DIFFERENTIAL EXPRESSION OF SERUM PROTEOME OF HYPERCHOLESTEROLEMIC RATS FOLLOWING TREATMENT WITH *Ficus deltoidea*

CHUO PECK HAM

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2018

DIFFERENTIAL EXPRESSION OF SERUM PROTEOME OF HYPERCHOLESTEROLEMIC RATS FOLLOWING TREATMENT WITH *Ficus deltoidea*

CHUO PECK HAM

DISSERTATION SUBMITTED IN FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2018

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: CHUO PECK HAM

Matric No: SGR 130061

Name of Degree: Master of Science (MSc.)

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

Differential Expression of Serum Proteome of Hypercholesterolemic Rats Following Treatment with *Ficus deltoidea*

Field of Study: Genetics and Molecular Biology

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date:

Subscribed and solemnly declared before,

Witness's Signature Date:

Name:

Designation:

DIFFERENTIAL EXPRESSION OF SERUM PROTEOME OF HYPERCHOLESTEROLEMIC RATS FOLLOWING TREATMENT WITH *Ficus deltoidea*

ABSTRACT

Cardiovascular disease (CVD) is one of the most common noncommunicable disease resulting in cardiac dysfunction and heart failure. In Malaysia, hypercholesterolemia or high blood cholesterol has emerged as the main risk factor for CVD. Currently, there is a paradigm shift of interest towards Eastern medicines as alternative approaches in prevention and therapeutic treatment for cholesterol lowering and cardioprotection. *Ficus deltoidea* (Mas Cotek) is amongst the medicinal plants traditionally consumed either as prevention or self-medication for various diseases like high blood pressure, diabetes and high blood cholesterol. This current study utilized proteomic application in determining the changes in series of proteins that are being differently expressed in response to *F. deltoidea* treatment in hypercholesterolemic and cardiac damaged–carbon tetrachloride (CCl_4) induced rats. Identification and quantification of proteins were conducted using relative quantitative techniques, such as 2D-GE along with Progenesis Samespots image analysis software and MALDI ToF/ToF mass spectrometry. Our study demonstrated a significant alteration of protein abundances following *F. deltoidea* treatment with the majority of these identified proteins were involved in lipid metabolism and transport, inflammation and immune response, anti oxidant protein, acute phase protein, cholesterol homeostasis and cholesterol binding as well in cytoskeletal and structural effects. The hypercholesterolemic related proteins (Apo A1, Apo E, RBP4, HAP, PDE1, C3, C1s, PROM1, TTR, MUP, CPI and PLG) identified are the key proteins responsible for the suppression of hypercholesterolemia following treatment with *F. deltoidea* extracts. Consequently, TPM2, MYH6, SUMO3, Apo A1 and Apo E are associated with

cardioprotective effects. Interestingly, Apo E and Apo A1 are the two profound putative proteins that exhibit both cholesterol lowering and ameliorate cardioprotection effects. The variations of these proteins may provide valuable new molecular insights into the mechanism of cholesterol lowering and cardioprotective effects of *F. deltoidea*. We speculated that long-term *F. deltoidea* consumption would set-back further extent of cardio atherosclerosis and myocardial infarction, as well subsequent cardiac function deterioration and mortality in hypercholesterolemic subjects. Thus, further development of this study will enable the finding of alternative treatments as well as preventions for CVD.

Keywords: Differential expressions, *Ficus deltoidea*, hypercholesterolemia, rats, MALDI-ToF/ToF Mass Spectrometry

PERBEZAAN TAHAP PENGEKSPRESAN PROTEOM SERUM BAGI TIKUS HIPERKOLESTEROLEMIA BERIKUTAN RAWATAN DENGAN *Ficus deltoidea*

ABSTRAK

Penyakit kardiovaskular (CVD) adalah sejenis penyakit tidak berjangkit paling umum yang menyebabkan disfungsi kardia dan kegagalan fungsi jantung. Di Malaysia, hiperkolesterolemia atau kandungan kolesterol yang tinggi di dalam darah merupakan faktor risiko utama penyakit kardiovaskular. Namun sejak akhir-akhir ini, perubatan alternatif secara timur telah diterima dengan penuh minat sebagai rawatan pencegahan dan terapeutik untuk menurunkan paras kolesterol darah dan juga sebagai perlindungan jantung (kardioprotektif). *Ficus deltoidea* (Mas Cotek) adalah di antara tumbuh-tumbuhan perubatan tradisional yang digunakan untuk tujuan pencegahan atau rawatan untuk penyakit tekanan darah tinggi, kencing manis dan kolesterol darah tinggi. Kajian ini menggunakan aplikasi kaedah proteomik dalam mengenalpasti protein yang menunjukkan perubahan ketara berikutan rawatan *F. deltoidea* ke atas tikus dalam keadaan hiperkolesterolemia dan infark jantung berikutan aruhan karbon tetraklorida (CCl_4). Pengenalpastian dan pengkuantitian protein telah dijalankan menggunakan teknik kuantitatif relatif seperti 2D-GE dengan bantuan perisian analisis imej *Progenesis Samespots* dan analisis spektrometer jisim MALDI ToF / ToF. Kajian kami menunjukkan terdapat perubahan protein yang ketara selepas rawatan *F. deltoidea*. Kebanyakan protein yang dikenalpasti terlibat dalam metabolisme dan pengangkutan lipid, tindak balas radang dan imun, anti pengoksidaan, protein fasa akut , homeostasis kolesterol, pengikatan kolesterol serta kesan struktur dan sitoskeletal. Protein-protein ini (Apo A1, Apo E, RBP4, HAP, PDE1, C3, C1s, PROM1, TTR, MUP, CPI and PLG) adalah protein utama yang berkaitan dengan hiperkolesterolemia dan bertanggungjawab menurunkan hiperkolesterolemia berikutan rawatan menggunakan ekstrak *F. deltoidea*. Di samping

itu, TPM2, MYH6, SUMO3, Apo A1 dan Apo E adalah protein yang dikaitkan dengan kesan perlindungan jantung. Yang menariknya, Apo E dan Apo A1 adalah dua protein yang menunjukkan kedua-dua fungsi dalam penurunan paras kolesterol darah dan perlindungan jantung. Penemuan protein-protein ini memberikan pandangan baru dalam tindakbalas molekul terhadap mekanisme yanng terlibat dalam penurunan kolesterol and perlindungan jantung berikutan rawatan menggunakan *F. deltoidea*. Kami menjangkakan bahawa penggunaan *F. deltoidea* dalam jangka masa panjang dapat mengurangkan kadar penyakit aterosklerosis dan serangan jantung, kemerosotan fungsi jantung dan kematian berikutan keadaan hiperkolesterolemia. Oleh yang demikian, perlanjutan kajian ini perlu diteruskan bagi penemuan pencegahan serta rawatan alternatif untuk penyakit kardiovaskular.

Kata Kunci: Perbezaan tahap pengekspresan, *Ficus deltoidea*, hiperkolesterolemia, tikus, spektrometer jisim MALDI-ToF/ToF

ACKNOWLEDGEMENTS

I wish to convey my gratitude to my supervisor **Associate Prof. Dr. Norhaniza Binti Aminudin,** Institute of Biological Sciences, University of Malaya, who deserves more thanks than I can give for her close supervision, revision, continuous encouragement and valuable advices during the study. Gratefully, I would like to owe great thanks to **Associate Prof. Dr. Puteri Shafinaz Akmar Binti Abdul Rahman,** Head of Unit at Medical Biotech Laboratory, Faculty Medicine, University of Malaya. I really appreciate her kindness, supervision, valuable support and equipment needed throughout this study.

Special acknowledgements go to **Atikah Haizum**, for her invaluable assistances in my experimental labworks, comments, and continuous encouragement during the study. Also great thanks to all my previous and present labmates in MUCED (**Lum Ai Theng, Khaniza Hasliza Abdul Khalil, Ayu and Shareena**) for their cooperation and scientific knowledge and discussion sharing. All this work has been carried out successfully to the end and I would acknowledge the help of staffs in MBL (**Nur Athirah Hussin and Nosrihah Ismail**) and HIR for their guidance and invaluable assistance in their laboratory.

Finally, my heartiest appreciation goes to my beloved family for providing such great loves and supports throughout the completion of this thesis and accomplishing my goals. And to my husband, Dennis, I am grateful for your never ending support and understanding has assisted me to this stage.

CHUO PECK HAM

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

%	percent
°C	degree celsius
µg	microgram
µl	microliter
µmol	micromole
1D	First dimension
2D-GE	two-dimensional gel electrophoresis
ACN	acetonitrile
Apo	Apolipoprotein
APS	ammonium persulfate
Ato	Atorvastatin
BME	beta mercaptoethanol
C	control
C1s	Complement C1s
C3	Complement C3
CCl_4	carbon tetrachloride
CHAPS	3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate
CHCA	α-cyano-4-hydroxycinnamic acid
CPI	Contrapsin-like protease inhibitor
CVD	cardiovascular disease
DAB	3,3'-diaminobenzidine
DTT	dithiothreitol
E250	250 mg/kg body weight *F. deltoidea* extract
E50	50 mg/kg body weight *F. deltoidea* extract
EDTA-Na_2H_2O	ethylenedinitrilotetraacetic acid disodium salt dihydrate (Titriplex III)
EE	ethanol extract
EFA	essential fatty acids
Est'd Z	estimated Z-score
F. deltoidea	*Ficus deltoidea*
g	gram

HAP	Haptoglobin
HC	Hypercholesterolemic control
HCl	hydrochloric acid
HDL	low-density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HRP	horseradish peroxidase
IAA	iodoacetamide
IEF	isoelectric focusing
ILDL	intermediate-density lipoprotein
IPG	immobilized pH gradients
kDa	kilo Dalton
L	Liter
LDL	low-density lipoprotein
M	Molar
m/z	mass to charge ratio
mA	miliAmpere
MALDI-ToF/ToF MS	Matrix-assisted laser desorption/ionization-Time-of -Flights/Time-of Flights Mass Spectrometry
mg	milligram
ml	mililiter
mM	miliMolar
MOWSE	Molecular Weight Search Engine
MUP	Major Urinary Proteins
MW	molecular weight
MYH6	Myosin heavy chain 6
NCD	noncommunicable disease
ND	not detectable
nm	nanometer
NS	nitrocellulose
PAGE	polyacrylamide gel electrophoresis
PDE1	Phosphodiesterase 1
pI	isoelectric point
PLG	Plasminogen
PMF	peptide mass fingerprint

ppm	parts per million
PROM1	Prominin 1
PUFA	Polyunsaturated Fatty Acids
PVDF	Polyvinylidene difluoride
RBP4	Retinol Binding Protein 4
RCT	reverse cholesterol transport
RT	room temperature
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
SELDI-ToF	Surface Enhanced Laser Desorption / Ionisation-Time of Flight
SHR	spontaneous hypertensive rat
SUMO3	Small Ubiquitin-Related Modifier Like 3
TBS	Tris buffer saline
TBST	Tris buffer saline-Tween
TC	total cholesterol
TEMED	tetramethylethylenediamine
TFA	triflouroacetic acid
TG	triglycerides
TPM2	Tropomyosin 2
TTR	Transthyretein
Vh	Volt-hour
VLDL	very low-density lipoproteins
W	Watt

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Cardiovascular disease (CVD), particularly coronary heart disease (CHD), is the leading cause of deaths in Malaysia for both gender according to Malaysia Clinical Practice Guideline (CPG) Management of Dyslipidemia (Ministry of Health Malaysia *et al.*, 2011). Factors that may contribute to this acute event include hypercholesterolemia, smoking, an elevated blood pressure and inflammation. In order to prevent future increases in sickness and disability of this noncommunicable disease (NCD), there is a need to tackle the challenges through the involvement of Research and Development sectors. The Malaysia Ministry of Health has committed enormous funds to address the issue of this NCD throughout the country as reported in Country Health Plan: 10th Malaysia Plan 2011-2015 (Ministry of Health Malaysia, 2010) and Malaysia National Health and Morbidity Survey (NHMS) 2015: Non-communicable diseases, risk factors and other health problems (Aris *et al.*, 2015).

The popularity of herbal medicine had emerged in various countries as an alternative to treating CVDs and other chronic diseases (Tachjian *et al.*, 2010). *F. deltoidea* has been used as alternative traditional medicine in some Asian countries and currently believed to have pharmacological properties that may help in reducing high cholesterol level (Kalman *et al.*, 2013; Taufik *et al.*, 2005). This plant is said to have been originated from Southeast Asia, Borneo and the Philippines (Brickell & Zuk, 1997). In Malaysia, this plant is popular in the east coast of Kelantan, the northern states of Kedah and Perlis and can be found in Sabah and Sarawak too. It is commonly known as Mas Cotek, Serapat Angin, Telinga Beruk, Emas Cotek, Sempit-sempit, mistletoe fig and many other names.

Several scientific studies had been carried out on *F. deltoidea* and have successfully demonstrated its potentials as antidiabetic (Aminudin *et al.*, 2007; Misbah *et al.*, 2013), anti-hypertensive (Razali *et al.*, 2013), antinociceptive (Sulaiman *et al.*, 2008),

anti-inflammatory (Abdullah *et al.*, 2009), antimelanogenic (Oh *et al.*, 2011), and photo-toxicity (Hasham *et al.*, 2013). This plant also has the ability to assist in uterine contraction (Amiera *et al.*, 2014) and possessed antioxidant activities (Hakiman & Maziah, 2009). *F. deltoidea* is also listed as ten commonly available indigenous medicinal plant in Malaysia consumed traditionally for its inexpensive, safety and vital effects on blood glucose and lipid levels (Sekar *et al.*, 2014).

Proteomics in CVD research has been extensively applied in discovering novel associated biomarkers and to elucidate mechanism involved (Shen *et al.*, 2014; Van Eyk, 2011). 'Proteomics' is focused on protein study of a cell or tissue to determine the protein identity, abundance as well as its biological functions (Thadikkaran *et al.*, 2005). Historically, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is one of the most widely used electrophoretic techniques for separation of complex mixtures of proteins in serum and plasma based on isoelectric point and molecular weight (Edwards *et al.*, 1979; Ivano Eberini *et al.*, 2000). The core combination in earlier proteomic study integrated isoelectric focussing (IEF) and SDS PAGE in protein separation and detection. Differential analysis of 2D gels were performed using image analysis software "Progenesis Samespots" to determine significant proteins with altered abundances under different conditions. The higher throughput with increased speed and automation development in proteomic technology, had subsequently enhanced quantification of proteins in a complex sample and protein identification using Matrix Assisted Laser Desorption / Ionisation-Time of Flight / Time of Flight (MALDI-ToF/ToF) mass spectrometry.

Adnan and Othman (2012) revealed the extensive use of plants for medicinal purpose since it was well thought-out as a healthy source of life. However, there have been no studies undertaken to comprehensively identify changes of serum proteome of hypercholesterolemic subject upon treatment with *F. deltoidea*. Despite numerous

literature has been published on pharmaceutical properties of the plant, little is known about its efficacy on anti-hypercholesterolemia activities. This study aims to characterize the serum proteome of the experimental animal using a 2D-GE approach combined with protein identification employing MALDI-ToF/ToF MS methodology. The identification of these altered proteins may help in understanding the mechanism involved in eliminating the increased cholesterol level that is exerted by *F. deltoidea*. The development of this study may be applicable in finding alternative treatments as well as preventions for CVD.

Objectives of study

a. To determine the altered profile of serum proteome in response to *F. deltoidea* treatment in hypercholesterolemic animals.
b. To identify proteins that are associated with hypercholesterolemia using relative quantitative proteomic techniques, such as 2D-GE and MALDI-ToF/ToF MS.
c. To correlate the changes of protein dysregulation that were observed to explain the biological process of disease progression and treatment response following the use of *F. deltoidea*.

CHAPTER 2: LITERATURE REVIEW

2.1 *Ficus deltoidea*

Ficus deltoidea, the scientific name of traditionally known Mas Cotek from family *Moraceae* is a kind of large or small tree (shrub) with aerial roots that grow as epiphytes. This medicinal plant differentiates into male and female species with a range of leaf shapes in accordance to its habitat. However, this male-female differentiation is not indicative of the plants gender; but more of the leaf and fruit morphologies. Female Mas Cotek has oval or round leaves with black spots at its lower surface. The leaves also bigger and thicker compared to the male Mas Cotek. Also, the leaf vein looked clearer compared to male Mas Cotek. It is recognized as Mas Cotek for its fine golden coloured spots on the upper surface of the leaves. On another note, distinguishing this plant as male-female is no longer favoured by the researchers; they are more into identifying them based on morphology and variety.

F. deltoidea contains five major phytochemicals that are essential in the human body system such as flavonoid, tannins, triterpenoids, proanthocyanins and phenols. All of these compounds can be found in different parts of the plant like leaves, stems and fruits. Ancient folks either consumed it as decoction or eaten raw. On the other hand, *F. deltoidea* are commercially available in the form of tea bags, coffee, massaging oil, formulated capsules and cordial juice throughout Malaysia (Ramamurthy *et al.*, 2014).

F. deltoidea as shown in Figure 2.1 had been scientifically reported to have antidiabetic potentials. Both the leaves and fruits aqueous extracts were shown to be able to reduce blood glucose level and inhibit the activity of α-glucosidase (Aminudin *et al.*, 2007; Misbah *et al.*, 2013). Water and methanolic extracts of *F. deltoidea* exhibited antihypertensive effects via different pathways in spontaneously hypertensive rats (SHRs). The water extract involves in endothelium-derived nitric oxide pathway, but not the prostacyclin pathway and the methanolic extract involves in nitric

oxide/cyclooxygenase pathway (Razali *et al.*, 2013). Besides being known as potential antidiabetic and antihypertensive agents, this plant has been reported to have potential in reducing total cholesterol, LDL-cholesterol and lower the risk of cardiovascular disease by decreasing the atherogenic index (LDL/HDL ratio) and increasing the percentage of HDL/total cholesterol ratio (Hadijah *et al.*, 2004). Kalman and colleagues (2013) revealed that the potential of *F. deltoidea* not only in lowering the glycemic level, but also lipid levels in human subjects with pre-diabetes. Another study performed using an aqueous extract of *F. deltoidea* leaves at different dosages assessed in several *in vivo* experimental subjects demonstrated potential antinociceptive activity (Ahmata *et al.*, 2008). Laboratory study conducted using *F. deltoidea* extract administered into female animal scientifically show the capability of this uterotonic plant in uterine contractions (Salleh & Ahmad, 2013).

Figure 2.1: *Ficus deltoidea* plant

Images are personal collection of Dr Norhaniza bt Aminudin

2.2 Cardiovascular disease

Cardiovascular diseases (CVD) are the major cause of mortality and morbidity worldwide and should be the focus of prevention efforts. The World Health Organization (WHO) reported that 17.3 million people (30% of global deaths) died from CVD in 2008 (Alwan *et al.*, 2011). It is expected to increase to 23 million death by the year 2030, mainly from heart disease and stroke (Mathers & Loncar, 2006). Current prevalence reported in National Health and Morbidity Survey 2015 on the other hand showed increasing trend of NCDs that contribute to 73% total mortality in Malaysia due to CVD.

Previous study carried out depicted that more than 75% of hospital admissions in diabetes subjects were complimentary to cardiovascular complications (Aronson, 2003). Interestingly, cardiovascular diseases are predominant human disease not only related to genetic, but also on diet-induced cholesterol abnormalities such as hypercholesterolemia, hypertriglyceridemia, HDL metabolism disease, and combined hyperlipidemias of which are more severe. Even in current lifestyles, diseases like hyperlipidemia, atherosclerosis, familial hypercholesterolemia, and diabetes are predominant in children too (Upadhyay, 2015).

Based on Global Atlas on Cardiovascular Diseases Prevention and Control (Mendis *et al.*, 2011), CVDs refer to the major heart disorders that include diseased vessels, structural problems, and blood clots (Table 2.1); which are strongly associated with health factors including behaviors, quality of health care, socioeconomic, physical environmental activities and inheritance (Table 2.2). CVD is listed as one of the major noncommunicable diseases (NCDs) beside other chronic disorders like chronic respiratory diseases, cancers and diabetes (World Health Organization, 2013). As defined by WHO, NCDs or chronic diseases are long term non-infectious and slow progressing diseases.

Table 2.1 Types of cardiovascular disease. Reprinted permission granted by World Heart Federation.

