UNIVERSITI PUTRA MALAYSIA

PHYTOCOMPONENTS, SAFETY, ANTIOXIDANT, ANTI-INFLAMMATORY, HYPOCHOLESTEROLEMIC AND ANTI-ATHEROSCLEROTIC PROPERTIES OF BASELLA ALBA L. LEAF EXTRACT

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PHYTOCOMPONENTS, SAFETY, ANTIOXIDANT, ANTI-INFLAMMATORY, HYPOCHOLESTEROLEMIC AND ANTI- ATHEROSCLEROTIC PROPERTIES OF *BASELLA ALBA* L. LEAF EXTRACT

By

BASKARAN GUNASEKARAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, In Fulfilment of the Requirements for the Degree of Doctor of Philosophy

June 2015

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

PHYTOCOMPONENTS, SAFETY, ANTIOXIDANT, ANTI-INFLAMMATORY, HYPOCHOLESTEROLEMIC AND ANTI-ATHEROSCLEROTIC PROPERTIES OF *BASELLA ALBA* L. LEAF EXTRACT

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June 2015

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Hypercholesterolemia is the major risk factor that leads to atherosclerosis, which is the primary cause of death in world population. Nowadays, alternative treatment using medicinal plants gained much attention since the treatment using synthetic drug, statins, lead to adverse health effects, especially liver and muscle toxicity. Thus, the focus of this study was on *Basella alba* (*B. alba*) leaf extract; its phytocomponents, toxicity, antioxidant, anti-inflammatory, hypocholesterolemic and anti-atherosclerotic properties.

In this study, 25 medicinal plants extracts were screened for anti HMG-CoA reductase activity. *B. alba* leaf extract showed the highest inhibitory effect, about 74%. Therefore, *B. alba* was examined in order to investigate its phytochemical components. Gas Chromatography Mass Spectrometry (GC-MS/MS) analysis detected 25 compounds while Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) revealed the presence of naringin, apigenin, luteolin, ascorbic acid and α-tocopherol. The major compounds of *B. alba* have been reported to possess antihypercholesterolemic effects and further investigation was conducted on *in vivo* model.

The toxicity evaluation of *B. alba* leaf extract was determined using cytoxicity test against Vero and WRL-68 cell lines, acute and subchronic toxicity test in rats. SRB assay revealed non cytotoxic effect of *B. alba* with IC_{50} value of more than 625 µg/ml. The hematological and biochemical analyses showed no significant elevation in the parameters of *B. alba* extract treated rats compared to the control group. Histopathological examination revealed no harmful effects noted in liver and kidney. Cytotoxicity and acute toxicity studies confirmed that *B. alba* extract is non toxic and can be utilized as alternative therapeutic agent.

The present study also seeks to investigate antioxidant, anti-inflammatory, proximate and mineral composition analyses of *B. alba* leaf extract, focusing on therapeutic potential relating to hypercholesterolemia. *B. alba* extract exhibits high antioxidant activity in inhibiting radicals like hydroperoxides, 2,2-diphenyl-1-picrylhydrazyl (DPPH), nitric oxide (NO) and ferric ions. Anti-inhibitory activity of *B. alba* extract on hyaluronidase, xanthine oxidase and lipoxygenase enyzmes demonstrated a desirable anti-inflammatory activities.

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Twenty New Zealand white rabbits were divided into 5 groups and fed with normal diet (G1), 2% high cholesterol diet (HCD) (G2), 2% HCD + 10 mg/kg simvastatin (G3), 2% HCD + 100 mg/kg *B. alba* extract (G4) and 2% HCD + 200 mg/kg *B. alba* extract (G5), respectively. The treatment with *B. alba* extract significantly lowered the levels of total cholesterol (TC), low density lipoptotein (LDL) and triglyceride (TG). The significant increase in high density lipoprotein (HDL) and antioxidant enzymes; superoxide dismutase (SOD) and glutathione peroxidase (GPx) levels observed in treatment with *B. alba* extract (G4 and G5) compared to the treatment with simvastatin (G3). The elevated levels of liver enzymes; alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and muscle enzyme; creatine kinase (CK) were noted in G2 and G3 indicate liver and muscle injuries. Treatment with simvastatin (G3) and *B. alba* extract (G4 and G5) significantly suppressed the aortic plaque formation. This is the first *in vivo* study on *B. alba* that suggests its potential as an alternative therapeutic agent for hypercholesterolemia and atherosclerosis.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

