



UNIVERSITI PUTRA MALAYSIA

***Amaranthus viridis L. AS ALTERNATIVE TREATMENT FOR
HYPERCHOLESTEROLEMIA AND ATHEROSCLEROSIS***

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By

SHAMALA SALVAMANI

**Thesis Submitted to the school of Graduate Studies, Universiti Putra Malaysia, in
fulfilments of the Requirements for the Doctor of Philosophy**

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DEDICATION

This thesis is dedicated to my dearest husband (Dr. Baskaran) and our both parents (Mr. Salvamani and Mrs. Mageswari) and (Mr. Gunasekaran and Mrs. Ramani) for their unconditional love, prayers and blessings throughout my studies. I also would like to dedicate this thesis to my supervisor (Dr. Siti Aqlima Ahmad) who has been a great support during my PhD study.



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

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May 2016

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Inflammation and oxidative stress are involved in the pathology of several chronic diseases including hypercholesterolemia and atherosclerosis. Oxidized low density lipoprotein (LDL) accumulation leads to atherosclerotic plaque formation, which contributes to myocardial infarction and cardiovascular diseases. Synthetic drug, statins, causes adverse effects on liver and muscles, thus 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors from plant origin are needed. *Amaranthus viridis* (*A. viridis*) has been used for its medically beneficial properties from ancient time. Thus, the focus of this study was on *A. viridis* leaf extract; its phytochemicals, safety, antioxidant, anti-inflammatory, hypocholesterolemic and anti-atherosclerotic properties. In this study, the different parts of *A. viridis* (leaf, stem and seed) were evaluated for anti-HMG-CoA reductase activity. *A. viridis* leaf extract showed the highest inhibitory effect, about 72%. Therefore, *A. viridis* leaf extract was examined in order to investigate its phytochemicals. Gas chromatography-tandem mass spectrometry (GC-MS/MS) analysis detected 30 compounds while reverse phase-high performance liquid chromatography (RP-HPLC) revealed the presence of ascorbic acid, rutin, quercetin and catechin. *In vitro* cytotoxicity effect of *A. viridis* extract was estimated using sulforhodamine B (SRB) assay on Vero and WRL-68 cells lines. SRB assay revealed non cytotoxic effect of *A. viridis* with IC₅₀ value of more than 1000 µg/ml. Acute and subchronic toxicity study in rats for 14 and 90 days, respectively, showed no significant elevation in biochemical and haematological parameters compared to the control group. Histopathological examination revealed no harmful effects observed in heart, lung, liver, kidneys and spleen. Cytotoxicity, acute and subchronic toxicity studies confirmed that *A. viridis* extract is non toxic and can be utilized as a therapeutic agent. The antioxidant and anti-inflammatory activities of the extract were analyzed in various *in vitro* assays. *A. viridis* 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 *A. viridis* extract on hyaluronidase, xanthine oxidase and lipoxygenase enzymes revealed a desirable anti-inflammatory properties. The experimental data indicated that *A. viridis* leaf is a potent antioxidant and anti-inflammatory agent. Animal model study was performed on twenty New Zealand white rabbits that were randomly divided into 5 groups and fed with normal diet, 2% high cholesterol diet (HCD), 2% HCD + 10 mg/kg

simvastatin, 2% HCD + 100 mg/kg *A. viridis* extract and 2% HCD + 200 mg/kg *A. viridis* extract, respectively. The supplementation with *A. viridis* extract significantly reduced total cholesterol, LDL and triglycerides levels, and increased high density lipoprotein (HDL) and antioxidant enzymes [superoxide dismutase (SOD) and glutathione peroxidase (GPx)] levels. The elevated levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatine kinase (CK) in hypercholesterolemic control and simvastatin-treated groups indicate liver and muscle injuries. Treatment with *A. viridis* extract also diminished the development of aortic plaque and decreased the intima: media ratio as observed in simvastatin-treated rabbits. The *in vivo* study on *A. viridis* leaf extract further confirms its potential as an alternative therapeutic agent for hypercholesterolemia and atherosclerosis.