Types	Descriptions	Disease
1. Rheumatic heart disease	Invasion of rheumatic fever caused by streptococcus which is common in children. Inflammation of the heart may lead to permanent damage to the heart valves, thus weakened the heart muscles.	• Rheumatic heart disease • Valvular heart disease
2. Hypertensive heart disease	Heart problems, mainly due to high blood pressure.	• Aneurysm • Atherosclerosis • High blood pressure (hypertension) • Peripheral arterial disease
3. Ischemic heart disease	A heart condition that affects the blood supply to the heart due to thinning of the coronary arteries. The narrowing mainly caused by fatty deposits in the arteries.	• Angina • Atherosclerosis • Coronary artery disease • Coronary heart disease • Heart attack
4. Cerebrovascular disease	Brain impairment due to disease of blood vessels, particularly the arteries to the brain.	• Atherosclerosis • Cerebral vascular disease • Stroke • Transient ischemic attacks (TIA)
5. Inflammatory heart disease	Inflammation of the heart muscles surrounding the heart due to infection of bacteria/virus or other common non-infectious causes.	• Atherosclerosis • Cardiomyopathy • Pericardial disease • Valvular heart disease
6. Other		• Congenital heart disease • Heart failure

Table 2.2: Risks that contribute to acute CVDs. Reprinted permission granted by American Heart Association.

	Risk factors
Modifiable	**Behavioral risk factors**: tobacco use (or second-hand smoker), physical inactivity, unhealthy diet (rich in salt, fat and calories) and harmful use of alcohol
	Metabolic risk factors: high blood pressure (hypertension), high blood sugar (diabetes), high blood cholesterol (hyperlipidemia), overweight and obesity.
Non-modifiable	Poverty, low educational status, aging, gender, genetically inherited disposition, poor mental health (stress /depression)

2.2.1 Hypercholesterolemia

Hypercholesterolemia (also known as dyslipidemia or high blood cholesterol) has emerged to correlate well as the main risk factor of cardiovascular disease in Malaysia. Lim e*t al.* (2000) in the National Health Survey conducted in 1996 reported the prevalence of hypercholesterolemia was 20% among individuals age 30 years old and above. However, the prevalence of hypercholesterolemia progressively increased to 47.7%, which include undiagnosed individuals with hypercholesterolemia (Aris *et al.*, 2015).

Hypercholesterolemia with raised total cholesterol (TC) and/or LDL-cholesterol or non-HDL-cholesterol (defined as the subtraction of HDL-cholesterol from total cholesterol) in the blood, is also often referred to as dyslipidemia, to encompass the fact that it might be accompanied by a decrease in HDL-cholesterol or an increase in triglycerides. Dyslipidemia is classified as serum TC, LDL-cholesterol, triglyceride, apolipoprotein B (Apo B), or lipoprotein(s) concentrations above the 90th percentile, or

HDL-cholesterol or apolipoprotein A1 concentrations below the 10th percentile for the general population (Heiss *et al.*, 1980). The elevated HDL levels denote efficient reverse cholesterol transport (RCT) and thus prevent atherosclerosis.

Dietary consumption containing high fat or cholesterol content may lead to both hypercholesterolemia and hypertriglyceridemia which are the key prognosis for CVD (Reiner & Tedeschi-Reiner, 2006). For that reason, high fat diets are undeniable a risk factor for coronary heart diseases, insulin resistance and obesity accompanied by systemic inflammation, which are the features of metabolic disorder (Lopez-Garcia *et al.*, 2005). The increased level either cholesterol or low density lipoprotein (LDL) is mainly a progress indicator of atherosclerotic lesions (Harrison *et al.*, 2003).

2.2.2 Atherosclerosis

Atherosclerosis is an inflammatory condition leading to the development of ischemic heart diseases, cerebrovascular diseases and peripheral vascular disease. One of the most important initial events in the development of atherosclerosis is the buildup of lipids within coronary arteries which mostly macrophages transformed monocytes that engulf oxidized-LDL to become foam cells or fat laden macrophages (Moore & Tabas, 2011). Figure 2.2 demonstrates the accumulation of plaque in the artery at the site of a tear in the lining of the vessel. The common risk factors for the development and aggravation of atherosclerotic cardiovascular disease included hypercholesterolemia, hypertension, smoking and diabetes mellitus; with or without family history, is related to pathophysiological vascular phenotype (Leopold & Loscalzo, 2009). However, Hansson and colleague (2006) emphasized the substantial role of hypercholesterolemia complementary with immune mechanisms in the pathogenesis of atherosclerosis.

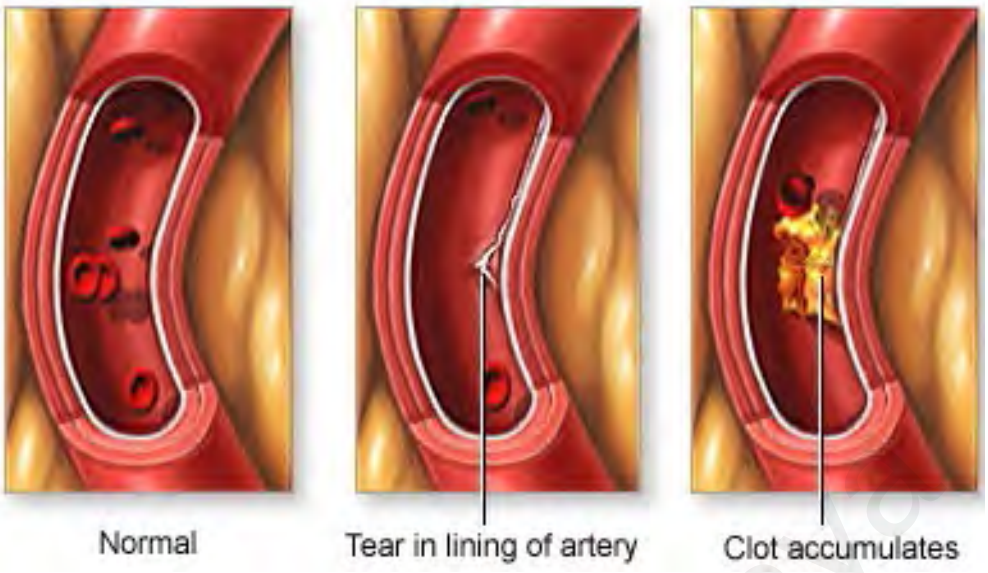

Figure 2.2: Development of plaque accumulation in a coronary artery.

Accessed from *http://www.healthcentral.com/encyclopedia/adam/progressive-build-up-of-plaque-in-coronary-artery-4007086/* on 13 Sept 2015

Atherosclerosis causes coronary heart disease because of the plaque buildup in the lining of the coronary arteries. These plaques which is made up of fat, cholesterol and other substances narrowed the arteries and obstruct the blood flow to the heart and other organs. Heart attack or stroke occurs when the plaque completely blocks the artery with blood clots.

2.2.2.1 Myocardial infarction

Myocardial infarction (MI) or mostly known as heart attack is a condition when the coronary artery is blocked, causing blood flow stops to the heart. Prolonged deprivation of oxygen to a portion of the heart muscle (myocardium) provokes the death of muscle cells (infarct) due to the blocked artery. The cause of MI often associated with atherosclerosis, a buildup of cholesterol plaque results in narrowing and hardening of the artery (Bolooki & Askari, 2010). Further narrowing of arteries may be due to the blood clot formation when the plaque lining ruptured. Recent proteomic findings by Haas and colleagues (2011) showed distinctive alteration of haptoglobin and its isoforms from MI sample group. Hypercholesterolemia is apparently the leading risk factor for MI. In sharp

contrast, prolonged hypercholesterolemia in animal studies further augmented myocardium injury (Girod *et al.*, 1999; Mączewski & Mączewska, 2006).

2.2.3 Lipoproteins

The hypothesis of lipid relevance to atherosclerosis in general predicated on the role of Apolipoprotein (Apo) A and E lipoproteins in the prevention of atherosclerosis, whereas Apo B lipoproteins, which promote it. Cholesterol is transported in the blood system via three types of lipoproteins: HDL (high density lipoproteins); LDL (low density lipoproteins) and VLDL (very low density lipoproteins).

HDL particles are amongst the five main lipoproteins, which are considered the good cholesterol. Apo A1 and Apo AII are abundantly found apolipoproteins in HDL particles (Kontush & Chapman, 2006; Rye & Barter, 2014). Other apolipoproteins found in HDL include Apo AIV, Apo AV, Apo CI, Apo CII, Apo CIII, Apo CIV, Apo D, Apo E, Apo F, Apo H, Apo LI, Apo M, and Apo O (Dahlbäck & Nielsen, 2006; Irshad & Dubey, 2005; Robinson & Freedman, 2016; Yu *et al.*, 2012). These particles help to eliminate plaque deposits on the artery wall, reverse the blockages and transfer cholesterol back to the liver. The distribution of the cholesterol into the bile by the liver subsequently drenches out with the bowel movement. In other words, it executes as arteries vacuum cleaner. In the previous study carried out by Heinecke (2009), HDL has been proposed for its cardioprotective effects in removing cholesterol from artery walls and the plausible anti-inflammatory properties inhibiting atherogenesis (Barter *et al.*, 2004). Despite the fact that HDL exhibits cardioprotective effects, it is dependable on the types of HDL particles produced *in vivo*. Research conducted on animals had found modification in proteins engaged in HDL metabolism significantly promote atherosclerosis, despite elevated HDL-cholesterol serum levels (Trigatti *et al.*, 1999).

Contrary, the LDL particles transporting cholesterol into body cells. Cholesterol is essential in constructing new body cell membrane which is waterproof and does not penetrate fat. Therefore, small LDL particles penetrate the artery wall more readily, causing damaging plaque formation compared to larger LDL particles. Apo B is the major apolipoprotein found in LDL particles and exists as Apo B48 (chylomicrons) and Apo B100 (LDL and VLDL) (Contois *et al.*, 2009). LDL is often referred as "bad" cholesterol for the prolonged elevated levels possibly will damage the heart and the blood arteries.

These lipoproteins however, are not cholesterol, but literally could cause heart disease. Lipoproteins are the carriers that transport cholesterol through the blood. Some lipoproteins cause cholesterol to penetrate the artery wall more easily, inflicting the artery to clog. These lipoprotein plaques become inflamed and rupture, causing a heart attack. Lipoproteins are available in different sizes and types.

Human diseases associated with lipid carried by very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) can be classified into dyslipidaemia, coagulation disorders, atherosclerosis and vascular disease. The human plasma VLDL and LDL do not only act as lipids carriage throughout the body, but also a method for protein transfer involved in inflammation and thrombosis from the site of synthesis to effector locations.

2.3 Hypercholesterolemia classification of lipid disorder

Familial hypercholesterolemia was first classified according to Fredrickson classification based on raised lipoprotein pattern on the lipid electrophoresis. Table 2.3 showed the classification adapted by WHO in distinguishing derangement in lipid profile.

2.4 Laboratory diagnosis

Fasting lipid profile, FLP (also known as lipoprotein profile) is used to diagnose the cholesterol levels in the blood. FLP gives the information about total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerides (TG). LDL is calculated based on Friedewald *et al.* (1972) calculation.

[LDL-chol] = [Total chol] - [HDL-chol] - ([TG]/2.2) (Unit: mmol/L)

However, Friedewald formula is not relevant when TG concentrations exceeds 4.6 mmol/l, the presence of chylomicrons and in patients with dysbetalipoproteinemia (type III hyperlipoproteinemia). Table 2.4 show normal blood lipid profile targets.

Table 2.3: Fredrickson classification (Fredrickson,1971). Reprinted permission granted by American College of Physicians.

Type	Elevated	Known case of / deficiency
I	Chylomicrons	Lipoprotein lipase deficiency, apolipoprotein C-II deficiency
IIa	LDL	Familial hypercholesterolemia, polygenic hypercholesterolemia, nephrosis, hypothyroidism, familial
IIb	LDL and VLDL	Familial combined hyperlipidemia
III	ILDL	Dysbetalipoproteinemia
IV	VLDL	Familial hypertriglyceridemia, familial combined hyperlipidemia, sporadic hypertriglyceridemia, diabetes
V	Chylomicrons and VLDL	Diabetes.

***LDL** = low-density lipoproteins; **TC** = total cholesterol; **TG** = triglycerides; **ILDL** = intermediate-density lipoprotein; **VLDL** = very low-density lipoproteins;

Table 2.4: Desirable lipid profile levels (Grundy *et al*.,2002). Reprinted permission granted by Elsevier B.V.

Lipids	Fasting values (mmol/l)	Interpretation
Total serum cholesterol	< 5.17	Desirable
	5.17-6.18	Borderline high
	≥ 6.18	High
Triglycerides	1.69-2.25	Borderline high
	2.26-5.63	High
HDL cholesterol	< 1.03	Low
	≥ 1.55	High
LDL cholesterol	> 1.55	Optimal
	1.55-3.34	Near optimal
	3.35-4.11	Borderline high
	4.12-4.89	High
	> 4.9	Very high

2.5 Prevention and treatments

2.5.1 Pharmacologic interventions

2.5.1.1 Drug therapy

To date, pharmacological intervention in cardiovascular treatment with statins is the most successful therapeutic approach in reducing cardiovascular risk in both primary and secondary prevention. Medical subject heading (MeSH) defines 'primary prevention' aimed towards delaying or preventing the onset of cardiovascular disease, whereas 'secondary prevention' seeks for early disease detection and imposed interventions to retard recurrence of the disease. List of some other cholesterol drugs therapy and its functions were tabulated in Table 2.5.

Statins or also known as 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA reductase) inhibitors are the drug of choice for LDL cholesterol-lowering agent (Ray *et al.*, 2014). Statins are used as first-line hypercholesterolemia therapy (Ostadal *et al.*, 2005). It is also a common drug for prevention and treatment of kidney disorder, hypertension and heart failure (Ludman *et al.*, 2009). Besides lowering cholesterol levels, statins have pleiotropic effects which include improved endothelial function, enhance the atherosclerotic plaque vulnerability, prevent oxidative stress by reducing inflammation amount and damage done to the cells through oxidation, and also to prevent thrombosis (Liao & Ulrich, 2005). Thus, various studies had been carried out and established on statins efficacy as cardioprotective drugs (Brugts *et al.*, 2009; Tonelli *et al.*, 2011). Statins disrupt production of cholesterol by blocking HMG-CoA reductase inside the liver cells. Hence, less cholesterol is released into the bloodstream. Some statins delay inflammatory process in the vessel wall inside an artery and produces strong and rigid stable plaque. There are two classes of statins, natural statins (e.g. Lovastatin@Mevacor, Pravastatin@Pravachol, and Simvastatin@Zocor) and synthetic statins (e.g. Atorvastatin@Lipitor, Fluvastatin@Lescol). All these commercial drugs are efficacious on cardiovascular disorders emerge from a reduction in cholesterol anabolism and thus decrease plasma cholesterol concentrations.

The newer drug in cholesterol lowering consequence prevents absorption of cholesterol into the intestines. Ezetimibe@Zetia is the first selective cholesterol lowering drug introduced in the year 2002 to treat hypercholesterolemia and inherited lipid aberration. Ezetimibe can be used alone or combined therapy with other statins to improve levels of total cholesterol, low-density lipoprotein cholesterol, non-HDL cholesterol, and apolipoprotein B.

Fibrates and Niacin (nicotinic acid) may be considered for increasing HDL and reducing triglycerides (TG) after LDL treatment goal has been achieved. Some

individuals may require combination therapy to achieve lipid target goals. Gemfibrozil@Lopid and Fenofibrate@Lipanthil Penta are commonly available drugs use.

Bile acid resins are another source of LDL-lowering drugs, where the substances bind with bile acids that contain cholesterol in the intestines and are then eliminated in the stool. The liver responds to make more bile and consequently more cholesterol are removed. Bile acid resins are sometimes combined with statin for patients with heart disease to further increase cholesterol reduction. Cholestyramine @ Questran and Colestipol @ Colestid are the two main bile acid resins currently available. These selective cholesterol absorption inhibitors are most effective at reducing LDL cholesterol and thus improving HDL cholesterol but moderate outcome on lowering triglycerides.

The summary of the metabolic effects on lipoproteins after lipid lowering drugs treatments are shown in Table 2.6.

2.5.1.2 Supplements

Marine-based polyunsaturated fatty acids (PUFA) and fatty acid ethyl esters are polyunsaturated fat that body derived from fish oils that are synthetically altered and purified. Both these Omega-3s and Omega-6s are essential fatty acids (EFAs) that are used as supplements to maintain good health and can be sourced from cold water fish which comprised of tuna, salmon and mackerel. These supplements are mainly consumed by individuals to reduce the content of triglycerides in the blood. However, individuals with seafood allergies may have severe adverse responses upon consuming this supplement. These fatty acids not only derived from cold water fishes, but also exist in dark green leafy vegetables, flaxseed oils and some vegetable oil too. These supplements found to benefit as anti-inflammatory, anti-blood clotting as well as in lowering blood cholesterol and triglycerides (Ellulu *et al.*, 2015).

Table 2.5: Recommendations for drug therapy for hypercholesterolemia (Ministry of Health *et al*., 2011). Reprinted permission granted by Ministry of Health.

Medications	Functions
Statins	Reduction of LDL-C. Increase dose till target levels are achieved or till tolerated.
Fibrates	As monotherapy to increase HDL-C and/or lower TG in individuals with mildly raised LDL-C As part of combination therapy with statins to increase HDL-C and lower TG after LDL-C target is achieved or almost achieved.
Nicotinic Acid	As monotherapy to increase HDL-C and/or lower TG in individuals with mildly raised LDL-C As part of combination therapy with statins to increase HDL-C and lower TG after LDL-C target is achieved or almost achieved
Ezetimibe	As an addition to statin if target LDL-C is not achieved As monotherapy in statin intolerant individuals

Table 2.6: Metabolic effects of lipid lowering agents on lipoprotein (Levy *et al*., 1993). Reprinted permission granted by Elsevier B.V.

Agents	LDL	HDL	VLDL
Bile acid sequestrants	⬆ Clearance	(Modest ⬆)	⬆ Secretion
Niacin	⬇ Synthesis	⬆ Clearance	⬇ Synthesis
Fibric acid derivatives	(Modest ⬇)	⬆ Synthesis	⬆ Clearance
HMG-CoA reductase inhibitors (statins)	⬆Clearance	(Modest ⬆)	⬆ Clearance ⬇ Synthesis*

* with Atorvastatin

⬆ Increase

⬇ Decrease

2.5.2 Lifestyle changes

Sedentary lifestyles complimented with the change of dietary patterns have attributed to the global prevalence of cardiovascular related risk factors. In the current study reported by Ghazali *et al.* (2015), hypercholesterolemia incorporated with other lifestyle risk factors statistically still contributed to this mortal chronic disease. Several reports had been published acknowledging the importance of lifestyle intervention by integrating state-of-art technologies in combating unhealthy behaviors like smoking beside maintaining healthy weight by proper food intake and active lifestyles; as well to improve cardiovascular indicators like blood pressure, body mass index, lipid profile and glucose level (Burke *et al.*, 2015; Eng, 2001). Abreast with these affordable gadgets like mobile phones, wearable devices, tablets and other health technologies, intervention in behavioral health is possible where assessment of health parameters can be done. In

addition to this, accessing and sharing health information is more conducive which enable prompt health decision making and indirectly engage individuals in positive health behaviors and practices (Ahern *et al.*, 2006; Gustafson *et al.*, 2002).

2.5.3 Medicinal plants as an alternative treatment

Medicinal plants are among the major and important group of crops (Rehm & Espig, 1991) which have been long standing use in delaying and healing of diseases (Wiart, 2002). In our current study using *F. deltoidea* plant, several studies had been conducted to investigate the potent medicinal properties as claimed for its usage throughout the centuries in prevention and treatment of diseases. As reported by Musa in 2005, *F. deltoidea*'s leaf decoction is traditionally consumed for general health. The decoction is used for post-natal treatment and to treat leucorrhoea disease. Several studies on this *Ficus* species reported its other medicinal properties such as being anti-inflammatory, anti-diabetic, anti-bacterial, anti-diarrhoea and anti-ulcer (Jabit *et al.*, 2005).

There has been a paradigm shift of interest toward Eastern medicines as alternative approaches in prevention and therapeutic treatment including for cholesterol lowering properties. There are about 7000 natural drug compounds being utilized in modern medicine in which these medicines had been practiced over the centuries (Eddouks *et al.*, 2014). In facts, many plant extracts have been reviewed and found to possess cardioprotective and cholesterol lowering properties (Fugh-Berman, 2000; Tappia *et al.*, 2013).

Laboratory study performed by Zar *et al.* (2012) revealed that the extract of a locally known medicinal plants named Kadok (*Piper sarmentosum*) extract may delay and improve cardiovascular system in diabetic rats. *Averrhoa bilimbi Linn.* (Belimbing buluh / belimbing asam) leaves originated from Malaysia and Indonesia has

hypoglycemic, hypotriglyceridemic, anti-atherogenic, and anti-lipid peroxidative efficacies (Pushparaj *et al.*, 2000). In the recent study carried out, the extract of *Gynura procumbens* (Sambung nyawa) possessed antidiabetic and antihyperlipidemic values (Zhang & Tan, 2000). The leaves are traditionally consumed by the ancient folks to reduce lipid abnormalities, stabilize glucose level, to control high blood pressure, as well as for cancer management. Another plant of interest in the medicinal plant research is *Hibiscus Rosa-sinensis* (locally known as Bunga Raya) as it revealed the potential of lowering total cholesterol (22%) and triglycerides (30%) and subsequently increased HDL-cholesterol by 12% (Sachdewa & Khemani, 2003). Others available evidence based reports published on medicinal plants which exert lipid lowering include licorice root (*Glycyrrhiza glabra*), Asteraceae (*Achillea wiohelmsii*), Silymarin (*Silybum marianum*) seed, *Terminalia arjuna* tree bark, and Berberine (*Coptis chinensis*). These therapeutic plants in fact worth plenty of natural substances in lowering blood cholesterol.