SIFAT-SIFAT FITOKOMPONEN, KESELAMATAN, ANTIOKSIDAN, ANTIKERADANGAN, HIPOKOLESTEROLEMIK DAN ANTI ATEROSKLEROSIS PADA EKSTRAK DAUN *BASELLA ALBA* L.

Oleh

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Hiperkolesterolemia merupakan faktor risiko utama yang boleh mengakibatkan aterosklerosis, iaitu punca utama kematian penduduk dunia. Kini, rawatan alternatif yang menggunakan tumbuhan ubatan semakin mendapat perhatian kerana rawatan yang menggunakan dadah sintetik iaitu statin boleh menyebabkan kesan kesihatan yang buruk, terutamanya ketoksikan hati dan otot. Oleh itu, fokus kajian ini adalah terhadap ekstrak daun *Basella alba* (*B. alba*); fitokomponen tersebut serta sifat-sifat ketoksikan, bahan antioksida, antiradang, hipokolesterolemik dan antiaterosklerosis oleh tumbuhan tersebut.

Dalam kajian ini, 25 ekstrak tumbuhan ubatan telah disaring untuk mengkaji aktiviti anti-*HMG-CoA* reduktase. Ekstrak daun *B. alba* menunjukkan kesan rencatan tertinggi, iaitu sebanyak 74%. Oleh itu, *B. alba* telah dikaji untuk menyelidik komponen-komponen fitokimianya. Analisis Kromatografi Gas-Spektrometri Jisim (*Gas Chromatography-Mass Spectrometry*, GC-MS/MS) mengesan 25 sebatian, manakala Kromatografi Cecair Prestasi Tinggi Fasa Berbalik (*Reverse Phase-High Performance Liquid Chromatography*, RP-HPLC) mendedahkan kehadiran naringin, apigenin, luteolin, asid askorbik, dan α-tokoferol. Sebatian-sebatian utama dalam *B. alba* didapati mempunyai kesan antihiperkolesterolemik dan siasatan lanjut telah dijalankan terhadap model *in vivo*. Penilaian ketoksikan ekstrak daun *B. alba* dilaksanakan dengan melakukan ujian kesitotoksikan terhadap titisan sel Vero dan WRL-68, ujian ketoksikan akut dan subkronik pada tikus. Asai sulforhodamin (SRB) menunjukkan kesan bukan sitotoksik *B. alba* dengan nilai IC_{50} yang lebih daripada 625 µg/mL. Analisis hematologi dan biokimia tidak menunjukkan sebarang peningkatan ketara pada parameter-parameter kumpulan tikus yang dirawat dengan ekstrak *B. alba* berbanding dengan kumpulan kawalan. Pemeriksaan histopatologi menunjukkan ketiadaan sebarang kesan berbahaya pada hati dan buah pinggang. Kajian kesitotoksikan dan ketoksikan akut mengesahkan bahawa ekstrak *B. alba* adalah tidak toksik dan boleh digunakan sebagai agen terapeutik alternatif.

Kajian ini juga bertujuan untuk menyiasat analisis bahan antioksida, antiradang, serta komposisi proksimat dan mineral pada ekstrak daun *B. alba* dengan menumpukan kepada potensi terapeutik yang berkaitan dengan hiperkolesterolemia. Ekstrak *B. alba* mempamerkan aktiviti antioksida yang tinggi bagi merencat bahan radikal seperti

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hidroperoksida, 2,2-difenil-1-pikrilhidrazil (*2,2-diphenyl-1-picrylhydrazyl*, DPPH), nitrik oksida (*nitric oxide*, NO), dan ion ferik. Aktiviti antiradang oleh ekstrak *B. alba* terhadap hialuronidase, oksidase xantina, dan enzim lipoksigenase mempamerkan aktiviti antiradang yang diingini.