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

***Amaranthus viridis* L. SEBAGAI RAWATAN ALTERNATIF UNTUK
HIPERKOLESTEROLEMIA DAN ATEROSKLEROSIS**

Oleh

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Keradangan dan tekanan oksidatif yang terlibat dalam patologi terdiri daripada beberapa penyakit kronik termasuk hiperkolesterolemia dan aterosklerosis. Pengumpulan lipoprotein berketumpatan rendah (*low density lipoprotein*, LDL) teroksida membawa kepada pembentukan plak atherosklerotik yang menyumbang kepada serangan jantung dan penyakit kardiovaskular. Ubat sintetik, statin, menyebabkan kesan buruk kepada hati dan otot, oleh itu perencat 3-hidroksi-3-metilglutaril-koenzim A (*3-hydroxy-3-methylglutaryl-coenzyme A*, HMG-CoA) reduktase daripada tumbuhan diperlukan. *Amaranthus viridis* (*A. viridis*) telah digunakan untuk ciri-ciri perubatan yang bermanfaat dari zaman purba. Oleh itu, fokus kajian ini adalah terhadap ekstrak daun *A. viridis*; sifat-sifat fitokimia, keselamatan, antioksidan, anti-radang, hipokolesterolemik dan anti-aterosklerosis. Dalam kajian ini, bahagian-bahagian *A. viridis* (daun, batang dan benih) telah dinilai untuk aktiviti anti-HMG-CoA reduktase. Ekstrak daun *A. viridis* menunjukkan kesan rencatan tertinggi, iaitu sebanyak 72%. Oleh itu, ekstrak daun *A. viridis* telah dikaji untuk menyelidik komponen-komponen fitokimianya. Analisis Kromatografi Gas-Spektrometri Jisim (*Gas Chromatography-Mass Spectrometry*, GC-MS/MS) mengesan 30 sebatian, manakala Kromatografi Cecair Prestasi Tinggi Fasa Berbalik (*Reverse Phase-High Performance Liquid Chromatography*, RP-HPLC) mendedahkan kehadiran asid askorbik, rutin, quercetin dan katechin. Ujian kesitotoksikan *in vitro* ekstrak *A. viridis* terhadap titisan sel Vero dan WRL-68 dijalankan dengan menggunakan asai sulforhodamin B (*sulforhodamine B*, SRB). SRB asai mendedahkan kesan bukan sitotoksik *A. viridis* dengan nilai IC₅₀ yang lebih daripada 1000 µg/ml. Ujian ketoksikan akut dan subkronik pada tikus selama 14 dan 90 hari, tidak menunjukkan sebarang peningkatan yang ketara pada parameter-parameter biokimia dan hematologi berbanding dengan kumpulan kawalan. Pemeriksaan histopatologi menunjukkan ketiadaan sebarang kesan berbahaya pada jantung, paru-paru, hati, buah pinggang dan limpa. Kajian kesitotoksikan, ketoksikan akut dan subkronik mengesahkan bahawa ekstrak *A. viridis* adalah tidak toksik dan boleh digunakan sebagai agen terapeutik. Aktiviti antioksidan dan anti-radang ekstrak dianalisis dalam pelbagai asai *in vitro*. Ekstrak *A. viridis* mempamerkan aktiviti antioksidan yang tinggi dalam merencat bahan radikal seperti hidroperoksida, 2,2-difenil-1-pikrilhidrazil (*2,2-diphenyl-1-picrylhydrazyl*, DPPH), nitrik oksida (*nitric oxide*, NO) dan ion ferik. Aktiviti anti