The usage of these herbal and medicinal plant products are currently consumed not only as a basic health line service, but also in areas where modern medicine is available (World Health Organization, 1998). In addition, WHO also reported higher demand (80% of the world population) of medicinal plants as an alternative treatment. With the effort from WHO, more pharmaceutical industries are now attracted to contribute funds or grants for research focusing biochemical characteristics of medicinal plant and pharmacological studies of the drug efficacy (Cheung, 2011). Thus, integrating traditional medicine into a national health care system has much to offer besides facilitating the efficient use of domestic medicinal plants.

2.6 Proteomics techniques

As cardiovascular remains the leading cause of death globally, protein identification by proteomics approach may aid in early diagnosis and monitoring of the

disease (von Hagen, 2008). The current trend in proteomics technologies and sample preparation have enabled in-depth analysis of many low abundant proteins/peptides, unlocking a large pool of potentially clinically valuable compounds (Xiao & Wong, 2010). Despite the move towards protein/peptide separation analysis using LC-MS, 2-dimensional gel electrophoresis (2D-GE) is still a very popular protein separation technique. Seibert *et al.* (2004) cited that proteomic analysis had become a valuable tool in mapping protein profiles to determine the high or low abundance of the proteins in a subject. Several researchers have reported the potential alternative of 2D-GE coupled with the high-throughput surface-enhanced laser desorption ionization (SELDI) technology to determine differentially expressed protein profiles by comparing the peak intensities (Fliser *et al.*, 2007).

2.6.1 Two dimensional gel electrophoresis (2D-GE)

The two dimensional gel electrophoresis or 2D-GE was first developed and established by O'Farrell (1975) and Klose (1975). It was well accepted and adopted by protein researchers in the study of protein complexity and this 2D-GE technique is globally applied in separating proteins according to their isoelectric point (pI), molecular weight, solubility and its relative protein abundance. Furthermore, this high resolution 2D-GE is able to resolve approximately 5000 proteins at once (≈2000 proteins routinely), and can detect less than 1 ng of protein per spot subjected to the gel size and pH gradient applied (Görg *et al.*, 2004).

The first dimension is the first step in separating proteins based on charge or isoelectric point, called isoelectric focusing (IEF). The isoelectric point is the pH at which the net charge of the protein molecule is neutral. Proteins varied in isoelectric points and achieved when it reached its steady state during iso-focusing. The range of the gradient determines the resolution of the separation. The use of commercialized ready-made IPG

dry strips is currently the choice for most laboratories for iso focusing in the first dimension electrophoresis as it offers easier handling, better reproducibility and higher throughput. Bjellqvist *et al.* (1982) also explained the advantages of pH gradient stability despite extended focusing runs using IPG strip. Samples can either be applied by cup-loading or by in-gel rehydration.

The selected IPG strips is about 3-mm wide and varied up to 24 cm long with a broad range of pH 3-10, medium (*e.g.* pH 4-7), narrow (*e.g.* pH 4.5-5.5), and/or ultra-narrow (*e.g.* pH 4.9-5.3) to get an overview of a more complex proteome (Görg *et al.*, 2004). Broad range IPG strips provide overview of spot distribution whereas narrow-range IPG strip allows greater spatial separation over a narrow pH range to increase the spots resolution. IPG strips varied in length depending to requirements. Shorter strips normally applied for sample screening and method optimization before switching to longer strips for better separation and resolution despite longer running time.

Samples of interest can be separated parallel up to 12 gels in a preset temperature to minimize the separation variations between individual gels. The dehydrated commercialized IPG strips need to be rehydrated to its original thickness at 0.5 mm upon use. Protein samples are prepared in/or diluted in rehydration solution and applied to IPG strip in the rehydration tray for subsequent rehydration for at least 12 hours prior to IEF. This step is highly recommended for efficient sample dissemination on the entire strip in the rehydration tray (Rabilloud *et al.*, 1994).

The rehydration buffer generally contains chaotropes, detergents (surfactants), reducing agents, ampholytes and tracking dye. These components are responsible to maintain protein solubility and allow tracking of the separation. Urea and thiourea are commonly used as chaotrope for 2D-GE. However, urea (8 M or up to 9.8 M) is necessary for sample solubility with / without 2 M thiourea by disrupting hydrogen links leading to protein unfolding and denaturation. Zwitterionic detergents like CHAPS or CHAPSO are

widely use for 2D-GE sample preparation for its compatibility with isoelectric focusing separation. Detergents are meant for breaking the hydrophobic interactions to enhance the solubility of proteins.

The IEF is the most critical step of the 2D-GE process. The proteins must be solubilized without charged detergents, usually in high concentrated urea solution, reducing agents and chaotrophs. It is essential to achieve low ionic strength conditions before the IEF to obtain high quality data. Since samples differ in their ion content, it is necessary to optimize the IEF buffer and the electrical profile to each type of sample.

After IEF, IPG can be stored preferably at -80^{o}C or the second dimension can be performed immediately. The proteins in the focused IPG strips are uncharged because they are at their pI and so they will not move into the SDS-PAGE gel. The IPG strips are then equilibrated with SDS buffer in the presence of urea, glycerol, dithiothrietol (DTT) and iodoacetamide (IAA), and applied onto horizontal or vertical SDS gels in the second dimension. The reductant agent DTT cleaved the disulfide bridges and alkylation of cysteine residues with IAA and the proteins are equilibrated with sodium dodecyl sulfate (SDS) so they become negatively charged for latter 2D-GE. SDS also linearized the proteins via strict separation by molecular weight. Anderson *et al*. (2001) stated that resolution obtained is not lost when the first-dimensional gel strip is mounted on top of the polyacrylamide gel during second dimension electrophoresis. A tracking dye like bromophenol blue is normally added to the sample buffer to track the progress of electrophoresis run and to make sure that proteins do not electrophorese out of the gel into the buffer tank.

After electrophoresis, the separated proteins are visualized by staining with silver stain, organic or fluorescent dyes, or autoradiography (or phosphor-imaging) of radiolabelled samples. Limitations (not only of 2D-GE, but of almost all current proteome analysis technologies) remain in the field of the analysis of very hydrophobic and/or

membrane proteins, as well as in the lack of highly sensitive and reliable techniques for detection and quantitation of low abundant proteins.

Yet, the recent introduction of more powerful chaotropes and detergents such as thiourea and sulfobetaines, as well as the advent of sensitive fluorescent dyes, in particular of dual label techniques for the visualization of differentially expressed proteins (Ünlü *et al.*, 1997) have improved the situation.

This coupling step involved first dimension (1D) or IEF, which separates the protein accordingly to its isoelectric point and subsequently 2D-GE where proteins are separated effectively accordingly to its molecular mass. Both separations are carried out in polyacrylamide gels containing sodium dodecyl sulfate (SDS). Hence, 2D-GE has contributed the birth of proteomics.

2.6.2 Protein identification by mass spectrometry: MALDI-ToF/ToF

The soft ionization technique matrix-assisted lasers desorption/ionization (MALDI) was primarily introduced by M. Karas, F. Hillenkamp and K. Tanaka in 1980s. MALDI is suitable in generating gas-phase ionization proteins and peptides for mass spectrometry detection. The time-of-flights (ToF) mass spectrometry (MS) analyzer, measure the acceleration of the ions by an electric field of known strength based to the flight times taken by the ions to travel in between two points. ToF/ToF is a tandem mass spectrometry method where two ToF MS are consecutively applied (Vestal, 2014). Protein identification using coupled MALDI-ToF/ToF mass spectrometer technology (Figure 2.3) had been extensively applied on 2D-GE gel spots after enzymatic digestion.

The commonly cited advantages of this technology include minimal sample volume, high speed measurement, accurate mass/charge (m/z) ratio, low cost, larger affinity surface and good reproducibility (Dreisewerd, 2014; Hidaka *et al.*, 2007).

Subjecting to the m/z ratio, lighter ions will reach the target earlier compared to heavier ions.

Matrix is an organic molecule with UV-absorbing capacity at laser wavelength. This matrix crystallizes together with the sample molecules and numbers of matrix molecules have to exceed those of the analytes. This solution is spotted onto a MALDI plate and air dried before analysis. The crystals are converted into the gas phase by the pulsed laser irradiation under vacuum conditions. The matrix absorbs the most of the energy of the laser and transfers the charge to the analyte. After all, α-cyano-4-hydroxycinnamic acid (CHCA) is considered as the gold standard in peptide analysis (Beavis *et al.*, 1992). Figure 2.4 shows commonly employed matrices to protein and peptide analysis structural formulas.

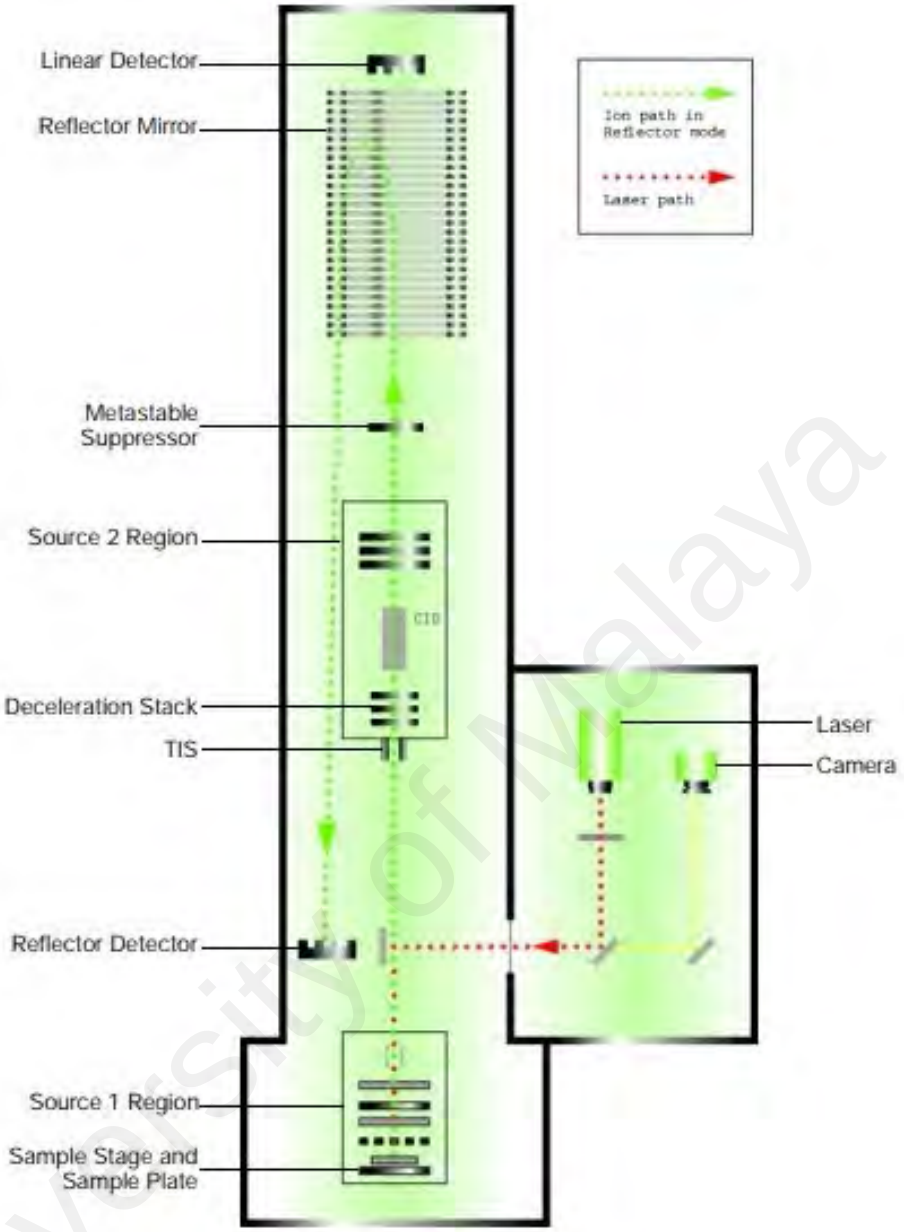

Figure 2.3: MALDI-ToF/ToF mass spectrometer. Reprinted permission granted by Institute of Tropical Disease, Universitas Airlangga

Accessed from http://itd.unair.ac.id/files/ABI/4800HardwareGuide.pdf on 13 Sept 2015

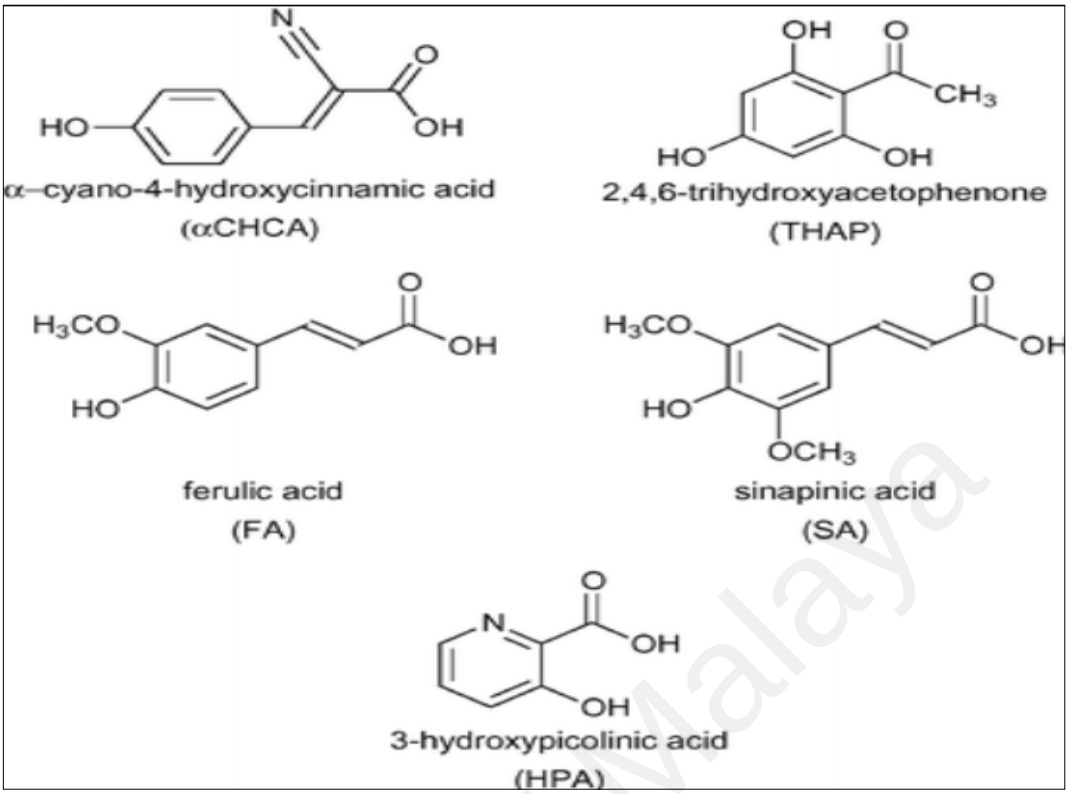

Figure 2.4: Commonly used matrices

2.6.3 Western blotting

Western blotting (also known as protein immunoblotting) had been routinely used in protein analysis for the past three decades. This procedure was initially introduced by Towbin *et al.* (1979) and later named by W. Neal Burnette (Burnette, 1981). The Western blot technique was used to detect the presence of specific proteins in a given sample and was frequently applied to validate the differential expression of proteins identified by MALDI-ToF/ToF MS. Detecting the spots with antibodies also validated the identity of the proteins determined using MALDI-ToF/ToF MS. The samples were resolved using 2D-GE as described in Section 3.7 and consequently cross-validated by Western blotting.

Western blotting procedure involves two phases: Protein transfer and protein detection on the membrane. The proteins are separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) where the electrical field moves

through the gel matrix before the protein is then transferred and immobilized onto solid membrane such as nitrocellulose (NS) or polyvinylidene difluoride (PVDF). PAGE is most commonly used analytical technique in characterizing and separating proteins to its molecular weights. The pore size of the gel is determined by the acrylamide to bisacrylamide ratio and acrylamide concentration. Polymerization of acrylamide-bisacrylamide monomers is induced by ammonium persulfate (APS). Tetramethylethylenediamine (TEMED) is added to promote polymerization. Briefly, the anionic detergent SDS denatures the protein structures and dissociates the disulphide bonding. Further heating to near boiling point for 5 minutes with the presence of reducing agent such as dithiothreitol (DTT) or beta-mercaptoethanol (BME), enhances denaturation process and reduces disulphide bonding. During SDS-PAGE, proteins of interests are separated accordingly to size. The rate migrations of SDS-treated proteins may differ, where lighter proteins with low molecular weight will navigate faster and farther to the bottom of horizontal gel compared to heavy molecular weight proteins.

Subsequently, in detection phase, adequate primary antibody is used to bind to the specific protein of interest and a conjugated horseradish peroxidase (HRP) secondary antibody is used to bind to the primary antibody. This is necessary because the HRP enzyme on the secondary antibody was made visible by catalyzing the reaction between two luminescence substrates.

CHAPTER 3: METHODOLOGY

3.1 Animal study materials:

- Albino Wistar-Kyoto-Rats (WKY) rats
- Carbon tetrachloride (CCl_4)
- Atorvastatin
- High cholesterol pellet
- Syringe and needles
- Syringe and oral gavage needle

3.2 Lists of chemicals / solvents / kits:

Protein content determination

Quick Start Bradford protein assay kits (Biorad)

2D electrophoresis

- 25% Glutaryldehyde
- 87% Glycerol (GE Healthcare)
- Acrylamide
- Agarose
- APS
- N,N'-methylenebisacrylamide
- Bromophenol blue
- CHAPS
- Drystrip cover fluid (GE Healthcare)
- Dithiothreitol (DTT)
- EDTA-Na_2H_2O (Titriplex)

- Ethanol
- Formaldehyde
- Glycine,
- IPG buffer
- n-butanol or t-butanol
- Paper wicks (GE Healthcare)
- sodium dodecyl sulphate (SDS)
- Silver nitrate
- Sodium thiosulphate
- Sodium acetate trihydrate
- Sodium carbonate
- Tetramethylethylenediamine (TEMED)
- Tris Base
- Urea
- Milli-Q water (sourced from Medical Biotechnology Laboratory, Faculty of Medicine, University of Malaya)

MALDI ToF/ToF MS

- α-cyano-4-hydroxycinnamic acid (CHCA)
- Acetonitrile (ACN)
- Ammonium bicarbonate
- DTT
- Formic acid
- Iodoacetamide (IAA)
- Potassium fericyanide

- Sodium thiosulphate
- Trypsin Gold (PROMEGA, EC: 3.4.21.4)
- Trifluoroacetic acid (TFA)
- C 18-ZipTips (Eppendorf)

Western blotting

- Nondairy fat milk
- β-mercaptoethanol (BME)
- Tween 20
- Nitrocellulose membrane
- Ponceau stain
- Sodium chloride
- Peroxidase Stain DAB Kit (Brown Stain) (Nacalai Tesque)
- Metal Enhancer for DAB Stain (Nacalai Tesque)
- BLUeye Prestained Protein Ladder (GeneDirex)
- Rabbit polyclonal Secondary Antibody to Chicken IgY H&L, ab31170 (ABCAM)
- Chicken polyclonal to Haptoglobin, ab117316 (ABCAM)
- Mouse monoclonal [G4E4] to Retinol Binding Protein RBP, ab24090 (ABCAM)
- Rabbit polyclonal Secondary Antibody to Mouse IgG H&L, ab6728 (ABCAM)

3.3 List of laboratory apparatus / equipments / software:

- 1D Ettan IPG phor 3 (GE Healthcare Biosciences)
- 2D EttanDalt six gel caster (GE Healthcare Biosciences)
- 2D EttanDalt six electrophoresis (GE Healthcare Biosciences)
- Heater (Thermolyne)
- Immobiline™ Drystrip Reswelling Tray (GE Heathcare Biosciences)
- IPGBox™ and Reswell Tray (GE Heathcare Biosciences)
- Image Scanner III (GE Healthcare Biosciences)
- MALDI plate (Applied Biosystems)
- MALDI-ToF/ToF mass spectrometer (Model: AB4800 Plus, Applied Biosystems)
- Microplate reader (Asys UVM 340)
- Microcentrifuge (Model: Micro 20 Hettich Zentrifugen)
- Orbital Shaker (Protech)
- Rotavapor R-114 (BUCHI, Switzerland)
- Milli-Q® Type 1 Ultrapure Water Systems (MERCK Millipore)
- SDS-Page gel set (Dual vertical mini gel unit Model: MGC-206, C.B.S Scientific Co.) including glass plates, spacer, comb and side rubber.
- Soxhlet apparatus (Favorit)
- Speed Vacuum Concentrator (SCANVAC)
- Stirer (Thermolyne)
- Temperature controller (Model: 631-Protech)
- TS Series Ultra-Low Temperature Freezer (Thermo Scienctific)
- Water bath (Memmert)

3.4 Animal studies

Albino Wistar-Kyoto-Rats (WKY) rats weighed 120-150 g were purchased from the Animal House, University Science Malaysia (USM). All methods and procedures used in this study were approved by the University Malaya - Institutional Animal Care and Use Committee (ISB/25/04/2013/NA(R)). The animals were acclimatized under standard laboratory conditions and normal photoperiod (12h light: dark cycle) for a period of two weeks. The animals were fed with commercial rat pellet and tap water *ad libitum*. At the end of the acclimatization period, the animals were randomly divided into three groups. The first group served as the control group (C) and received normal diet and water. The other two groups were subgrouped into hypercholesterolemia and carbon tetrachloride (CCl_4) - induced groups before further subgrouping to respective treatment procedures as tabulated in Table 3.1. Drug administration was performed via intraperitoneal.