Dua puluh ekor arnab putih New Zealand telah dibahagikan kepada 5 buah kumpulan, dan masing-masing diberikan makan dengan diet yang normal (G1), 2% diet berkolesterol tinggi (HCD) (G2), 2% HCD + 10 mg/kg simvastatin (G3), 2% HCD + 100 mg/kg ekstrak *B. alba* (G4), dan 2% HCD + 200 mg/kg ekstrak *B. alba* (G5). Rawatan dengan ekstrak *B. alba* menurunkan paras jumlah kolesterol (*total cholesterol*, TC), lipoprotein ketumpatan rendah (*low density lipoprotein*, LDL) dan trigliserida (*triglyceride*, TG). Peningkatan ketara pada lipoprotein ketumpatan tinggi (*high density lipoprotein*, HDL), dan enzim antioksida; tahap superoksida dismutase (*superoxide dismutase*, SOD) dan glutation peroksidase (*glutathione peroxidase*, GPx), diperhatikan dalam rawatan yang mengandungi ekstrak *B. alba* (G4 dan G5) berbanding dengan rawatan yang mengandungi simvastatin (G3). Peningkatan tahap enzim hati; alanina aminotransferase (*alanine aminotransferase*, ALT) dan aspartat aminotransferase (*aspartate aminotransferase*, AST), serta enzim otot; kreatina kinase (*creatine kinase*, CK) yang diperhatikan dalam G2 dan G3 menandakan kecederaan hati dan otot. Rawatan dengan simvastatin (G3) dan ekstrak *B. alba* (G4 dan G5) menyekat pembentukan plak aorta dengan amat ketara. Kajian *in vivo B. alba* ini merupakan kajian pertama yang mencadangkan potensinya sebagai agen terapeutik alternatif bagi merawat hiperkolesterolemia dan aterosklerosis.

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I certify that a Thesis Examination Committee has met on 16 June 2015 to conduct the final examination of Baskaran a/l Gunasekaran on his thesis entitled "Phytocomponents, Safety, Antioxidant, Anti-Inflammatory, Hypocholesterolemic and Anti- Atherosclerotic Properties of *Basella alba* L. Leaf Extract" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

Ab_b	absorbance of blank
Ab_c	absorbance of control
Ab_s	absorbance of sample
ACE	angiotensin converting enzyme
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANOVA	analysis of variance
AOAC	Association of Official Analytical Chemists
AST	aspartate aminotransferase
ATP	adenine trinucleotide phosphates
B. alba	*Basella alba*
°C	degree Celcius
°C /min	degree Celcius per minute
CETP	cholesterol ester transfer protein
CK	creatine kinase
cm	centimetre
CO_2	carbon dioxide
CVDs	cardiovascular diseases
Da	dalton
dH_2O	distilled water
DMEM	dulbecco's modified eagle medium
DNA	deoxyribonucleic acid

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DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	dry weight
EDTA	ethylenediaminetetraacetic acid
eV	electron volt
GAE	gallic acid equivalent
FDA	Food and Drug Administration
FID	flame ionization detector
Fig	figure
fl	femtoliters
FPP	fornesylpyrophosphate
FRAP	ferric-reducing antioxidant power
FRIM	Forest Research Institute Malaysia
FTC	ferric thiocyanate
g	gram
GAG	glcosoaminoglycans
GC-MS/MS	gas chromatography mass spectrometry
GGPP	geranylgeranyl pyrophosphate
g/L	gram per litre
GPx	glutathione peroxidase
HA	hyaluronan
Hb	haemoglobin
HCD	high cholesterol diet
HDL	high-density lipoprotein
H&E	hematoxylin and eosin

