver to cells (Basile-Borgia & Abel, 1996). Elevated level of cholesterol is known as hypercholesterolemia and it is usually associated with an increased risk of coronary

heart disease such as atherosclerosis, angina, heart attack and stroke. Instead of transporting cholesterol to the cells in the body, excess LDL deposits cholesterol into the arteries which cause the formation of the thick and hard plague called atheroma which finally leads to atherosclerosis. On the other hand, HDL lipoprotein transports the cholesterol from the atheroma plague back to the liver to be metabolized, thus reducing the atheroma plague. So, it is important to maintain an ideal level of HDL (>60mg/dl) and LDL (<100mg/dl) (Stapleton, Goodwill, James, Brock & Frisbee, 2010)

Cholesterol metabolism occurs mostly in the liver via LDL receptor. LDL receptor, a cell surface membrane protein, recognizes apolipoprotein E (apoE) and apolipoprotein B100 (apoB100) in the lipoprotein. VLDL, IDL and LDL contain apoB100 where as apoE is found on most of the lipoprotein especially VLDL and HDL. Even so, the primary ligand for the LDL receptor is low-density lipoprotein, followed by beta-migrating form of VLDL (ß-VLDL) (Lagor & Millar, 2009). After binding of the LDL to LDL receptor via apo B100, LDL is taken into the cells through receptor mediated endocytosis pathway. Then the LDL receptor is recycled back to the cell surface. Like HMG-CoA reductase, synthesis of LDL receptor is regulated by intracellular cholesterol through the SREBP-2 pathway. Mutation of LDL receptor is related to the familial hypercholesterolemia where by the LDL metabolism is disrupted, causing a high level of cholesterol in the blood (Goldstein & Brown, 2009; Goldstein, Brown, Anderson, Russell, & Schneider, 1985)

Apart from regulating LDL receptor and HMG-CoA, SREBP-2 also regulates the synthesis of proprotein convertase subtilisin-like kexin type 9 (PCSK9). PCSK9 is

identified as a new member of proprotein convertase family and has a role in the degradation of the LDL receptor. Since liver is the major organ that regulates cholesterol metabolism due to high level of LDL receptor, thus it is natural that PCSK9 is also highly expressed in liver. Synthesized as an inactive zymogen, PCSK9 undergoes autocatalytic cleavage at the Golgi apparatus. However, even though it is cleaved, PCSK9 prodomain remain associated to the mature PCSK9, assisting in escorting PCSK9 into the secretory pathway. Once outside the cells, the prodomain dissociates from the mature PCSK9. PCSK9 does not degrade the LDL receptor directly but binds to the LDL receptor on the cell surface, forming PCSK9-LDL receptor complex. This complex is then taken into the cells through receptor mediated endocytosis pathway. However, once inside the cells, the PCSK9 pump the LDL receptor into the lysosome for degradation. Like LDL receptor, PCSK9 mutation is also related to hypercholesterolemia. Gain-of-the function mutation shows a decrease in the LDL receptor where as loss-of-the function mutation shows an increase in the LDL receptor abundance in the liver (Kwon, Lagace, McNutt, Horton, & Deisenhofer, 2008; Lagor & Millar, 2009; Peterson, Fong, & Young, 2008).

Studies have shown that high cholesterol diet can leads to the formation of non-alcoholic fatty liver disease (NAFLD). An increase in dietary cholesterol is suggested to have increased the *de novo* synthesis of fatty acids in hepatocytes via Liver X Receptor Alpha (LXRα)-SREBP-1c pathway. Synthesized fatty acids in hepatocytes can form triglycerides, thus causing accumulation of triglyceride in liver, forming NAFLD. NAFLD is closely associated with metabolic syndrome, which is the risk factor for cardiovascular disease. Patients with NAFLD usually have an

increment in liver weight, proatherogenic lipid profile with elevated LDL, reduced HDL and postpandrial hypertriglyceridemia (Cohen, Horton, & Hobbs, 2011; Smith & Adams, 2011; Zelber-Sagi, Ratziu, & Oren, 2011).

Hypercholesterolemia is defined as cholesterol level higher than the recommended level (>200mg/dl). Hypercholesterolemia will lead to chronic heart disease. Thus, hypercholesterolemic condition must be treated. Currently there are synthetic drugs that are available in the market that are used to treat the hypercholesterolemic condition such as statins, cholesterol absorption inhibitors, resins, fibrates and niacin. Simvastatin, a statin drugs used in this experiment as a positive control is a HMG-CoA reductase inhibitor. HMG-CoA is a rate-limiting enzyme for cholesterol biosynthesis. Apart from inhibiting HMG-CoA reductase, simvastatin has also been reported to be able to upregulate hepatic LDL receptor and PCSK9. However, like all synthetic drugs, simvastatin can cause side effects. Thus, herbs and plants may have potential as alternatives in medicine to control hypercholesterolemia as they have lesser side effects. One of the herbs currently used is *Phaleria macrocarpa* (*Pm*) fruit. *Pm* which originates from Papua, Indonesia is a traditional herb that has been used for a few decades to treat diabetes mellitus, hypertension, hypercholesterolemia and others (Sugiwati, Leonardus, & Bintang, 2006). Even though *Pm* fruit had been proven to be able to reduce cholesterol level (Adnyana, Yulinah, Sigit, & Fitriani, 2005; Armenia, Ermilda, Widya, & Rusdi, 2006) but the mechanism is still unknown. Thus, this study was conducted to determine the possible mechanism of anti-hyperholesterolemic property of *Pm* fruit aqueous extract.

1.1 Objectives

1.1.1 General Objectives

To assess the anti-hypercholesterolemic property of *Pm* fruit aqueous extract *in vivo* and *in vitro*.

1.1.2 Specific Objectives

1.1.2.1 *In vivo* study

1. To determine the effects of *Pm* fruit aqueous extract on the body weight, liver weight and liver weight-to-body weight ratio in hypercholesterolemic rats.
2. To determine the effects of *Pm* fruit aqueous extract on the blood lipid profiles profiles [Total Cholesterol (TC), Triglycerides (TG), HDL and LDL]
3. To determine the effects of *Pm* fruit aqueous extract on the expression of hepatic LDL receptor and PCSK9 proteins

1.1.2.2 *In vitro* study

1. To determine the effects of *Pm* fruit aqueous extract on the expression of LDL receptor and PCSK9 proteins in HepG2 cells.
2. To determine the effects of *Pm* fruit aqueous extract on the expression of LDL receptor and PCSK9mRNA transcript in HepG2 cells.

1.2 Research Hypothesis

Pm fruit aqueous extract posses anti-hypercholesterolemic property by reducing blood TC, TG and LDL level but increases HDL of hypercholesterolemic diet-induced rats by upregulating LDL receptor and PCSK9 protein and mRNA expression. Apart from that, *Pm* fruit also reduces body weight gain, liver weight and liver weight-to-body weight ratio of diet induced hypercholesterolemic rats.

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