3.4.1 Induction of hypercholesterolemia using high fat diet

Hypercholesterolemic state was induced by giving the rats a high fat diet (a mixture containing roasted Bengal flour, groundnut flour, milk powder, health mix and butter) as a routine daily diet for a period of 1 month (30 days). Rats received normal tap water for drinking. Following the cholesterol-induction state, rats were given different doses of EE and drugs through oral gavage. Normal rats received only distilled water. Group 2 (HC) served as hypercholesterolemic control or diseased state received distilled water; group 3 (E50) received 50 mg/kg body weight of EE; group 4 (E250) received 250 mg/kg body weight of EE and group 5 (Ato) received Atorvastatin (10 mg/kg body weight) once daily for a period of 21 days.

Table 3.1: Treatment groupings for the experimental animals

	Group	Treatment
	C	Normal diet, untreated rats (normal control)
CHOLESTEROL LOWERING	**HC**	High fat diet, untreated rats (disease control)
	HC-E50	High fat diet, treated with low dose (50 mg/kg) *F. deltoidea* ethanolic extract
	HC-E250	High fat diet, treated with high dose (250 mg/kg) *F. deltoidea* ethanolic extract
	HC-Ato	High fat diet, treated with statin drug (positive control)
CARDIOPROTECTIVE	**CCl_4**	Normal diet, CCl_4 induced rats (disease control)
	CCl_4-E50	Normal diet, CCl_4 induced rats pre-treated with low dose (50 mg/kg) *F. deltoidea* ethanolic extract
	CCl_4-E250	Normal diet, CCl_4 induced rats pre-treated with high dose (250 mg/kg) *F. deltoidea* ethanolic extract
	CCl_4-Ato	Normal diet, pre-treated with statin drug (positive control)

3.4.2 Induction of cardiac infarction using CCl_4

Rats received normal tap water for drinking. Rats were given different doses of EE and drugs through oral gavage. Group 2 (CCl_4) served as cardiac infarct control or diseased state received distilled water; group 3 (CCl_4-E50) received 50 mg/kg body weight of EE; group 4 (CCl_4-E250) received 250 mg/kg body weight of EE and group 5 (CCl_4-Ato) received Atorvastatin (10 mg/kg body weight) once daily for a period of 30 days. Cardiac infarction was induced using subcutaneous injection of 1mg/kg body weight CCl_4 on day 31.

3.4.3 Sample analysis

The rats were then sacrificed by CO_2 asphyxiation and the bloods were immediately collected through an incision made in the jugular vein. Serums obtained were used for lipid profile analysis as well as proteomics analysis whereas tissues samples were used for cardiac enzymes analysis. The analysis was outsourced to Clinical Diagnostic Laboratory (CDL), Faculty of Medicine, University of Malaya

3.5 Extraction of *F. deltoidea*

The leaves of *F. deltoidea* var *kunstleri* were harvested from a plantation in Rembau, Negeri Sembilan, Malaysia. A voucher specimen was kept in the Herbarium, Rimba Ilmu, University of Malaya (KLU 046470). The leaves were air-dried before being ground into fine mesh. Extraction was conducted with 90% ethanol using a Soxhlet apparatus (Favorit, Malaysia). The solvent was evaporated under vacuum using rotary evaporator (BUCHI Rotavapor R-114, Switzerland) at 45^{o}C and the resulting ethanolic extract (EE) was then stored in tight-capped containers until further use.

3.6 Sample preparation

3.6.1 Protein determination using Bradford protein assay kit

The protein content of plasma samples was carried out by the Bradford method using protein assay dye reagent concentrate (Biorad, Hercules, CA, USA) and bovine serum albumin (BSA) was employed as the standard. The absorbance readings were obtained using a ELISA reader (Mikrowin 2000, ASYS UVM 340). The concentrations of the proteins were determined and generated using a standard curve plotted against absorbance reading at 595 nm

3.7 2D- electrophoresis

3.7.1 Rehydration of immobiline pH gradient (IPG) dry strip

Eighty µg of serum protein was mixed with 450 µl of rehydration solution and centrifuged at 5000 rpm for 5 minutes. Sample mixture was then pipetted into the respective reservoir channel of the IPG Reswell Tray (GE Healthcare, Uppsala, Sweden) which had been leveled on IPGBox^{TM}. After sample loading, a 24 cm immobilized linear gradient IPG Dry Strips (pH 4-7) was positioned with gel side down onto the solution. Further precaution was needed to prevent bubble trapping under the IPG Dry Strips gel. The strip was allowed to rehydrate at room temperature for 18 hours or overnight to ascertain complete uptake of protein by IPG strip.

Urea Rehydration Stock Solution

(8 M Urea, 2% CHAPS, 2% IPG Buffer, 0.002% Bromophenol blue)

Twelve g of urea, 0.5 g of CHAPS, 500 µl of the IPG buffer of the same range as IPG strip and 50 µl of 1% Bromophenol blue were dissolved in double distilled water (ddH_2O). The solution was further topped up to 25 ml using double distilled water. The solution was aliquoted into 1 ml bullet tubes and stored below -20^{o}C.

Urea Rehydration Buffer

(8 M urea, 2% CHAPS, 0.5% IPG buffer, 0.002% bromophenol blue, 0.0028% DTT)

0.1 g of DTT was added into 1 ml of urea rehydration stock to obtain 10% DTT buffer solution. This stock solution was kept at -20^{o}C. Twenty-eight µl of this solution was added into 1 ml of urea rehydration stock solution together with protein sample, each time prior to rehydration of IPG dry strip.

3.7.2 First dimension run

The temperature of the Ethan IGPhor 3 Electrophoresis Unit (GE Healthcare, Uppsala, Sweden) was set at 20^{o}C using thermostatic Eyela CA-1310 (Tokyo Rikakikai Co., Tokyo, Japan). The Ethan IGPhor 3 Manifold was placed onto the Ethan IGPhor 3. Then, the IPG dry strip was rinsed with double distilled water before transferred to the manifold with gel side up and anodic (+ve) end of the strip on the bottom of the manifold tray. The precut electrode pads were moistened before applied on both cathodic and anodic ends of the IPG strips. Next, the electrode assembly was positioned over the top of all pads to seat the electrodes in place. The immobiline dry strip cover fluid was then poured evenly on each track to ensure good thermal contact and to prevent rehydration. The Ethan IGPhor 3 electrophoresis unit was securely connected to the power supply before closing the lid. The first dimension isoelectric focusing conditions were run accordingly as in Table 3.2.

Table 3.2: IEF focusing conditions on Ethan IGPhor 3 electrophoresis unit

Phase	Voltage (V)	Time (Hour)
Step 1: Step and Hold	500V	1:00 hour
Step 2: Gradient	1000V	7:00 Hrs
Step 3: Gradient	8000V	3:00 Hrs
Step 4: Step and Hold	8000V	7:10 Hrs

After completing the first dimension electrophoresis, the focused IPG dry strips gel either to proceed immediately to second-dimension electrophoresis or preserved in the equilibration tube individually. The preserved strips kept in -80^{o}C to be used within 2 weeks.

3.7.3 Second-dimension electrophoresis

Stock solutions:

Monomer stock (Solution A)

(30% acrylamide, 0.8% N, N'-methylenebisacrylamide)

Both 75 g of acrylamide and 3 g of N, N'-methylenebisacrylamide were mixed and the solution was made up to 250 ml with double distilled water and kept in the dark.

4x resolving gel buffer (Solution B)

(1.5 M Tris-HCl, pH 8.8)

45.425 g of Tris base was dissolved in 187.5 ml of double distilled water before adjusting to pH 8.8 with concentrated HCl. The solution was made up to 1 L with double distilled water.

10% sodium dodecyl sulphate (SDS) (Solution C)

5.0 g of SDS was dissolved in 50 ml of double distilled water. Solution C can be stored at room temperature.

10% ammonium persulfate (APS) (Solution D)

0.1 g of APS was dissolved in 1 ml of double distilled water. The solution was freshly prepared prior to use.

N, N, N', N' – tetramethylethylenediamine (TEMED) (Solution E)

TEMED solution was taken directly from the bottle.

SDS equilibrium buffer solution

(6 M Urea, 75 mM Tris-HCl pH 8.8, 29.3% Glycerol, 2% SDS, 0.002% Bromophenol blue)

72.1 g of urea, 10 ml of Tris-HCl pH 8.8, 69 ml of glycerol, 4.0 g of SDS and 400 µl of 1% bromophenol blue were mixed and made up to 200 ml using double distilled water. The solution was stored at -20^{o}C. The first SDS equilibration buffer was prepared by adding 0.1 g dithiothreitol (DTT) per 10 ml of SDS equilibrium buffer solution. The second SDS equilibration buffer was ready by adding 0.25 g of Iodoacetamide (IAA) into the SDS equilibrium buffer solution. Each strip required 10 ml of solution during equilibration prior to 2D electrophoresis.

10x Laemmli SDS electrophoresis buffer

(250 mM Tris Base, 1.92 M Glycine, 1% SDS)

30.3 g of Tris base, 144.1 g of glycine and 10 g of SDS were mixed and dissolved with double distilled water up to 1 L. This solution was prepared 1x as running buffer for 2D electrophoresis.

Agarose sealing solution

(25 mM Tris base, 192 mM glycine, 0.1% SDS, 0.5% agarose, 0.002% bromophenol blue)

100 ml of 1x Laemmli SDS electrophoresis buffer, 0.5 g of agarose and 200 µl of 1% bromophenol blue stock solution were mixed. Concentrated 10x Laemmli SDS electrophoresis buffer was diluted to 1x before mixing all together. The mixture was then swirled and heated in the microwave oven until the agarose had completely dissolved.

Bromophenol blue stock solution

(1% Bromophenol blue, 0.6% Tris base)

0.1 g of bromophenol blue and 0.06 g of Tris base were mixed and dissolved with 10 ml of double distilled water. The solution was aliquoted and kept under -20^{o}C.

Overlaying solution (water saturated-butanol)

50 ml of either n- butanol or t- butanol was added with 5 ml of double distilled water.

Gel storage buffer

250 ml of Tris-HCl pH 8.8 was mixed with 10 ml of 10% SDS and topped up to 1 L with double distilled water.

3.7.3.1 Preparation of 11% homogenous SDS-polyacrylamide gel

A vertical 11% gel was prepared by mixing all the stock solutions according to the gel recipe below (Table 3.3):

Table 3.3: The gel recipe for 11% 2D large gel

Stock solution	Volume of stock solution
Solution A (ml)	148
Solution B(ml)	100
Double distilled water (ml)	146
Solution C (ml)	4
Solution D (ml)	2
***Solution E (µl)**	133.2
Total volume (ml)	**400**

*TEMED to be added immediately once Solution D was added to the solution.

Once all solutions were mixed thoroughly, the gel solution was poured slowly into the filling channel of the gel caster within 10 minutes. Subsequently, each gel was overlaid with water-saturated-n-butanol. The homogenous gels were allowed to polymerize for a minimum of one hour.

3.7.3.2 Equilibration of IPG strip prior SDS PAGE

First equilibration was carried out with 10 ml SDS-Equilibration buffer containing 0.1 g dithiothreitol (DTT). Subsequently, second equilibration was performed with SDS-Equilibration buffer containing 0.25 g iodoacetamide (IAA). Both procedures were conducted with a gentle shake on a shaker for 15 minutes with IPG strips facing upwards. The IPG strips were then washed with SDS PAGE running buffer (1x) before loading to the second-dimension gel in order to get rid of the free DTT and IAA. Next, the strip was sealed with 0.5% agarose sealing solution and let solidified for 10 to 15 minutes.

3.7.3.3 Second-dimension run

The SDS buffer in the GE Ettan DALTSix tank (GE) was maintained at 25^{o}C using a thermostatic circulator. SDS-PAGE was carried out at a constant current in 2 steps as below:

Phase	Voltage(V)	Miliampere (mA)	Watt (W)	Time (Hour)
1	80	10	1	1
2	500	40	13	7.5

-value per gel

The electrophoresis system was terminated once the blue indicator was 1 cm away from the bottom. The gels were then removed and spots visualized by silver staining.

3.7.4 Silver staining

Visualization of proteins was conducted accordingly as in Table 3.4. The gels were individually fixed overnight prior to silver staining in a solution containing ethanol and acetic acid. Each gel required at least 250 ml of solution volume to cover the surface of the bath gel. Subsequently, sensitization with thiosulfate solution aimed to increase image formation. The gels were then washed and retained to its origin size. The image development procedure involved silver impregnation for 20 minutes. Short step rinsing was required to eliminate the excessive silver solution before development process. The developer solution contained formaldehyde, carbonate and thiosulfate. The brown precipitate of silver carbonate formed and immediately agitated to prevent deposition and dark background formation. Background reduction by thiosulfate achieved by brief incubation in thiosulfate prior to development (Wiederkehr *et al.*, 1985) or by inclusion in the developer. When the desired image level was obtained, stop with stopping solution containing acetic acid and ethanol to reach a pH of 7. This was done to ensure maximal and reproducible sensitivity. Final stabilization of the image proceeds by thorough rinsing with water to eliminate all the compounds present in the gel. After staining, the gel was captured by Image Scanner III (GE Healthcare Biosciences) in a digital format.

Table 3.4: Silver stain stock solutions

Steps	Solutions	Volume	Duration
Fixation	Ethanol Acetic acid glacial Double distilled water	100 ml 25 ml Up to 250 ml	30 mins (no limits@overnight)
Sensitizing (or Incubation)	Ethanol #*25% Glutaraldehyde Sodium thiosulfate Sodium acetate Double distilled water	75 ml 1.25 ml 0.5 g 17 g Up to 250 ml	30 mins
Washing with double distilled water, 3 x 5 mins			
Silver reaction	Silver nitrate #*37% Formaldehyde Double distilled water	0.625 g 0.1 ml Up to 250 ml	20 mins
Washing with double distilled water, 2 x 1 min			
Developing	Sodium carbonate *37% Formaldehyde *5% Sodium thiosulfate Double distilled water	6.25 g 0.1 ml 7 µl Up to 250 ml	4 mins
Stopping	Titriplex III (EDTA-Na_2H_2O) Double distilled water	3.65 g Up to 250 ml	10 mins
Washing with double distilled water, 3 x 10 mins			
Preserving	Ethanol Glycerol (87%) Double distilled water	75 ml 11.5 ml Up to 250 ml	

*only to be added immediately prior to use

#Glutarylaldehyde (sensitizing) and formaldehyde (silver reaction) were omitted from the staining procedure for gels that were subjected for MS analysis.

3.7.5 Image analysis

Prior to protein identification using mass spectrometry, 2D gel protein patterns were scanned and analyzed using computerized image analysis software. The significant protein detection can be precisely performed by aligning the overlapping gel images with the reference gel before spotting (Magdeldin *et al.*, 2014) using Progenesis SameSpots.

The proteome profiles of all tested groups were compared to the proteome of normal group. Subsequently, significant cutoff spots can be programmed to identify significant protein spots changes. The aligned images were grouped into their respective treatment group and evaluated statistically in the review step based on ANOVA p value less than 0.05 and displaying fold higher than 1.5. Significantly altered protein levels were then excised for protein identification by matrix-assisted laser desorption/ionization *(*MALDI).

3.8 MALDI ToF/ToF mass spectrometry

3.8.1 Preparation of reagents

*All working solutions must be prepared fresh immediately before use.

200 mM ammonium bicarbonate

0.791 g of ammonium bicarbonate was dissolved in 50 ml of Milli-Q water for further use.

Destaining solution: 15 mM potassium ferricyanide in 50 mM sodium thiosulfate

0.05 g of potassium ferricyanide and 0.12 g of sodium thiosulfate were mixed and dissolved in 10ml with Milli-Q water.

Reduction solution: 10 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate

7.7 mg of DTT was added into 5 ml of 200 mM ammonium bicarbonate and topped up to 10 ml with Milli-Q water.

Alkylation solution: 55 mM iodacetamide (IAA) in 100 mM ammonium bicarbonate

50.86 mg of IAA was added into 5 ml of 200 mM ammonium bicarbonate and topped up to 10 ml with Milli-Q water.

Washing of gel plugs: 50% acetonitrile in 100 mM ammonium bicarbonate

Acetonitrile (ACN) volume was added equally with 200 mM ammonium bicarbonate.

Dehydration solution

Gel plugs incubation was carried out using pure 100% ACN.

In-gel digestion (7 ng/µl trypsin in 40 mM ammonium bicarbonate)

5 µl (1 µg/ml trypsin) was dissolved with 695 µl of 40 mM ammonium bicarbonate.

a) 40 mM ammonium bicarbonate

0.2 ml of 200 mM ammonium bicarbonate was diluted with 0.8 ml of Milli-Q water.

Extraction of peptides

a) 50% ACN solution

ACN solution volume was added equally with 200 mM Milli-Q water.

b) 100% ACN

100% pure ACN was used.

3.9 MALDI-ToF/ToF MS protein identification

3.9.1 Proteolytic trypsin digestion

Following optimized protocol by Shevchenko *et al.* (2006), 2D gels were stained without crosslinking reagents such as glutaralydehyde. Silver stained spots that showed significant alteration in their abundance were excised manually and gel plugs were later subjected to digestion procedure. For the purpose of protein identification using MALDI ToF/ToF MS, 240 µg protein amount was loaded on each gel. Excisions were performed using clean tips (1000 µl) to avoid any contamination. Tips were cut with scissors accordingly to the area of the spots to be excised. Each gel plug obtained was

placed in a clean Eppendorf tube, layered with enough water to cover the plug and freeze below -20^{o}C. The proteolytic digestion was carried out according to Table 3.5 prior to mass spectrometer analysis. Pure trypsin is unstable, especially in non-acidic state (Chauvet *et al.,* 1976), therefore the stock solution is constituted into vials before being frozen at -80^{o}C. The trypsin solution will be thawed and diluted prior to use.

Table 3.5: In gel trypsin digestion

Steps	Time / Frequency
Destaining 100 µl of 15 mM potassium ferricyanide in 50 mM sodium thiosulphate	15 mins (x 2) (shake)
Reducing 150 µl of 10 mM DTT in 100 mM ammonium bicarbonate	30 mins @ 60^{o}C (shake)
Alkylation 150 µl of 55 mM IAA in 100 mM ammonium bicarbonate	20 mins in dark
Washing 500 µl 50% ACN in 100 mM ammonium bicarbonate	20 mins (x 3)
Dehydration 50 µl 100% ACN	15 mins (dry in speedvac for 15 mins @ 1000 rpm)
Digestion 25 µl of 7 ng/µl trypsin in 50 mM ammonium bicarbonate	Overnight @ 37^{o}C
Extraction 1) 50 µl 50% ACN 2) 50 µl 100% ACN	 15 mins (shake) 15 mins (shake)

3.9.2 Mass spectrometry analysis

Following digestion, proteins were extracted from the digested gel plugs using acetonitrile. Dried extracted protein samples were subjected to clean-up step using C18 Ziptip**.** An equivalent volume of eluted samples and matrix (6 mg/ml α-cyano-4-hydroxy-transcinnamic acid) were mixed and transferred onto MALDI-ToF/ToF sample plate (384 Opti-ToF 123x81mm, ABSciex). The samples were allowed to air dry and then analysed using ABI 4800 MALDI-ToF/ToF Plus (Applied Biosystems, Foster City, USA) mass spectrometer. Preceding to protein identification by using MALDI-ToF/ToF MS, all procedures are mandatory to be conducted in clean environment to prevent any human (e.g. keratin) contaminations.