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HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HPLC	high performance liquid chromatography
IACUC	Institutional Animal Care and Use Committee
IC_{50}	inhibitory concentration 50%
ICAM -1	intercellular cell adhesion molecule-1
ICP-OES	inductively couple plasma-optical emission spectrometry
ID	inner diameter
IDL	intermediate density lipoprotein
IL	interleukin
kcal	kilocalorie
kg	kilogram
L	liter
LCAT	lecithin-cholesterol acyltransferase
LDL	low density lipoprotein
LOXs	lipoxygenases
LTB4	leukotriene B4
m	metre
mm	millimetre
mM	millimolar
mmol/l	millimol per litre
mg	milligram
mg/dl	milligram per deciliter
mg/kg	miligram per kilogram
mg/ml	milligram per milliliter

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min	minute
ml	milliliter
ml/min	milliliter per minute
Mw	molecular weight
m/z	mass-to-charge ratio
NaCl	sodium chloride
nm	nanometer
NO	nitric oxide
OECD	Organization for Economic Cooperation and Development
O2-	superoxide
ONOO-	peroxynitrite
pg	pictogram
PGE2	prostaglandin E2
pH	*Puissance hyrogene*
ROS	reactive oxygen species
RP-HPLC	reverse phase-high performance liquid chromatography
rpm	revolution per minute
RT	retention time
s	second
SD	standard deviation
SOD	superoxide dismutase
SPSS	statistical package for social sciences
SRB	sulforhodamine B
SREBP	sterol regulatory element binding protein

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TBA	thiobarbituric acid
TBIL	total bilirubin
TC	total cholesterol
TFC	total flavonoid content
TG	triglyceride
TPC	total phenolic content
µg	microgram
µg/ml	microgram per millilitre
µl	microlitre
U/l	unit per litre
Mm	micromolar
U/ml	unit per milliliter
µmol/l	micromol per litre
VCAM -1	vascular cell adhesion molecule-1
VLDL	very low density lipoprotein
v/v	volume per volume
WHO	World Health Organization
w/v	weight per volume

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CHAPTER 1

INTRODUCTION

Hypercholesterolemia is known to play a crucial role in the development of atherosclerosis and is generally identified as a risk factor for cardiovascular diseases (CVDs), which is a primary cause of morbidity and mortality in most developed countries (Kaup *et al*., 2011; Vogel, 1997). Significant elevation of blood cholesterol can lead to the progression of fatty liver (Assy *et al.*, 2000) and renal injury (Quyyumi, 1998), as indicated by the increased levels of liver and kidney enzymes. Accumulation of cholesterol impairs endothelial dysfunction, which leads to low density lipoprotein (LDL) oxidation, vasoregulation, monocyte and platelet adhesion and smooth muscle cell proliferation (Shaila *et al*., 1995). Although several factors like lifestyle, age, improper diet and hypertension have been reported to cause heart failure (Schaefer *et al*., 1995), hypercholesterolemia due to elevated cholesterol level, mainly LDL (Krieger, 1998), is principally responsible for CVDs. Therefore, lowering the prevalence of hypercholesterolemia conditions is considered to be an essential therapeutic approach (Ali *et al.*, 2000).

The current treatment for hypercholesterolemia is a synthetic drug, statins. Statins competitively inhibit HMG-CoA reductase, the enzyme that catalyzes the rate-limiting step in the biosynthesis of cholesterol. Basically, statins are well tolerated and effective in cholesterol lowering. The most common adverse effects of statins are liver and muscle toxicity (Bradford *et al.*, 1991). This occurs due to increase of liver transaminases (greater than three-fold) in the body. If this happens, the statins therapy should be stopped; transaminases levels usually take about three months to return to the baseline (Maron *et al.*, 2000). Another side effect of statins is myopathy, defined as muscle weakness or pain associated with the increased level of creatine kinase (CK) more than ten times the upper limit of normal. Symptoms may include increased serum concentration of the statin, malaise and fever. Acute renal failure and rhabdomyolysis may result if myopathy is not recognized and the drug is continued (Bellosta *et al.*, 2004).