radang oleh ekstrak *A. viridis* terhadap enzim hialuronidase, oksidase xantina dan lipoksigenase mendedahkan aktiviti anti-radang yang diingini. Data eksperimen menunjukkan bahawa daun *A. viridis* adalah agen antioksidan dan anti-radang yang kuat. Kajian model haiwan telah dilakukan ke atas dua puluh ekor arnab putih New Zealand yang telah dibahagikan secara rawak kepada 5 buah kumpulan dan masing-masing telah diberikan makanan dengan diet normal, 2% diet berkolesterol tinggi (HCD), 2% HCD + 10 mg/kg simvastatin, 2% HCD + 100 mg/kg ekstrak *A. viridis* dan 2% HCD + 200 mg/kg ekstrak *A. viridis*. Suplementasi dengan ekstrak *A. viridis* menurunkan paras jumlah kolesterol, LDL dan trigliserida dengan ketara, dan meningkatkan paras lipoprotein berketumpatan tinggi (*high density lipoprotein*, HDL) dan enzim antioksidan, superoksida dismutase dan glutathion peroksidase (*superoxide dismutase*, SOD; *glutathione peroxidase*, GPx). Peningkatan tahap aspartat aminotransferase (*aspartate aminotransferase*, AST), alanina aminotransferase (*alanine aminotransferase*, ALT) dan kreatina kinase (*creatine kinase*, CK) dalam kumpulan kawalan hiperkolesterolemik dan kumpulan simvastatin menunjukkan kecederaan hati dan otot. Rawatan dengan ekstrak *A. viridis* juga mengurangkan pembentukan plak aorta dan mengurangkan nisbah intima dan media seperti yang diperhatikan dalam kumpulan simvastatin. Kajian *in vivo* daun *A. viridis* mengesahkan potensinya sebagai agen terapeutik alternatif bagi merawat hiperkolesterolemia dan aterosklerosis.

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Shamala Salvamani, 2016

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirements for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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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
<i>A. viridis</i>	<i>Amaranthus viridis</i>
°C	Degree Celcius
CETP	Cholesterol ester transfer protein
CK	Creatine kinase
cm	Centimetre
CO ₂	Carbon dioxide
CVDs	Cardiovascular diseases
Da	Dalton
dH ₂ O	Distilled water
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
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
fl	Femtoliters
FPP	Fornesylpyrophosphate
FRAP	Ferric-reducing antioxidant power
FRIM	Forest Research Institute Malaysia
FTC	Ferric thiocyanate
g	Gram
GAG	Glucosaminoglycans
GC-MS/MS	Gas chromatography mass spectrometry
GGPP	Geranylgeranyl pyrophosphate
GPx	Glutathione peroxidase
HA	Hyaluronan
Hb	Haemoglobin
HCD	High cholesterol diet
HDL	High-density lipoprotein
H&E	Hematoxylin and eosin
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
LTB ₄	Leukotriene B ₄
M	Metre
mm	Millimetre
mM	Millimolar
mg	Milligram
min	Minute
ml	Milliliter
M _w	Molecular weight
m/z	Mass-to-charge ratio
NaCl	Sodium chloride
nm	Nanometer
NO	Nitric oxide
OECD	Organization for Economic Cooperation and Development
O ₂ ⁻	Superoxide
ONOO ⁻	Peroxynitrite
pg	Pictogram
PGE ₂	Prostaglandin E ₂
pH	<i>Puissance hyrogene</i>

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
TBA	Thiobarbituric acid
TBIL	Total bilirubin
TC	Total cholesterol
TFC	Total flavonoid content
TG	Triglyceride
TPC	Total phenolic content
µg	Microgram
µl	Microlitre
U	Unit
µm	Micromolar
VCAM -1	Vascular cell adhesion molecule-1
VLDL	Very low density lipoprotein
v	Volume
WHO	World Health Organization
W	Weight