Matrix solution:

6 mg/ml Alpha-cyano-4 hydroxycinnamic acid (α-CHCA) (70% ACN in 0.1% TFA)

Six mg of α-cyano-4-hydroxy-cinnamic acid was dissolved in 700 µl 100% ACN in addition of 300 µl 0.1% TFA

a**) 0.1% TFA**

Mix 10 µl TFA with 990 µl Milli-Q water.

b) **100% ACN**

Use pure ACN.

3.9.3 Database searching

Proteins were identified by peptide mass fingerprint (PMF) databases search using the Mascot (Matrix Science, USA) and ProFound (Proteometrix, USA) programs.

3.9.3.1 Mascot search engine based on probability scoring

The MS along with MS/MS spectra were matched with the theoretical peptide masses using the Mascot™ database search engine v2.2.03 (Matrix Science Ltd., London, UK) and searched against SwissProt 51.6 database with the following setups: Trypsin cleavage; allowed one missed cleavage; fixed modification: carboxymethylation of cysteine (C); variable modification: oxidation of methionin (M); MS precursor mass tolerance was set to ±100 ppm, mass tolerance set at ±0.2 Da and monoisotopic with MH+ mass value.

3.9.3.2 ProFound search based on Bayesian calculation

ProFound applied Bayesian theory in identifying proteins from the protein databases. The unmatched peptides were analysed using ProFound software accessible online: http://prowl.rockefeller.edu/prowl-cgi/profound.exe with *Rattus* as taxonomy category; mass range of 0 to 100 kDa; protein pI in between 4 to 7; allowed one missed cleavage and MH^+ charge state with mass tolerance of 0.5 to 1.15 Da.

3.10 Western blot analysis

3.10.1 Sodium dodecyl sulphate (SDS) gel preparation

3.10.1.1 Preparation of reagents/solutions:

All solutions required for SDS-gel preparation and running buffer were as described in Section 3.6.3 **(**except Solution F and loading buffers as below).

0.5 M Tris-HCl , pH 6.8 (Solution F)

12.11 g of Tris base was dissolved with double distilled water before adjusting to pH 6.8 with concentrated HCl. The solution was made up to 200 ml with double distilled water and kept at 4^0C.

SDS-PAGE sample loading buffer:

(62.5 mM Tris-HCl pH 6.8 buffer (Solution F), 20% (w/v) glycerol, 10% (w/v) SDS, 1.0% (w/v) Bromophenol blue, β-mercaptoethanol)

2.0 ml of Solution F, 1.6 ml of Glycerol, 3.2 ml of 10% SDS and 40 µl of 1.0% Bromophenol blue were all mixed well. The aliquoted buffers were then kept in -20ºC. 10% β-mercaptoethanol (BME) was added prior to use.

Prestained protein marker (Molecular Weight: 11 – 245 kDa)

The BLUeye pre-stained protein ladder (GeneDirex) was vortexed well before aliquoted into separate tube and kept in -20ºC.

3.10.1.2 Sample preparations

7 µg of samples were denatured and ionized by adding 3 µl of 2x sample loading buffer. The mixture was heated at 95°C for 5 minutes. Finally, the mixture was spun to bring condensation to bottom prior to gel loading.

3.10.1.3 Preparation of 12.5 % separating gel

All the SDS gel apparatus and glass plates were cleaned thoroughly and wiped with 70% ethanol. All solutions for separating gel as shown in Table 3.6 were mixed thoroughly. APS and TEMED were added last prior to gel casting. The separating gel (≈ 6 ml) was then poured at once into the gel caster for Dual vertical mini-gel unit Model: MGV-200 SDS-PAGE (CBS Scientific Co.) with a serological pipette and overlaid with distilled water. The gel was allowed to polymerize for at least an hour before decanting water out onto Kimwipes. Subsequently, the separating gel surface was dried using filter paper.

3.10.1.4 Preparation of 4% stacking gel

The stacking gel solution was prepared as shown in Table 3.6 by adding APS and TEMED last. The stacking gel solution (≈ 1 ml) was overlaid immediately on top of separating gel. A comb was inserted at an angle to avoid trapping bubbles in the stacking gel.

Prior to performing polyacrylamide gel electrophoresis, the wells were rinsed with running buffer to eliminate any residual of unpolymerised stacking gel. The wells are filled up with running buffer to avoid drying up as well to ease sample loading. Seven microlitres of GeneDirex BLUeye pre-stained protein ladder (Appendix A) was loaded onto the first well and subsequently wells with 7 µg samples. Serum samples were reduced by 2-mercaptoethanol prior to loading onto the wells on 4% stacking gel and 12.5% SDS-PAGE. Allow a gap on both side of the comb to ensure all wells had complete walls. Electrophoresis was performed using the Dual vertical mini-gel unit Model: MGV-200 SDS-PAGE (CBS scientific Co.) set constantly at 60 mA, and later increased to 120 mA once it reached the separating gel (approximately half an hour later).

Table 3.6: 4% Stacking gel and 12.5% separating gel recipes

	Gel density	
	Stacking gel (4%)	**Separating gel (12.5%)**
Solution A	0.65 ml	8.34 ml
Solution B	-	5.00 ml
Solution C	50 µl	200 µl
Double distilled water	3.05 ml	6.36 ml
Solution D	25 µl	100 µl
Solution E	5 µl	6.60 µl
Solution F	1.25 ml	-

3.10.2 Protein transfer

Trans-Blot® SD Semi-Dry electrophoretic transfer cell (Bio-rad, USA) was used to transfer the proteins from 12.5% homogenous gel onto a 0.45 µm nitrocellulose membrane. The filter papers and the membrane were cut accordingly to the dimension of the gels before soaking in the transfer buffer for 15 mins. Sequentially, the gel was transferred onto the nitrocellulose membrane with the filter papers on top and bottom of the sandwiched membrane and gel. The formations of bubbles between the layers were excluded by rolling out air bubbles on each layer. The mini gels were then transferred for 15 mins at 15 V accordingly to semi-dry transfer system manufacturer's instructions.

Semi-dry (Transfer buffer)

(48 mM Tris, 0.39 M glycine, 20% v/v methanol)

5.82 g of Tris and 2.93 g of Glycine were mixed in 800 ml double distilled water and stored in 4^{o}C. 20 ml methanol (20% methanol) was added to 80 ml Tris-Glycine solution prior to use.

10x Tris Buffer Saline (concentrated TBS)

24.23 g of Tris base and 80.06 g of sodium chloride were dissolved with 800 ml of double distilled water before adjusting to pH 7.6 with concentrated HCl. The solution was made up to 1 L with double distilled water and kept at 4^{0}C.

Tris Buffered Saline-Tween (TBST)

100 ml of 10x tris buffered saline were diluted with 900 ml of double distilled water before adding 1 ml of Tween-20 into the solution.

5% Skimmed milk (Blocking buffer)

5 g of skimmed milk were dissolved in 95 ml of TBST. The solution was filtered and kept in 4^0C.

3.10.3 Immunoblotting

After protein transfer, the nitrocellulose membrane was stained with commercialized Ponceau red solution (Sigma) for 1 minute to visualize the protein bands as well to confirm that the proteins were equally loaded. The red Ponceau stain on the membrane was then washed with copious amounts of double distilled water. The membranes were first blocked with 5% non-fat milk in TBS/0.1%Tween-20 (TBST) overnight at 4°C for HAP and one hour at room temperature for RBP4. The membranes were consequently washed twice with fresh TBST for 5 and 10 minutes respectively. Antibody solutions were prepared containing 5% non-fat milk in TBST. For detection of HAP, the antibody binding procedure involved overnight incubation at 4°C with the primary chicken anti-haptoglobin antibody (1:1000 dilution, Abcam, Cambridge, UK), followed by horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000 dilution rabbit anti-chicken, Abcam, Cambridge, UK) incubation for two hours at room temperature. For detection of RBP4, membrane was probed for one hour at room temperature with primary rabbit anti-RBP4 antibody (1:200 dilutions, Abcam, Cambridge, UK) and then incubated at room temperature for two hours with secondary antibody (1:1000 dilution rabbit anti mouse, Abcam, Cambridge, UK). The primary and secondary antibodies used for HAP and RBP4 protein immunoblottings were summarized in Table 3.7.

Bands on the membrane were developed with a substrate, 3,3'-diaminobenzidine (DAB Nacalai Tesque, Kyoto, Japan) consolidated with the metal enhancer for DAB stain

in the presence of hydrogen peroxide. The intensity of both HAP and RBP4 bands were visually compared against the protein ladder loaded.

Table 3.7: Summary of primary and secondary antibody used for protein immunoblot

	Primary antibody			**Secondary anti primary antibody-HRP conjugated**		
Analyte [MW (kDa)]	**Dilution**	**Description**	**Company**	**Dilution**	**Description**	**Company**
Haptoglobin [≈ 11]	1:1000 (O/N at 4°C)	Chicken polyclonal antibody to Haptoglobin	Abcam	1:10000 (2h at RT)	Rabbit polyclonal antibody to chicken antibody	Abcam
RBP4 [≈ 21-25]	1:200 (1h at RT)	Mouse monoclonal antibody to RBP4	Abcam	1:1000 (2h at RT)	Rabbit polyclonal antibody to mouse antibody	Abcam

CHAPTER 4: RESULTS

4.1 Anti-hypercholesterolemic effect of *Ficus deltoidea*

4.1.1 Serum lipid profile

The lipid profile of the sera from rats (Table 4.1) showed an obvious difference between healthy and the diseased-state groups. Hypercholesterolemic-induced group (HC) demonstrated an increase in all of the observed parameters; triglycerides and total cholesterol levels were significantly increased by 61.5% and 52% respectively, while HDL was increased by 25.2% compared to the control. The increase in LDL was also shown as it appeared to reach the detectable value as oppose to the control. Treatment with Atorvastatin demonstrated a reduced pattern for TG, TC and LDL compared to HC. A similarly reduced trend was also observed following the administration of *Ficus* sample, E50 and E250 compared to HC with an exception to the increase in HDL level in the Ato-treated sample. Treatment with E50 appeared to give a better normalizing effect compared to E250. The *F. deltoidea* extracts believed to have definite cholesterol reducing action by restoring serum HDL level concentration which efficaciously prohibited the increase of serum cholesterol and markedly normalized serum LDL in *F. deltoidea* treated groups.

Table 4.1: Serum lipid profile of hypercholesterolemic-induced rats

Group	Lipid parameters			
	TG (mg/dL)	**TC (mg/dL)**	**HDL (mg/dL)**	**LDL (mg/dL)**
C	0.52 ± 0.08	1.46±0.13	1.35±0.16	ND
HC	0.84 ± 0.21*	2.22±0.48*	1.69±0.13	0.06±0.10
E50	0.50 ± 0.14**	1.7±0.39	1.56±0.32	ND
E250	0.62 ± 0.16	1.75±0.33	1.52±0.23	0.02±0.05
Ato	0.50 ± 0.07	2.04±0.18	1.76±0.14	0.05±0.08

Values are presented as mean ± SD.

* indicates statistical significance against C ($p < 0.05$).
** indicates statistical significance against HC ($p < 0.05$).

TG: Triglycerides, **TC**: Total cholesterol, **HDL**: High density lipoproteins, **LDL**: Low density lipoproteins, **ND**: Not detected, below threshold level, **C**: Control, **HC**: High cholesterol, **E50**: 50 mg/kg *F. deltoidea* extract, **E250**: 250 mg/kg *F. deltoidea* extract, **Ato:** Atorvastatin

4.1.2 2D-GE proteome maps of hypercholesterolemic-induced rats

Total protein concentration (x-axis, µg/ml) in rat samples was determined using calculated formula, y=0.0005x + 0.0507, generated from the protein standard curve with R=0.98 (as shown in Appendix B). Serum proteins from rats were separated by 2D-GE and silver-stained. The proteome maps of rat serums as shown in Appendix C. Normal/Control rats denote healthy rats with normal cholesterol concentration.

High fat-diet fed rats were all given treatments except one group with hypercholesterolemia induced condition (HC). All these HC induced rats were treated with Atorvastatin (HC-Ato), 50 mg/kg body weight of *F. deltoidea* extract (HC-E50) and 250 mg/kg body weight of *F. deltoidea* extract (HC-E250). Consequently, rat groups with cholesterol lowering drug, Atorvastatin (Ato) served as positive control (HC-Ato).

4.1.3 Image acquisition and analysis using Progenesis software

2D-GE analysis of serum proteomes from hypercholesterolemic-induced rats (treated and non-treated) were performed. Nearly 1000 individual spots ranging from 7 to 210 kDa with pI 4 to 7 were discovered using Progenesis Samespots software. Close to 30 protein spots were significantly different ($p < 0.05$) when compared with those in control (normal) group. The identified proteins were presented in the 2D-GE maps of master gel visualized by silver staining (Figure 4.1).

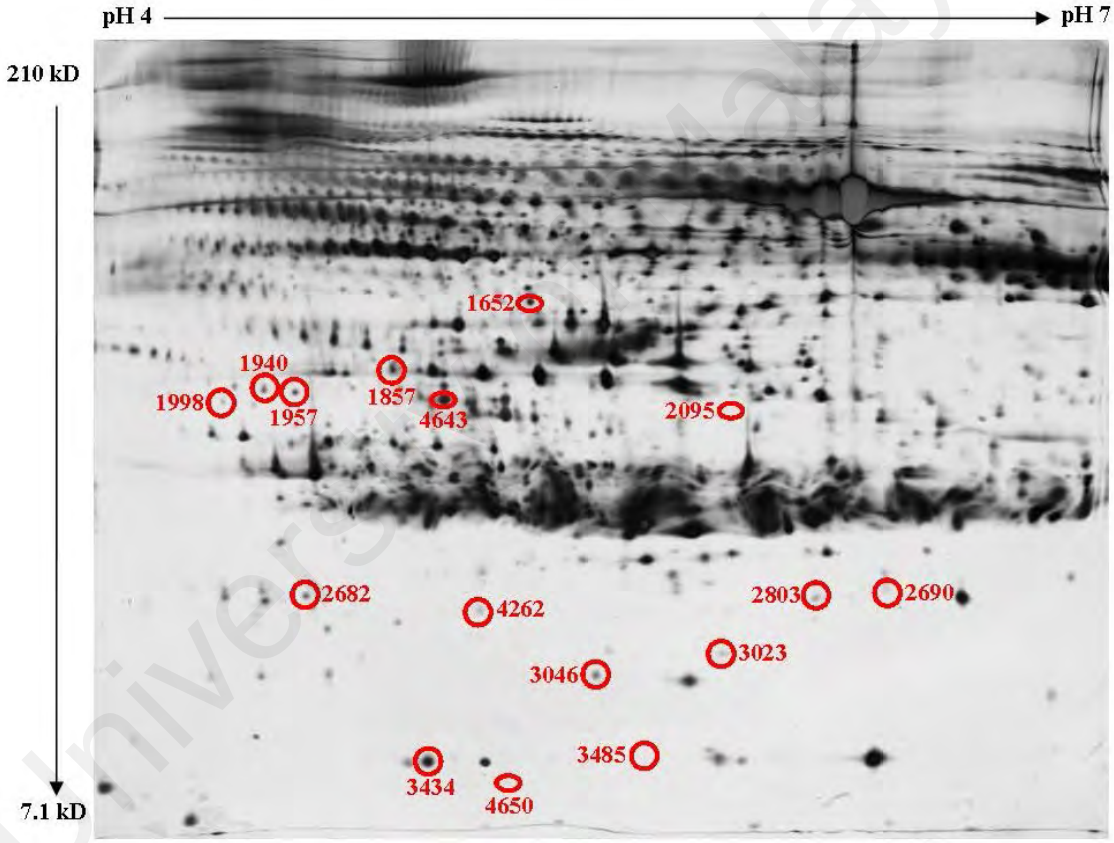

Figure 4.1: A representative of 2D gel proteins from rat serum induced with high fat diet ranged between pH 4-7

Differentially expressed protein spots and each spot numbers are tabulated in Table 4.2 and Table 4.3.

4.1.4 Database mining for protein identification (ID) of differentially altered proteins

4.1.4.1 MASCOT database and ProFound software

MASCOT search engine uses mass spectrometry data to identify proteins from primary sequence databases. The preliminary step of a Molecular Weight Search Engine (MOWSE) search is analyzing the calculated peptide masses for each entry in the sequence database with the obtained experimental datas. ProFound is another capable search engine that provides accurate protein identification even when the data quality is comparatively low or the presence of the target protein is in a complicated mixture. ProFound can concurrently identify a single or multiple proteins in a search accurately. The putative proteins identified are then ranked with decreasing probability score matched to the experimental data.

Among all proteins observed in the HC-induced rats' serum, 30 protein spots showed significant differences ($p < 0.05$). Based on MALDI/ToF/ToF MS data search, 12 spots from HC group Table 4.2 were matched with known proteins in MASCOT database. The identified proteins were matched using mass values either peptide masses or MS/MS fragment ion masses in which are based on a probability-based scoring. Four other protein spots with low MOWSE score from this search were reanalysed using the ProFound software as in Table 4.3, using data of the non-matched peptides. A significant Z-score (Est'd Z) of 2.43 with high probability of 1.0e+000 denotes 99.5% confidence level with only 0.5% false positive. The other 14 protein spots were unidentified.

Table 4.2: List of proteins with significantly altered expressions in hypercholesterolemic rats after treatments, as identified by MALDI-ToF/ToF MS based 2D-GE.

Spot	Protein identification	Acc number/ SWISS PROT	MASCOT[a]		Sequence coverage (%)	Theoritical		Observed	
			Mascot score	Matched peptide		Mw (kDa)	pI	Mw (kDa)	pI
1652	Complement C3 precursor (**C3**)	P01026	60	21 / 85	24.7	186.5	6.1	35	5.4
1957	Spectrin (**SPECTRIN**)	Q9QWN8	23	5 / 9	55.6	12.0	7.1	31	4.5
1998	Contrapsin-like protease inhibitor 1 precursor (**CPI**)	P05545	75	2 / 18	11.1	44.6	5.3	46.8	5.3
2095	Complement C1s (**C1s**)	Q6P6T1	45	3 / 24	7.1	75.3	4.7	28	5.9
2690	Apolipoprotein A1 precursor (**Apo A1**)	P04639	25	5 / 40	12.5	27.4	5.5	13	6.5
2803	Retinol Binding Protein 4 precursor (**RBP4**)	P04916	242	10 / 72	13.8	21.4	6	14	6.2
3046	Major Urinary Proteins (**MUP**)	P02761	71	7 / 104	6.7	17.6	5.4	11	5.5
3434	Haptoglobin (**HAP**)	P06866	108	2 / 2	100	36.6	6	8	4.9
3485	Transthyretin (**TTR**)	P02767	30	6 / 70	8.6	13.6	5.8	7.1	5.6
4262	Plasminogen (**PLG**)	Q01177	29	2 / 16	12.5	88.3	6.8	14	5.1
4643	Apolipoprotein E (**Apo E**)	Q6PAH0	259	19 / 35	54.0	33.8	5.2	29	5.0
4650	Prenylcysteine oxidase precursor (**PCYOX**)	Q99ML5	34	5 / 28	17.9	53.5	6.3	<7.1	5.2

Database search using Mascot : http://www.matrixscience.com

[a] denotes Mascot probability based molecular weight search score calculated for PMF. Protein score is -10 x log [P], where P is the probability that the observed match is a random event. The scores are based to Swiss-Prot database using the MASCOT searching program in MS/MS spectra.

Table 4.3: List of proteins with significantly altered expressions in hypercholesterolemic rats after treatments, as identified by ProFound software.

Spot ID	Protein identification	Acc number/ SWISS PROT	ProFound (Est'd Z score)	Sequence coverage (%)	Theoritical		Observed	
					Mw (kDa)	pI	Mw (kDa)	pI
1857	Prominin-1 isoform 2 precursor (**PROM1**)	Q7TSL4	2.43	14.0	93.2	6.1	31	4.9
3023	Alkaline phosphodiesterase (**PDE1**)	P97675	2.43	14.0	99.2	5.9	90	5.9
2682	Islet cell autoantigen 1-like protein (**Ica1l**)	Q6RUG5	2.43	35.0	48.6	5.2	15	4.6
1940	Ectoderm-neural cortex protein 2 (**ENC2**)	Q4KLM4	2.07	22.0	65.8	6.3	31	4.5

Database search using ProFound: http://prowl.rockefeller.edu/prowl-cgi/profound.exe

*Z-Score estimation correspond with the percentile of the search with significant certainty: Z-score 1.282 = 90%; 1.645 = 95%; 2.326 = 99%; 3.09 = 99.9% confidence.