Due to the adverse effects of synthetic drugs, attention is now directed to alternative medicine of plant origin (Loke *et al*., 2010). Over the decades, the use of medicinal plants represents the interaction between human with the environment (Sasidharan *et al*., 2011). Medicinal plants have been widely reported on its medicinal properties, nutritional values and pharmacological activities like antioxidant, anti-thrombotic, anti-inflammatory, anti-atherogenic and cardioprotective effects (Manach *et al*., 2005). Medicinal plants that can inhibit or scavenge free radicals and reduce serum cholesterol have gained wide therapeutic benefits. Great efforts have been made to lower the risk of CVDs through the regulation of cholesterol, and the therapeutic effects of plants have been the main focus of many dietary studies (Zhang *et al*., 2007a; Prasad, 2005).

Accordingly, attempts were made to identify the antihypercholesterolemia effects of various medicinal plants. *B. alba* has been identified as a potent inhibitior of HMG-COA reductase. *B. alba* is known as Remayung locally, belongs to the family of Basellaceae. *B. alba* is a wildly cultivated vegetable that has been utilized for its various pharmacological activities such as antimicrobial, anti-ulcer, antimutagenic,

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antihypertensive and many more (Adhikari *et al*., 2012). Therefore, this study focuses on the potential of *B. alba* leaf extract as an alternative source of therapeutic against hypercholesterolemia and atherosclerosis. The specific objectives of this study were:

1) To screen the HMG COA reductase inhibitory activity of 25 medicinal plants.

2) To examine the phytochemical components of *B. alba* leaf methanol extract using Gas Chromatography Mass Spectrometry (GC-MS/MS) and Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) analyses.

3) To evaluate the toxicity of *B. alba* leaf extract using cytotoxicity test on Vero and WRL-68 cell lines, acute and subchronic oral toxicity on rats.

4) To determine the antioxidant and anti-inflammatory activities, proximate and mineral composition, and heavy metal analysis of *B. alba* leaf extract.

5) To investigate the hypocholesterolemic and antiatherosclerotic effects of *B. alba* leaf extract in hypercholesterolemia-induced rabbits.

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BIODATA OF STUDENT

Baskaran Gunasekaran was born in Mentakab, Pahang in the year of 1985. He is the youngest in the family. In year 2004, he completed his pre-university studies in Saint George Institution. In year 2006, he enrolled in Universiti Putra Malaysia to pursue his first degree. He obtained Bachelor Science (Hons) Biochemistry, with Second Class Upper. In September 2009, he furthered his second degree at the Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. He obtained Master of Science in Biochemistry in the year of 2011. During his education period, he was awarded numerous awards and honors in both academic and sports.

He furthered his Ph.D. in the field of Biochemistry under supervision of Assoc. Prof. Dr. Mohd Yunus B. Abd. Shukor. His research focuses on the development of alternative medicine for treatment of atherosclerosis. Besides being active in research, he has participated in several local and international conferences. He was appointed as the President for postgraduate students in the Faculty of Biotechnology and Biomolecular Sciences.

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LIST OF PUBLICATIONS

Baskaran Gunasekaran, Shamala Salvamani, Noor Azmi Shaharuddin, Siti Aqlima Ahmad , Mohd Yunus Shukor (2014). Anti-atherosclerotic effects of plant flavonoids, *BioMed Research International Journal*, 480258. (IF: 2.88, Q2)

Baskaran, Gunasekaran, Shamala Salvamani, Siti Aqlima Ahmad, Noor Azmi Shaharuddin, Parveen Devi Pattiram, and Mohd Yunus Shukor (2015). HMG-CoA reductase inhibitory activity and phytocomponent investigation of *Basella alba* leaf extract as a treatment for hypercholesterolemia. *Drug Design, Development and Therapy* 9:509-517. (IF: 3.026, Q2)

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Baskaran, Gunasekaran, Shamala Salvamani, Siti Aqlima Ahmad, Mohd Khalizan Hasbullah and Mohd Yunus Shukor (2015). Antioxidant, anti-inflammatory and properties analysis of *Basella alba* leaf extract (Submitted to BioMed Research International).

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PATENT

Mohd Yunus Shukor, **Baskaran Gunasekaran**, Shamala Salvamani, Azrina Azlan, Md Zuki Abu Bakar and Siti Aqlima Ahmad. Novel compound for use as medicament for treatment of hypercholesterolemia and atherosclerosis (Patent file-PI 2014704025).

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