CHAPTER 1

INTRODUCTION

Hypercholesterolemia is a primary cause of morbidity and mortality in most developed countries (Kaup *et al.*, 2011; Vogel, 1997). Hypercholesterolemia plays an essential role in the development of atherosclerosis and is generally identified as a risk factor for cardiovascular diseases (CVDs). The significant elevation of blood cholesterol leads to the increased levels of liver and kidney enzymes which indicates the development of fatty liver (Assy *et al.*, 2000) and renal injury (Quyyumi, 1998). Accumulation of cholesterol causes endothelial dysfunction which further leads to low density lipoprotein (LDL) oxidation, monocyte and platelet adhesion, vasoregulation and smooth muscle cell proliferation (Adaramoye *et al.*, 2005; Shaila *et al.*, 1995). Although several factors like age, improper diet, lifestyle and hypertension have been reported to cause heart failure (Schaefer *et al.*, 1995), hypercholesterolemia is principally responsible for CVDs. Therefore, reducing the prevalence of hypercholesterolemia conditions is considered to be a crucial therapeutic approach (Ali *et al.*, 2000).

Statins are widely prescribed synthetic drugs that competitively inhibit HMG-CoA reductase, the enzyme that catalyzes the rate-limiting step in the cholesterol biosynthesis. Generally, statins are well tolerated and effective in cholesterol lowering. The most common adverse effects of statins are muscle and liver 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 immediately stopped; the elevated levels of transaminases usually take about three months to return to the baseline (Maron *et al.*, 2000). Another side effect of statins is myopathy, defined as muscle pain or weakness associated with the increased level of creatine kinase (CK) more than ten times the upper limit of normal. Symptoms may include fever, malaise and increased statin concentration in the serum. Rhabdomyolysis and acute renal failure may result if myopathy is not recognized and the statin is continued (Bellosta *et al.*, 2004).

Due to the serious 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 that can scavenge or inhibit free radicals and lower serum cholesterol have gained wide therapeutic benefits. The therapeutic effects of plants have been the main focus of many dietary studies and great efforts have been made to decrease the risk of CVDs through the regulation of cholesterol (Baskaran *et al.*, 2016b; Zhang *et al.*, 2007a; Prasad, 2005).

Consequently, attempts were conducted to identify the antihypercholesterolemia effects of various medicinal plants. *Amaranthus viridis* L. (*A. viridis*) is a branched glabrous herb that belongs to the family of Amaranthaceae. *A. viridis* which is known as green amaranth and *Bayam pasir* locally is distributed in most of the tropical countries (Girija

and Lakshman, 2011). *A. viridis* has been traditionally used to reduce labour pain and as antipyretic in India and Nepal. *A. viridis* has been traditionally used as analgesic, antiulcer, antirheumatic, antileprotic and antiemetic (Agra *et al.*, 2008). It is also believed to treat eye diseases, respiratory problems, asthma, eczema and psoriasis (Kumar *et al.*, 2010b). *A. viridis* has been scientifically proven to possess medicinal effects like antimicrobial, antidiabetic, antinociceptive and antipyretic activities (Sowjanya *et al.*, 2014).

The hypocholesterolemic effects of *A. viridis* have not been well researched up to date. Therefore, this study focuses on the potential of *A. viridis* 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 of different parts of *A. viridis* (leaf, stem and seed).
- 2) To examine the phytochemicals of *A. viridis* leaf methanolic extract using gas chromatography-tandem mass spectrometry (GC-MS/MS) and reverse phase-high performance liquid chromatography (RP-HPLC) analysis.
- 3) To determine the toxicity of *A. viridis* leaf extract using cytotoxicity test on Vero and WRL-68 cell lines, acute and subchronic oral toxicity on rats.
- 4) To evaluate the antioxidant and anti-inflammatory activities, proximate and mineral composition, and heavy metal analysis of *A. viridis* leaf extract.
- 5) To investigate the hypocholesterolemic and anti-atherosclerotic effects of *A. viridis* leaf extract in hypercholesterolemia-induced rabbits.

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