4.1.5 Functions of identified proteins

These proteins were successfully identified by MS analysis using the Mascot search engine as the primary search and Profound search engine as the complementary searching tool. The significantly altered proteins were mainly associated with lipid metabolism and transport, inflammation and immune response, anti-oxidant protein, acute phase protein, cholesterol homeostasis and cholesterol binding. Other identified proteins were involved in pheromone binding and coagulation. Based on the literatures searched, the categorized functions of these proteins are depicted in Figure 4.2.

4.1.6 Protein abundancy fold change comparison

Comparison between normal (control) and hypercholesterolemic-induced (HC) rats showed differences in term of protein abundances. HC or diseased state rats demonstrated an increased in abundance of C3 (7.0 fold), PDE1 (6.3 fold), C1s (5 fold), CPI (2.8 fold), RBP4 (1.7 fold), PROM1 (1.4 fold), Apo E (1.4 fold), HAP (1.3 fold) PLG (1.3 fold), PCYOX (2.0 fold) and decreased in abundance for TTR (-4.6 fold), MUP (-2.8 fold), Apo A1 (-1.8 fold) and Spectrin (-3.6 fold). Atorvastatin (Ato), the common cholesterol lowering drug demonstrated distinguished increase of Apo A1 (5.2 fold), TTR (4.3 fold), HAP (2.4 fold), CPI (2.1 fold) and Spectrin (1.87 fold); and hence in contrast, decreased abundance of Apo E (-3.2 fold), PROM1 (-1.9), RBP4 (-1.6 fold), PLG (-1.5 fold), MUP (-1.5 fold), C1s (-1.4 fold), PCYOX (-1.4 fold) and C3 (-1.1 fold) when compared to HC. Not much differences noted on PDE1 following treatment with Atorvastatin.

With comparison to HC, treatment with higher dosage of *F. deltoidea* extract (250 mg/kg-E250) markedly induced increase in abundance of Apo A1 (7.3 fold), HAP (2.4 fold), PCYOX (2.1 fold), spectrin (2.5 fold) and CPI (1.2 fold); and decrease in abundance for PDE1 (-6.0 fold), C3 (-4.0 fold), TTR (-2.7), Apo E (-2.3), C1s (-2.2), PROM1 (-2.1),

MUP (-1.8 fold), PLG (-1.6) and near normalization for RBP4. Treatment with low dosage of *F. deltoidea* extract (50mg/kg- E50) however, nearly normalized TTR, PCYOX and Spectrin level in hypercholesterolemic-induced rats. Other proteins like CPI, PROM1, RBP4 and PLG demonstrated similar pattern as observed in E250. However, HAP, PDE1, MUP and C3 exhibited spots with greater intensities when compared to E250. Comparative analysis of the fold change and spot abundancy patterns of each identified protein are shown in Figure 4.3 and Figure 4.4 respectively.

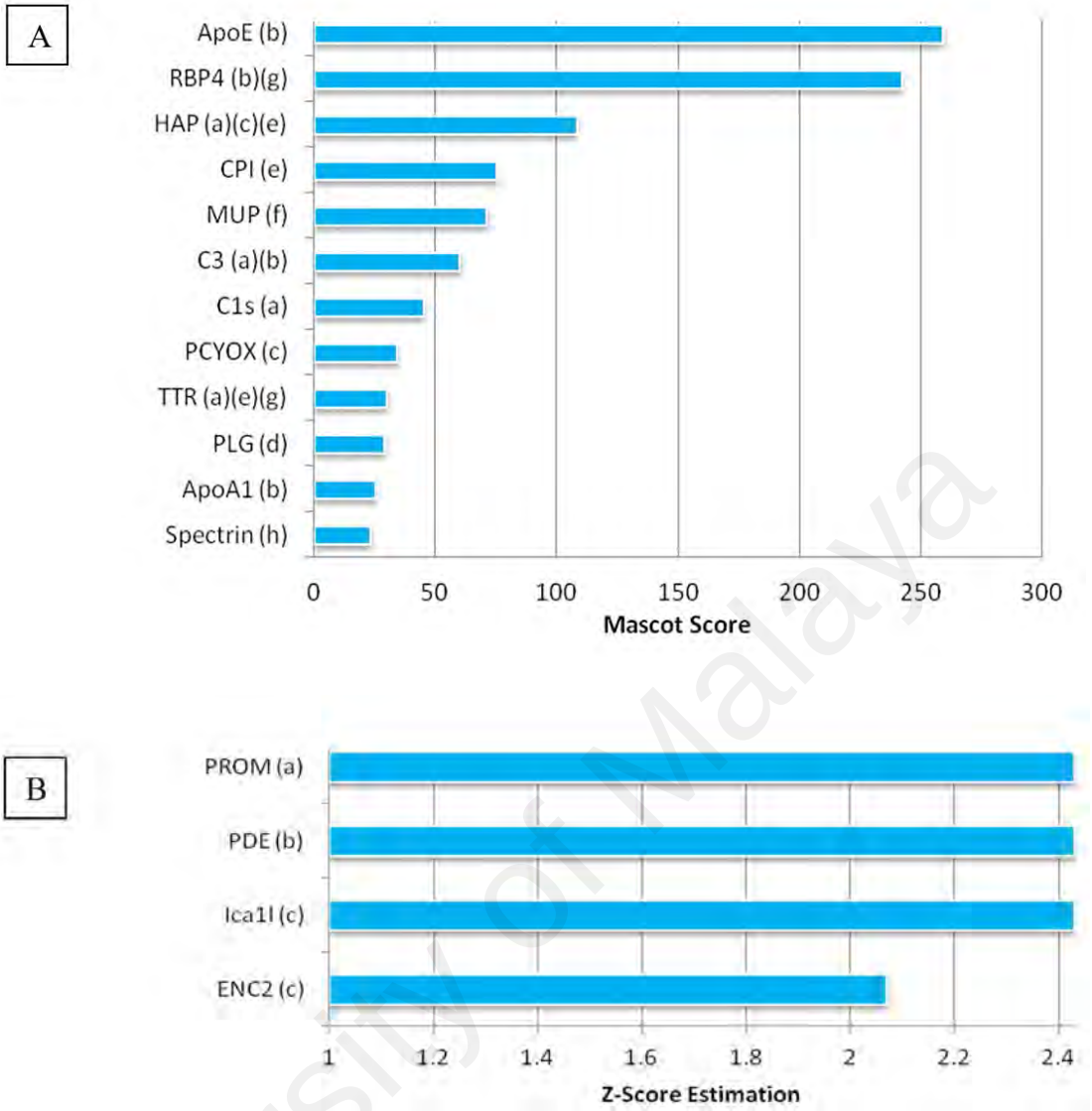

Figure 4.2: Categorized functions of identified proteins acquired by data search

A - data search using MASCOT database. The identified proteins were displayed horizontally with respective categorized functions (a) Inflammation and immune response; (b) Lipid metabolism and transport; (c) Anti-oxidant protein; (d) Coagulation/fibrinolysis; (e) Acute phase protein; (f) Pheromone binding; (g) Cholesterol homeostasis, (h) Unknown
B - data search using ProFound. Identified proteins were displayed horizontally with categorized functions (a) Cholesterol binding; (b) Inflammation and immune response; (c) unknown

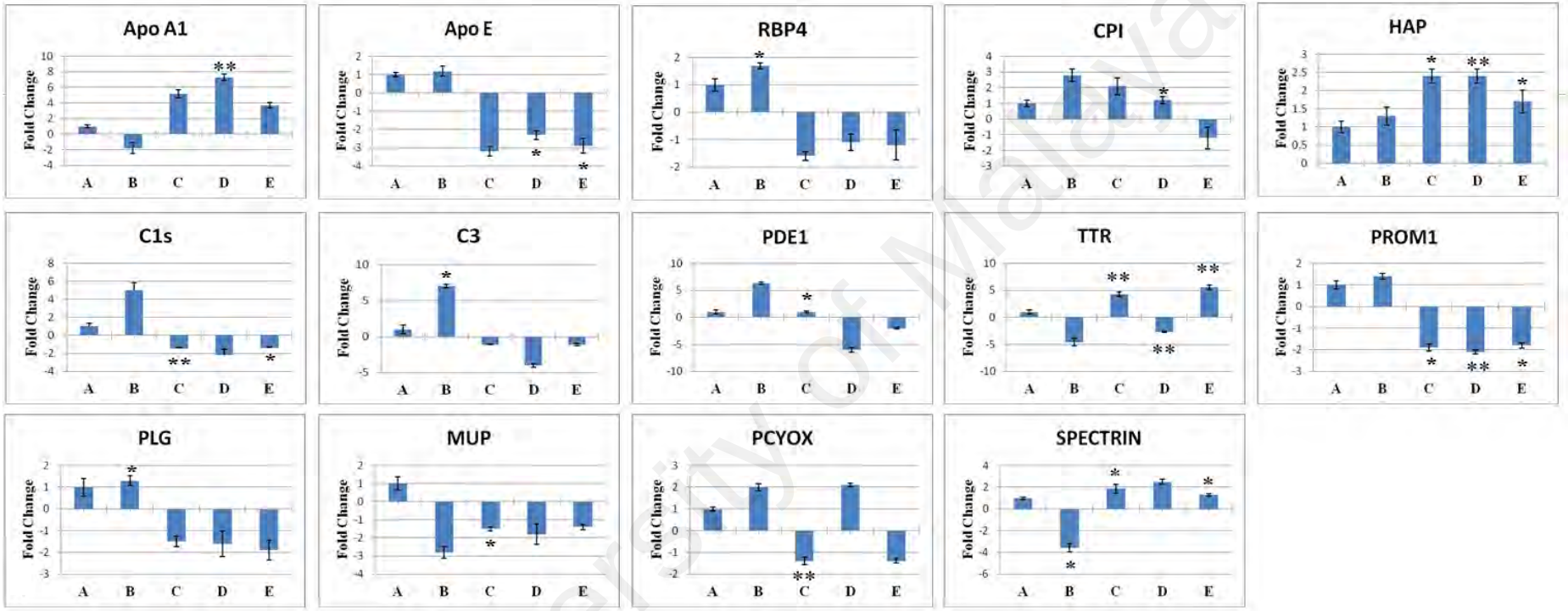

Figure 4.3: Comparative fold change patterns of the identified protein spots in hypercholesterolemic-induced rats

A: Control; **B**: Hypercholesterolemic-induced rats (HC); **C**: Atorvastatin-treated HC (Ato); **D**: *Ficus*-treated HC (E250); **E**: *Ficus*-treated HC (E50).

Fold change was calculated by dividing the average normal spot volumes from each of the two groups. Hypercholesterolemic-induced (HC) was compared to control (C); whereas fold change for the treated groups were measured against HC to denote recovery to normalize value (if presence). Data are expressed as mean±SEM of 4 experiments with 4 replicates. The mean difference is significant at fold change of ≥ 2 fold with $p < 0.05$ (*) and $p < 0.01$ (**), where applicable.

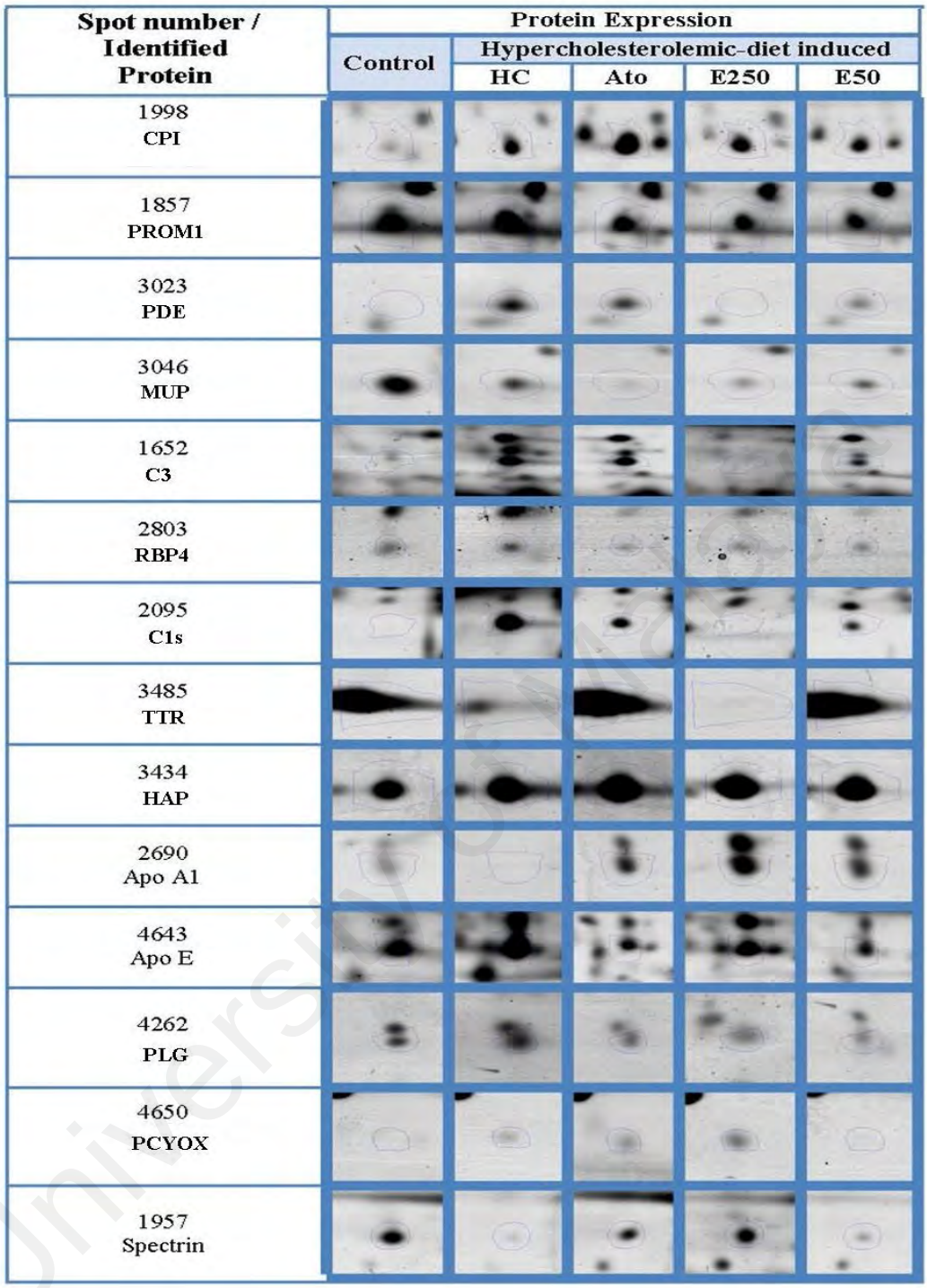

Figure 4.4: Protein spots differential abundancy patterns for treated and non treated hypercholesterolemic-induced rats

Magnified spots of 2D-GE images represent differential abundancy patterns between the different rat groups. **C**: Control/normal rats; **HC**: hypercholesterolemic-induced rats; **Ato**: Atorvastatin-treated rats; **E250**: *Ficus*-treated rats (250 mg/kg body weight); **E50**: *Ficus*-treated rats (50 mg/kg body weight).

4.2 Cardioprotective effect of *Ficus deltoidea*

4.2.1 Serum lipid profile and cardiac tissue marker enzymes analysis

Serum lipid profile (Table 4.4) showed an obvious difference between healthy and diseased-state groups. Elevated cardiac enzymes (ALP, AST, ALT, CK and LDH) levels as tabulated in Table 4.5 were observed in CCl_4 induced rats compared to the control. Increased level of these enzymes was due to the oxidative stress and myocardial cell necrosis caused by CCl_4. On the other hand, pre-treatment with *F. deltoidea* extract demonstrated that the cardiac enzymes were almost near to normal levels, indicating the cardioprotective potential of *F. deltoidea* extracts.

4.2.2 2D-GE proteome maps of CCl_4-induced MI rats

Serum proteins from rats were separated by 2D-GE and silver-stained. The proteome maps of rat serums were shown in Appendix C. Normal/Control denoted healthy rats with normal cholesterol concentration. The control group was further subgrouped into four individual groups and induced with CCl_4 to mimic cardiac infarct effects. All these induced groups were given common cholesterol lowering drug, Atorvastatin (CCl_4-Ato), 50 mg/kg body weight for *F. deltoidea* extract (CCl_4-E50) and 250 mg/kg body weight for *F. deltoidea* extract (CCl_4-E250) respectively, except one group with myocardial infarct served as disease control (CCl_4). Consequently, rat groups with cholesterol lowering drug, Atorvastatin (Ato) served as positive control (CCl_4-Ato).

4.2.3 Image acquisition and analysis using Progenesis software

2D-GE analysis of serum proteomes from both CCl_4 and hypercholesterolemic-induced rats (treated and non-treated) were performed. Nearly 1000 individual spots ranging from 7 to 210 kDa with pI 4 to 7 were discovered using Progenesis Samespots software respectively from the gels. Proteins that exhibited differentially altered profiles

were manually excised from silver-stained gels prior to overnight trypsin digestion. All samples were prepared using α-CHCA matrix before MALDI ToF/ToF MS peptides analyses. The high-end mass spectrometry had been routinely used for rapid and precise protein identification by Peptide Mass Fingerprinting (PMF) or protein fingerprinting. Two software packages which were available online, Mascot datasearch and ProFound were used to identify protein spots. The significantly altered protein spots were presented in the 2DE maps of master gel visualized by silver staining (Figure 4.5).

Table 4.4: Serum lipid profile of CCl_4-induced rats

Group	Lipid parameters			
	TG (mg/dL)	**TC (mg/dL)**	**HDL (mg/dL)**	**LDL (mg/dL)**
C	0.52 ± 0.08	1.46±0.13	1.35±0.16	ND
CCl_4	0.84 ± 0.21	1.90±0.37	1.20±0.30	0.11±0.22
E50	1.40 ± 0.12*	2.05±0.19	0.90±0.05*	0.42±0.08*
E250	1.20 ± 0.00	1.70±0.00	1.01±0.04	0.65±0.03
Ato	1.40 ± 0.56	2.43±0.67	1.06±0.43	1.21±0.51

Values are mean ± SD.

* indicates statistical significance against CCl_4 ($p<0.05$).

TG: Triglycerides, **TC**: Total cholesterol, **HDL**: High density lipoproteins, **LDL**: Low density lipoproteins, **ND**: Not detected, below threshold level, **C**: Control, **CCl_4**: Carbon tetrachloride, **E50**: 50 mg/kg *F. deltoidea* extract, **E250**: 250 mg/kg *F. deltoidea* extract, **Ato**: Atorvastatin

Table 4.5: Cardiac tissue marker enzymes in rats serum of CCl_4-induced rats

Group	Cardiac tissue marker enzymes				
	ALP (IU/L)	**AST (IU/L)**	**ALT (IU/L)**	**CK (IU/L)**	**LDH (IU/L)**
C	290.17 ± 2.43	104.17 ± 1.57	69.17 ± 1.05	1148.67 ±21.38	321.5 ± 4.97
CCl_4	398.67 ± 1.37*	170.67 ± 3.23*	84 ± 2.23	2912 ± 26.32*	693.67 ± 15.3*
E50	258.5 ± 2.33**	109.5 ± 1.26**	72.5 ± 1.1	1389.5 ± 12.21**	329.5 ± 4.86**
E250	270 ± 2.41**	110.75 ± 1.06**	67.25 ± 1.46	1116.25 ± 9.55**	367.25 ± 4.98
Ato	301.83 ± 1.94**	118 ± 1.28**	70.67 ± 0.97	1235.17 ±17.36**	354.17 ± 6.79**

Note: All cardiac tissue marker enzymes were outsourced to Clinical Diagnostic Lab (CDL), Faculty of Medicine, University of Malaya

Values are mean ± SD.

* indicates statistical significance against C ($p < 0.05$).
** indicates statistical significance against CCl_4 ($p < 0.05$).

ALP – Alkaline phosphatase, **AST** – Aspartate aminotransferase,
ALT – Alanine aminotransferase, **CK** – Creatine kinase,
LDH – Lactate dehydrogenase. **C** – Control, **CCl_4** – Carbon tetrachloride,
E50 – 50 mg/kg *F. deltoidea* extract, **E250** – 250 mg/kg *F. deltoidea* extract,
Ato – Atorvastatin

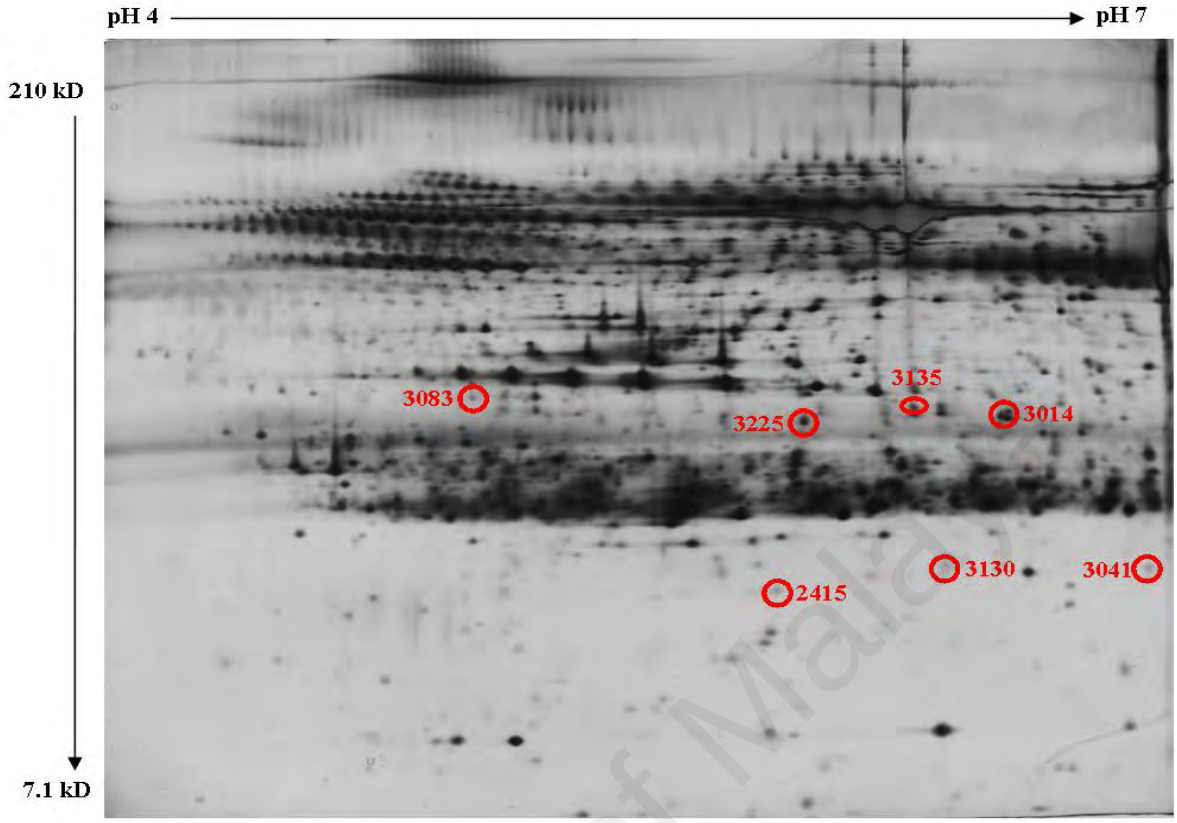

Figure 4.5: A representative of 2D-GE protein profile from rat serum induced with CCl_4 , ranged between pH 4-7

Differentially expressed protein spots and each spot numbers are tabulated in Table 4.6.

4.2.4 Database mining for protein identification (ID) of differentially altered proteins

4.2.4.1 MASCOT database

Among all proteins observed in the serum of CCl_4-induced rats, 10 protein spots showed significant differences ($p < 0.05$). Based on MALDI-ToF/ToF MS data search, seven spots from CCl_4-induced group in Table 4.6 was matched with known proteins in MASCOT database. The identified proteins were matched using mass values either peptide masses or MS/MS fragment ion masses in which are based on a probability-based scoring. The other three protein spots were unidentified. None of the low MOWSE score proteins from CCl_4 group were successfully identified using ProFound software.

Table 4.6: List of proteins with significantly altered abundancies in CCl_4-induced rats, as identified by MALDI-ToF/ToF MS-based 2D-GE

Spot	Protein identification	Acc number/ SWISS PROT	MASCOT[a]		Sequence coverage (%)	Theoritical		Observed	
			Mascot score	Matched peptide		Mw (kDa)	pI	Mw (kDa)	pI
3041	Tropomyosin (**TPM2**)	TPM2_RAT	54	16/145	49	32.9	4.7	16	6.9
3225	Myosin heavy chain-6 (**MYH6**)	MYH6_RAT	47	20/61	12	224	5.6	27	6.0
2415	Small-ubiquitin related modifier 3 (**SUMO3**)	SUMO3_RAT	48	4/23	29	12.6	5.6	14	5.9
3014	Small-ubiquitin related modifier 3 (**SUMO3**)	SUMO3_RAT	39	4/40	30	12.6	5.6	24	6.5
3135	Small-ubiquitin related modifier 3 (**SUMO3**)	SUMO3_RAT	50	4/22	30	12.6	5.6	28	6.3
3130	Apolipoprotein A1 precursor (**Apo A1**)	P04639	25	5 / 40	12.5	27.4	5.5	13	6.5
3083	Apolipoprotein E (**Apo E**)	Q6PAH0	259	19 / 35	54.0	33.8	5.2	30	5.0

Database search using Mascot : http://www.matrixscience.com

[a] denotes Mascot probability based molecular weight search score calculated for PMF. Protein score is -10 x log [P], where P is the probability that the observed match is a random event. The scores are based to Swiss-Prot database using the MASCOT searching program in MS/MS spectra.

4.2.5 Functions of identified proteins

The significantly altered proteins were mainly associated with lipid metabolism and transport, inflammation and immune response, anti-oxidant protein, acute phase protein, cytoskeletal and structural proteins, and myosin-complex related proteins. The biological process based on UniProt denotes the involvement of these proteins in cholesterol transport, muscle contraction and remodeling, anti-oxidant protein as well in immunity. The proposed categorized functions of these proteins are depicted in Figure 4.6.

4.2.6 Protein abundancy fold change comparison

Comparison between normal (control) and CCl_4-induced (CCl_4) rats showed differences in term of protein abundancies. CCl_4-induced or disease-state rats demonstrated an increased in abundance of SUMO3 (2.3 to 4.6 fold), MYH6 (2.1 fold), TPM2 (3.4 fold) and decreased in abundance for Apo A1 (-0.8 fold) and Apo E (-0.3 fold). Atorvastatin (Ato), the common cholesterol lowering drug markedly increased protein abundancy of Apo A1 (2.6 fold) and hence contrary, decreased abundance of SUMO3 (-1.3 to -4.6 fold), MYH6 (-2.6), TPM2 (-2.2 fold) when compared to CCl_4 – induced group. Not much expressions noted on Apo E with Atorvastatin treatment.

With comparison to CCl_4-induced group, pre-treatment with higher dosage of *F. deltoidea* extract (250 mg/kg-E250) markedly induced increase in abundance of Apo A1 (1.7 fold) and Apo E (2.7 fold); and decrease in abundance for SUMO3 (-1.3 to -2.5 fold), MYH6 (-2.2 fold), TPM2 (-3.1 fold). Treatment with lower dosage of *F. deltoidea* extract (50 mg/kg-E50) however, almost leads Apo A1 to normal level in treated CCl_4-induced rats. Other proteins however exhibited similar pattern as observed in E250 with exception in greater intensities. Comparative analysis of fold change and spot abundancy patterns of each identified protein are shown in Figure 4.7 and Figure 4.8 respectively.

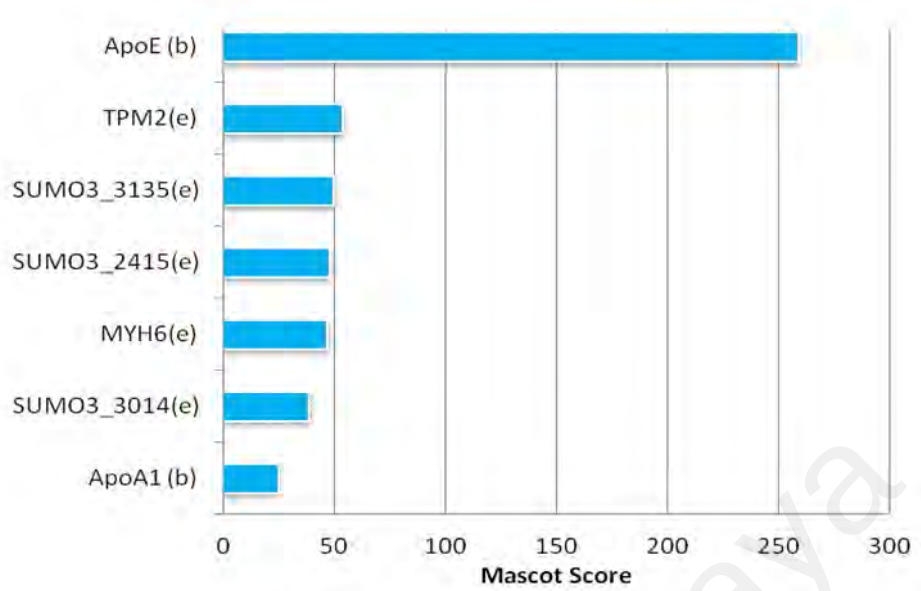

Figure 4.6: Categorized functions of identified proteins acquired by data search using MASCOT database

The identified proteins were displayed horizontally with respective categorized functions (a) Inflammation and immune response; (b) Lipid metabolism and transport; (c) Anti-oxidant protein; (d) Acute phase protein; (e) cytoskeletol and structural function.

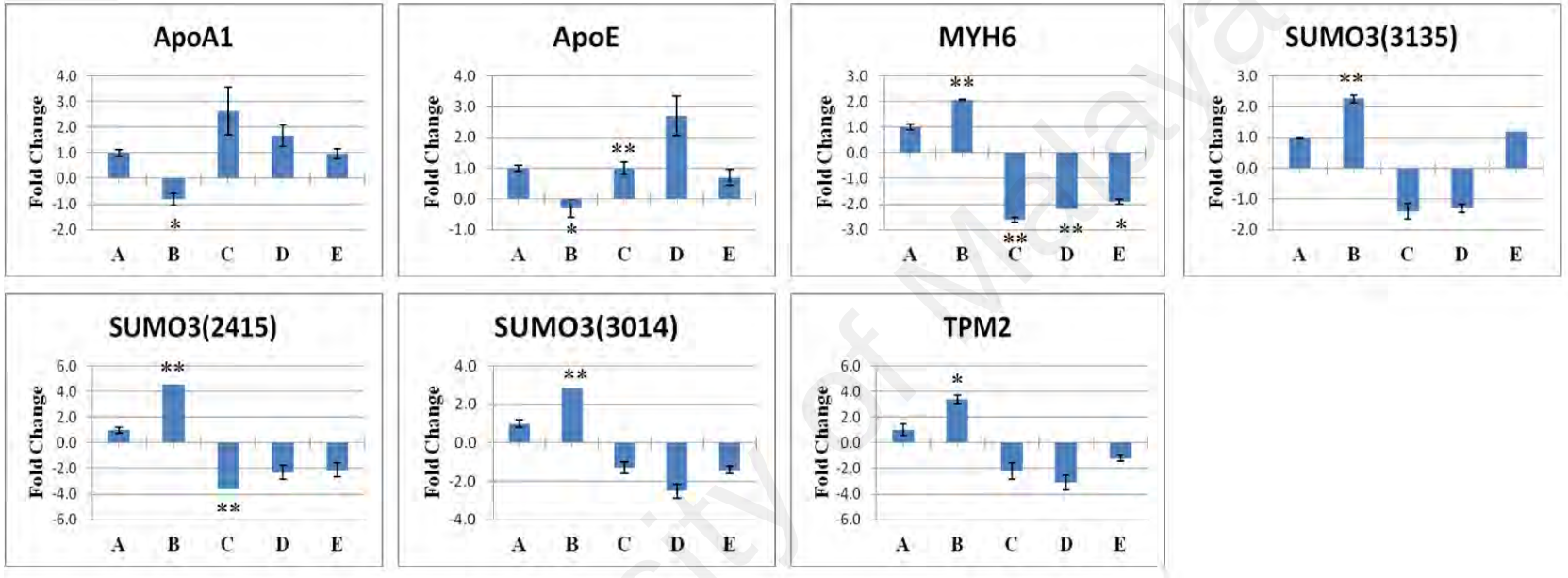

Figure 4.7: Comparative fold change patterns of the identified protein spots in CCl_4-induced rats

A: Control; **B**: CCl_4-induced MI rats (CCl_4); **C**: Atorvastatin-treated CCl_4 (Ato); **D**: *Ficus*-treated CCl_4 (E250); **E**: *Ficus*-treated CCl_4 (E50).

Fold change was calculated by dividing the average normal spot volumes from each of the two groups. CCl_4-induced (CCl_4) was compared to control (C); whereas fold change for the treated groups were measured against CCl_4 to denote recovery to normalize value (if present). Data are expressed as mean±SEM of 4 experiments with 4 replicates. The mean difference is significant at fold change of ≥ 2 fold with $p < 0.05$ (*). and $p < 0.01$ (**), where applicable.

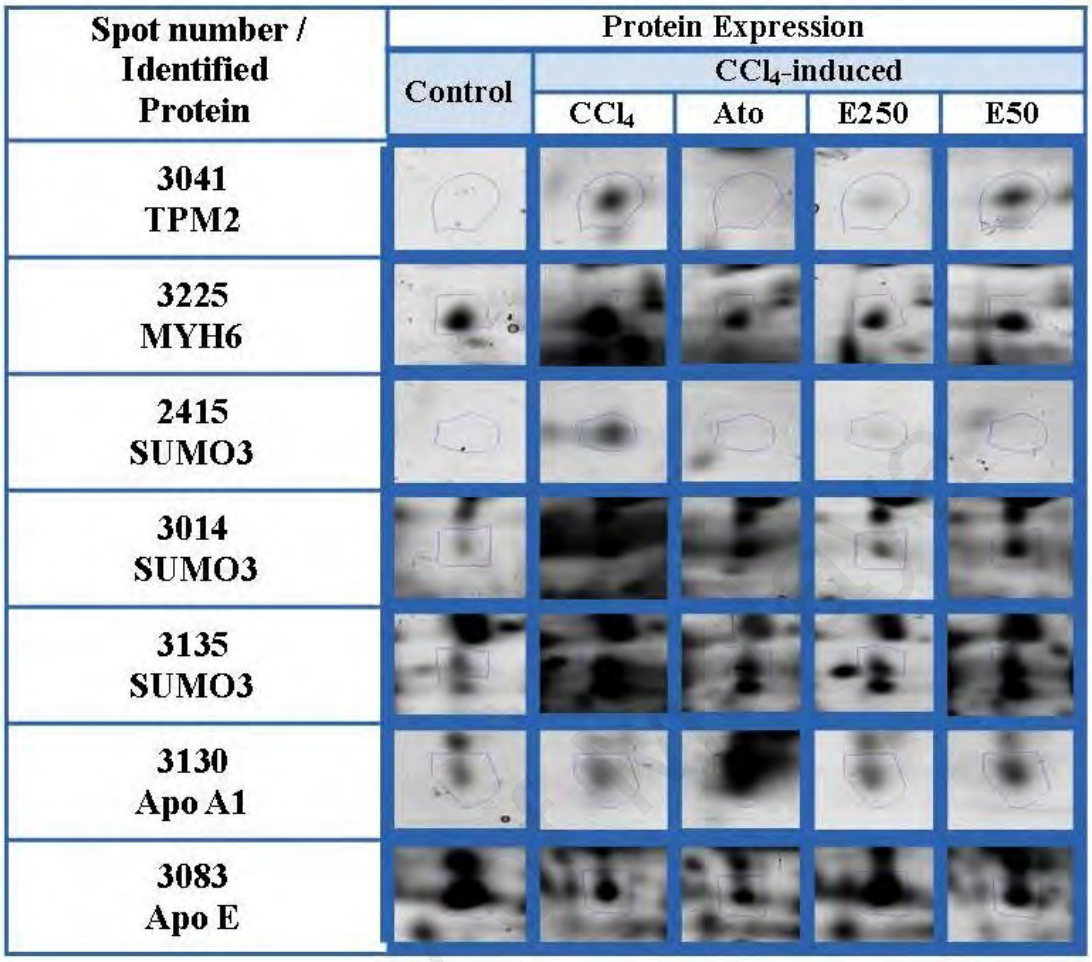

Figure 4.8: Protein spots differential abundancy patterns for treated and non-treated CCl_4-induced rats.

Magnified spots of 2D-GE images represent differential abundancy patterns between the different rat groups. **C**: Control/normal rats; **CCl_4**: CCl_4-induced rats; **Ato**: Atorvastatin-treated rats; **E250**: *Ficus*-treated rats (250 mg/kg body weight); **E50**: *Ficus*-treated rats (50 mg/kg body weight)

4.3 Validation by Western immunoblotting

Consecutively to validate the differentially altered proteins abundancies detected by the proteomics analysis, western blotting of HAP (MW ≈ 11 kDa) and RBP4 (MW ≈ 21-25 kDa) were performed. The protein quantification of HAP and RBP4 were randomly selected respectively from two different categorized functions, and since the ease in obtaining both antibodies for western blot validation. Pooled normal serums (Lane A) were used as the control. Protein expression levels of both HAP and RBP4 were increased

abundantly in hypercholesterolemic-induced rats. Following Atorvastatin and *F. deltoidea* extract treatments, HAP was further increased in abundance and in contrary, RBP4 was decreased. As shown in Figure 4.9, the alterations of these differentially expressed proteins were in agreement with 2D-GE results, which implicated the coherency of 2D-GE result.

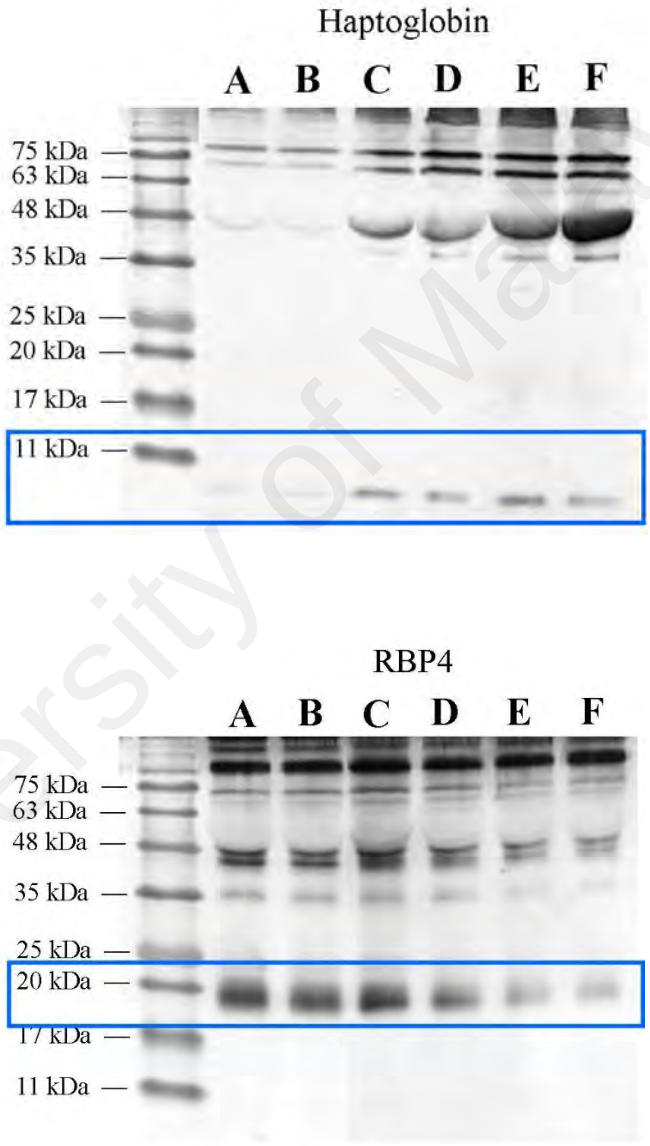

Figure 4.9: Western immunoblot validation for serum levels of HAP and RBP4 in hypercholesterolemic-induced rats

Lane **(A)** Pooled normal serum;**(B)** Control/normal rats; **(C)** Hypercholesterolemic-induced rats, HC; **(D)** HC Atorvastatin-treated rats; **(E)** HC *Ficus*-treated rats (50 mg/kg body weight) and **(F)** HC *Ficus*-treated rats (250 mg/kg body weight).

CHAPTER 5: DISCUSSIONS

5.1 Anti-hypercholesterolemic effect of *Ficus deltoidea*

5.1.1 Serum lipid profiles

The cholesterol-lowering effect of *F. deltoidea* based on lipid profile and proteomics analysis in hypercholesterolemic-induced rats was evaluated in this study. Different extract dosages were given based on human consumption on a commercially available *F. deltoidea* tea bag at 5 mg/kg multiply with the rats body weights. Lipid profile which included total cholesterol (TC), triglyceride (TG), HDL and LDL levels were determined prior to proteomics analysis. The outcome of lipid profiling revealed that *F. deltoidea* extract at 50 mg/kg of body weight of EE concomitant with cholesterol lowering drug (Atorvastatin) in decreasing TC, TG and LDL levels when compared to the HC group. Both E50- and Atorvastatin-treated groups demonstrated a normalizing effect as the reduced levels of TC, TG and LDL were almost nearing the baseline values of control rats.

Alongside the marked reduction in both TC and TG levels, these treatments showed elevated HDL cholesterol level (compared to control). At the same time, these treatments also lowered the HDL level compared to the HC-group which implied the normalizing effect. Both observation indicate the beneficial effects of *F. deltoidea* extract administration in preventing or reducing the risk of CVD as it showed the potential to maintain the HDL level within certain range. Hayek and team (1993) suggested in their study that elevated HDL was accordance with the build-up of HDL flux through the HDL pathway following high fed-high cholesterol dietary loaded. Hayek *et al.* (1993) subsequently concluded that inability to reciprocate HDL increment after HC-induced may be at high risk proceeding to CVD. Similarly to Zhukova *et al.* (2014) study on delayed HC induced rats; they ascribed the raised HDL accumulation duly to lipid metabolism consequence to overloaded high fat diet effect. These observations suggested

that *F. deltoidea* extract feeding may influence the suppression of cholesterol synthesis, thus exaggerate receptor-mediated catabolism of LDL-cholesterol which will then elevate blood LDL-cholesterol uptake and increased fecal bile acid secretion in rats (Khanna *et al.*, 1996).

To date, hypercholesterolemia is still the main risk factor in contributing the progress of cardio atherosclerosis. In an earlier finding by Wang and the team (1998), it was notably that hypercholesterolemia level indeed determines the severity of myocardial infarction. Pretreatment of statins (10 mg/kg body weight) and *F. deltoidea* plant extracts (50 and 250 mg/kg body weight) to HC rats however markedly reduced HDL levels, preventing elevation of other serum lipid profiles. The propensities of these lipid profiles to normalize in post treated group upon statins uptake exhibit concomitance mechanisms of statins drug and *F. deltoidea* extracts.

5.1.2 Proteome analysis of rats serum

In previous studies carried out, serum proteins were abundantly detected in a pI between 4 to 7 and these acidic proteins demonstrated better resolution in gels covering pH 4–7 (Chiaradia *et al.*, 2012; Gong *et al.*, 2008). Therefore, in this study, the proteome analysis was performed using a 24cm length of the linear IPG strip (pH 4-7). Protein electrophoresis were conducted to separate serum proteins accordingly to their isoelectric point (IEF) during first dimension and subsequently to their molecular weight (Mw) in second dimension electrophoresis. All gel images were then aligned with Progenesis Software to obtain a reproducible profile of 800-1000 protein spots per gel. The spots were scattered with a molecular mass between 7.1 – 210 kDa and pI range 4 to 7. Rats were used in this study due to their feasibility and susceptibility to atherosclerosis upon high fat diet induction (Leong *et al.*, 2015). This is due to the impairment of the cholesterol and bile acid metabolism where the rats are devoid of cholesteryl ester transfer

protein (CETP) that function to commute cholesterol ester and triglycerides between the LDL and HDL cholesterol components (Conn, 2013). As a consequence, the animal study is important in evaluating anti-hypercholesterolemic drug's efficacy (Zaragoza *et al.*, 2011) as well in understanding the biological process in disease progression and treatment response using medicinal plants. Altered serum proteome levels of HC rats upon treatment with *F. deltoidea* therefore may help in understanding the mechanism of *F. deltoidea* in eliminating the cholesterol build-up.

Intriguingly, most literatures on proteomic study advocate abundant protein depletion during sample preparation in finding novel biomarkers on certain diseases. This enables higher yield of low abundant proteins by masking all high abundant proteins in the plasma through proteomic approach. In our study, depletion of high abundant proteins is not performed for the aim of this study is to conduct comparative analysis of the whole proteome between normal rats, non-treated and treated induced-rats.

The high fat diet-induced hypercholesterolemia as diet regime itself composed of both cholesterol and lipid, which play an important role in the development of cardiovascular lesions (Ram *et al.*, 2014). Prolonged hypercholesterolemia not only will lead to cardiovascular disease, but also chronic complications that include Type 2 diabetes mellitus, strokes, kidney failure and diabetic retinopathy (Phuong *et al.*, 2014). The prolonged cholesterol accumulation in macrophages and immune cells in advance stimulate inflammatory responses of Toll-like receptor (TLR) signaling, production of the phagocytes (monocytes and neutrophils) in the spleen and marrow along with inflammasome activation. Hence, the exaggeration of TLR signaling in cellular alleviated cholesterol efflux resulted in further cholesterol accumulation and raised up inflammatory responses (Tall & Yvan-Charvet, 2015). The inflammation may be beneficial in response to infection; however, condition exacerbated for those with chronic metabolic diseases.

Comparative observation between proteome profiles of the serum derived from HC-induced rats with serum from statin and *F. deltoidea* extracts treated rats resulted in the identification of several proteins with specific / unspecific relationship with hypercholesterolemia.

5.1.3 Potential hypercholesterolemia putative proteins

Identification of the altered serum proteome from HC rats upon treatment with *F. deltoidea* may help in understanding the mechanism of cholesterol lowering properties of *F. deltoidea*. This present study discovered 30 significant protein spots that showed >1.5 fold differential expressions. Among the identified differential proteins, focus are directed to these 12 proteins (Apo A1, Apo E, RBP4, HAP, PDE1, C3, C1s, PROM1, TTR, MUP, CPI and PLG) which are associated with inflammation and immune response, lipid transport and metabolism, cholesterol homeostasis and cholesterol binding. The putative functions of these proteins in hypercholesterolemic are briefly elaborated below

5.1.3.1 Haptoglobin (HAP)

Haptoglobin (HAP) is a tetrameric structure with two alpha (α) chains and two beta (β) chains bonded by disulphide. It is produced in abundance in the liver before being released into blood plasma to combine with free plasma haemoglobin (Hb) or red blood cell (RBC). HAP is an acute phase protein that helps to bind hemoglobin and lower the risk of iron loss, thus, acting as an antioxidant (Morimatsu *et al.*, 1991; Roguin *et al.*, 2001). An elevated level of HAP triggers the accumulation of plasma haemoglobin or red blood cell (RBC), hence indirectly upregulate plasma lipids and lipoproteins in experimental hypercholesterolemia subjects (Weng *et al.*, 1998).

Previous studies relate the several fold increase of plasma HAP with systemic inflammatory stimuli on infection, malignancy and cardiovascular events (Bergamini *et*

al., 2014; Carbone & Montecucco, 2015; Ray *et al.*, 2015). In this study, HAP in HC group was increased in abundance compared to the control. This increment could be suggested as the anti-inflammatory and antioxidant responses of HAP during the progression of cardiovascular disease. Treatments of Atorvastatin and *Ficus* extracts maintained the same pattern as in HC suggesting the corrective effort of the treatments post-hypercholesterolemic induction.

While hemolysis occurs in the artery, the oxidative stress will be elevated to promote the initiation of LDL oxidation and destruction of vascular endothelium. Following the internalization of oxidative LDL particles in macrophages through specific scavenger receptors, the accumulation of oxidized LDL initiates the formation of atherosclerosis. Therefore, HAP may serve as an important role in prevention of atherosclerosis due to its potent antioxidant property. Since HAP was significantly increased after Atorvastatin and *F. deltoidea* extracts treatment, there is plausible reason of antioxidant roles that raised the antioxidant response in the hypercholesterolemia rats (Alonso-Orgaz *et al.*, 2006).

5.1.3.2 Retinol Binding Protein 4 (RBP4)

It has been revealed that RBP4, a fat mimicking adipokines in transporting retinol (Vitamin A) is a potential metabolic and cardiovascular biomarker associated with hypercholesterolemia (Carbone *et al.*, 2015; Christou *et al.*, 2012). Aguilar and Fernandez (2014) revealed in their study regarding the susceptibility of adipose tissue attributed in cholesterol metabolism. As a consequence, hypercholesterolemia leads to adipocyte cholesterol overload and thus, causing inflammation.

There are rarely proteomic study approaches conducted on hypercholesterolemia animals on *F. deltoidea* extract treatments. Our study experimentally demonstrated an increase of abundance in RBP4 level in HC rats. Contrary, a recent study by Calò *et al.*

(2014) employing *in vitro* and *ex vivo* vasolidation demonstrated a reduced in RBP4 in high cardiovascular risk subject. Among the metabolic syndromes, hypercholesterolemia with non-metabolic disorder like obesity or diabetes elucidated the greater affiliation with RBP4 (Ingelsson *et al.*, 2009; Kim *et al.*, 2011).

Post *F. deltoidea* treatments experimentally exhibited normalized RBP4 expression in our animal study. The results obtained from 2D-GE and Western blotting clearly showed correspondent results (see Figure 4.4 and Figure 4.9) prior and after *F. deltoidea* treatment, indicating the treatment possess cholesterol lowering activity along with remarkable impact on high fat-diet given. In addition to this, Graham *et al.* (2007) also reported the 'gold standard' assessment for RBP4 using Western blot.

5.1.3.3 Transthyretin (TTR)

Transthyretin, TTR also known as Prealbumin is a tetrameric structured protein that derives mainly from the liver and the choroid plexus of the brain. TTR functions to transport protein for thyroxine (T4), triiodothyronine (T3) and retinol (Vitamin A) (van Bennekum *et al.*, 2001). TTR (≈ 55,000 da) interacts with the small molecular weight (≈ 21,000 da) RBP4 from lipocalin protein family forming a protein complex prevents the loss of RBP4 by permeation through kidney glomeruli. In previous study carried out, TTR is a potent protein in cholesterol metabolism (Liz *et al.*, 2007) and exhibits anti-inflammatory properties (Borish *et al.*, 1992). It is also suggested that TTR is a marker of hypercholesterolemia in humans (Yoshida *et al.*, 2006). Reduced TTR levels also exhibited in several pathological condition like Alzheimer's disease (Velayudhan *et al.*, 2012), Type 1 diabetes (Zhang *et al.*, 2013), ovary malignancy (Goufman *et al.*, 2006) and myocardial infarction (Cubedo *et al.*, 2012). However, treated group with cholesterol lowering drugs (Ato) and *F. deltoidea* extract (E50) significantly increased TTR abundantly when compared to HC rats. This finding also suggests further up regulated TTR levels may have cardio protective effect. Intriguingly, TTR is regulated by high

density lipoprotein (HDL) in correspondence of lipid-free Apo A1, and the stability of the HDL fragments are not affected by the existence or absence of TTR (Sousa *et al.*, 2000).

5.1.3.4 Prominin 1 (PROM1)

Prominin 1 (PROM1) also known as Fudenine or CD133 was first identified in mouse neuroepithelial stem cells is a cholesterol interacting pentaspan membrane glycoprotein. The molecular and physiological functions of PROM1 remain unclear. However, in an earlier work in 2007, Yang and colleagues reported that the hyperglycemic event in their animal study induced the expression of PROM1 in rat's skeletal muscle by raising glyceraldehyde 3-phosphate dehydrogenase (GADPH) enzyme which further attached to the LDLs in the blood, forming the atheromatous or sticky plaques. In our present study, PROM1 in HC group was slightly increased in abundance. Upon treatments with Atorvastatin and *F. deltoidea* extracts, it could be observed that PROM1 was reduced.

5.1.3.5 Apolipoproteins (Apo A1 and Apo E)

Apolipoprotein A1 (Apo A1) and Apolipoprotein E (Apo E) play important roles in cholesterol transport and metabolism. These 2 lipoproteins levels are usually contrary, where a low level of HDL and elevated LDL contributes to coronary artery disorder (CAD).

Apo E is the ligand for LDL receptor which is triglyceride-rich lipoproteins and strongly correlated with cardiovascular disease and an emergent risk factor for Alzheimer's disease (Lane-Donovan & Herz, 2014; Mahley & Rall, 2000; Weisgraber, 1994). Apo E transports lipoproteins, cholesterol and fat soluble vitamins to the lymph system and subsequently to the blood plasma. On the other hand, Apo E promotes anti atherosclerotic property by regulating lipoprotein metabolism and exerting cellular

cholesterol efflux (Zanotti *et al.*, 2011). Previous study has shown the susceptibility of Apo E within the progression of cardiovascular disease as a result to elevated HDL-cholesterol (Khan *et al.*, 2013). The up regulated Apo E is related to abnormal LDL-R and may cause hypercholesterolemia, a distinguished feature in atherosclerosis.

Apo A1 is the fundamental apolipoprotein linked with HDL and can be found in chylomicrons. Recent study carried out by Majek *et al.* (2011) revealed that elevated level of Apo A1 may play an important role in prevention of further heart diseases and contrary, lower level of Apo A1 may contribute to cardiovascular complications. HDL in the form of Apo A1 displays several functions in the reverse transport of cholesterol (RCT) between tissues and liver by promoting cholesterol efflux (Quaye, 2008). Contrary in our animal study, a hypercholesterolemic diet increased the lipid profiles including HDL cholesterol but decreased of abundance in Apo A1 suggesting the impairment of reverse cholesterol transport and possess an increase in cardiovascular risk.

In line with this, a decrease in abundance was observed for Apo A1 in HC serum proteome in comparison to control rats whereas Apo E was elevated. Treatment with *F. deltoidea* extract caused an increase in Apo A1 and reduced Apo E abundancies. These observations reflect the lipid profile pattern following E50 and E250 treatments. Changes in abundances of Apo A1 and Apo E following treatment with Atorvastatin showed an almost similar manner as E50, which also in agreement with the lipid profile analysis. This study suggests that the use of Atorvastatin and *F. deltoidea* may lead to the elevation of serum HDL that consequently will inhibit cholesterol build-up and clearance of cholesterol-rich proteins from plasma respectively.

5.1.3.6 Complement Factor (C3 and C1s)

Significant differences were also observed in proteins relevant to inflammation and immune system. Both complement components (C3 and C1s) were markedly

increased in HC rats due to the progression of complement activation in atherosclerotic lesions beyond the foam cell stage. C3 isolated from hypercholesterolemic subjects were apparently enriched in HDL and related to innate immunity as well as HDL's cardioprotective effect (Vaisar *et al.*, 2007). Hence, many studies concluded the elevated serum C3 level in inflammatory condition is associated with myocardial infarction (Muscari *et al.*, 1995), hypertension (Engström *et al.*, 2006) and atherosclerosis (Alwaili *et al.*, 2012). However, no apparent difference was observed in HC rats treated with lipid lowering drug (Atorvastatin). This may due to inadequate treatment period (Muscari *et al.*, 2001) to reduce the elevated C3 levels. In contrary, the C3 level in E250 treated HC decreased in abundance. This indicated that *F. deltoidea* extract may also involve in non-lipid lowering effects and postulated anti-inflammatory mechanisms when higher dosage of *F. deltoidea* extract consumed.

5.1.3.7 Phosphodiesterase 1 (PDE1)

In mammals, PDE enzymes are diversely categorized into 12 big families (PDE1 –PDE12) along with their specific structure and functional properties. Phosphodiesterase type 1 (PDE1) is wholly calcium- and calmodulin- (Ca^{2+} / CaM) dependent PDE enzyme for its activity, with 3 subtypes (PDE1A, PDE1B and PDE1C) and further classified to its isozymes (Lugnier, 2006). The PDE1 isomers are mainly deposited in skeletal muscles, smooth muscle, liver, coronary heart, lung, brain and testis, with PDE1A expression dominant in mice (Johnson *et al.*, 2012) and PDE1C in human beings (Vandeput *et al.*, 2007). In addition to this, PDE1C not only have a functional role in insulin homeostasis (Dousa, 1999) but also regulates smooth muscle cell proliferation due to atherosclerotic lesions or recurrent of stenosis episode (Rybalkin *et al.*, 2002). In our present study, the abundance of PDE1 was increased in HC rats. Nonetheless, no significant difference was noted on hypercholesterolemic rats treated with common

cardiovascular (Atorvastatin) drug as compared to *F. deltoidea* extracts (50 and 250 mg/kg). Thus, there is probable effect of *F. deltoidea* extracts inhibiting the action of PDE and consequently declining the breakdown of cyclic adenosine monophosphate (cAMP). Apparently *F. deltoidea* extracts may have diverse therapeutic benefits as phosphodiesterase inhibitor (PDE1) on the heart, lung, brain, testis, skeletal muscles and smooth muscles.

5.1.3.8 Major Urinary Protein (MUP)

MUP or Alpha-2 microglobulin (A2UM) from lipocalin family is a unique low molecular weight secreted protein binds to lyphophilic pheromones that mediate chemical attraction in rodent species. Other than this, physiological functions of MUP as glucose regulator and lipid catabolism via autocrine or paracrine mechanisms in liver of both genetics and high fat diet regime induced obesity and diabetic rodent were also reported (Zhou *et al.*, 2009). MUP is abundantly synthesized in the liver before discharged into bloodstream and emitted in the urine (Roy & Neuhaus, 1966). The published report demonstrated a decrease in abundance of MUP in both genetics and high fat diet regime induced obesity and diabetes rodents. Baynes (1991) however, stressed the correlation between long term diabetes and lipid peroxidation elevation in tissues and plasma. Treatment in experimental HC rats with statin drug and *F. deltoidea* extracts in our study exhibited further significant decrease in abundance of MUP, hence there is plausible mechanism in inhibition of free radical generation and oxidative damage to the cell membrane. Our findings coincide with the existing result by Steiner and colleague (2000) where high dosage of Lovastatin drug decrease the abundance of alpha-2-microglobulin in the liver thus reduce its synthesis alpha 2-microglobulin-related protein from rats, a protein so far unknown in humans.

5.1.3.9 Contrapsin (CPI)

Another interesting protein identified is CPI, which is a serine protease appeared to be restricted in rodents, but also homologue with human alpha-1-antitrypsin (Hill *et al.*, 1984). The name evolved from the combination of "contra" and "trypsin" (Takahara & Sinohara, 1982). CPI is another remarkably acute phase reactant involved in atherosclerosis (Wait *et al.*, 2005) that triggered inflammatory disorder.

Hypercholesterolemia tends to attenuate and blunt endothelial dysfunction through deposition of matrix on arterial wall which later accelerate the atherosclerotic lesions. Hence, this inflammatory process consequently impaired angiogenesis where new vessels intended to form consecutively throughout the cardiac cell regenerative process. Our current study noted markedly reduced CPI abundancy in all treated group rats with the most in E250 group when compared to HC rats. The ability of E250 extract in lowering CPI providing the potential of natural remedies in combating atherosclerosis related inflammations.

5.1.3.10 Plasminogen (PLG)

PLG is another cardiovascular related protein in this study, which play crucial role in arterial thrombosis (Carmeliet & Collen, 1995; Deitcher & Jaff, 2002). Furthermore, common risk factors for atherosclerosis, including hypercholesterolemia, obesity, Type 2 diabetes, hyperinsulinemia, and high blood pressure parallel with the increment of Plasminogen Activator Inhibitors (PAI) levels which is suggestive risk of cardiovascular disease (Sehestedt *et al.*, 2011). PLG and the derived serine protease, plasmin, together with the PAI is postulated to influence cardiovascular inflammatory responses (Plow & Hoover-Plow, 2004). This effect also occurred in *F. deltoidea* extracts treatment in which PLG markedly downregulated on HC rats. It is noteworthy to demonstrate pleiotropic (non-lipid lowering) effects of statin mechanisms where both anti

thrombotic and anti-platelet effect ameliorate in response to statin therapy (Lefer *et al.*, 2001).

5.2 Cardioprotective effect of *Ficus deltoidea*

5.2.1 Cardiac tissue markers enzyme analysis

Carbon tetrachloride (CCl_4) infusion were used in our animal study to induce cardio injury in mice. The assessment of lipid profile which included total cholesterol (TC), triglyceride (TG), HDL and LDL levels and cardiac tissue biomarkers (AST, ALT, ALP, CK and LDH) were carried out prior to proteomics analysis. The outcome of lipid profiling revealed the minimal effect for *F. deltoidea* extract against CCl_4-induced cardio toxicity in mice. However, the risk of cardiac infarct in our investigation is concordance with progression of cardio atherosclerosis following significant leakage of the cardiac tissue biomarkers from cells into serum. Higher levels of TC and LDL were observed in CCl_4-induced group when compared to the normal group. With the elevated TC and LDL levels, treatments with Atorvastatin drug and both E50- and E250-treated groups did not show much reduction levels of TC and LDL in comparison to CCl_4-induced groups.

Both *F. deltoidea* extracts and Atorvastatin-treated groups on the other hand demonstrated a normalizing effect as the reduced levels of ALP, AST, ALT, CK and LDH were almost nearing the baseline values of control rats. *F. deltoidea* extract at higher concentration concomitant with Atorvastatin in decreasing ALP, AST, ALT and CK levels when compared to the CCl_4-induced group. In this study, both E50- and E250-treated group show better performance in cardioprotection compared to cholesterol lowering effect. This study indicated the potential cardioprotective activity of *F. deltoidea* extracts against the CCl_4-induced cardiotoxicity in rats.

5.2.2 Proteome analysis of rats serum

Mammals are extensively used to mimic human cardiac models in studying tissue damage upon exposure to toxic agents (Dominguez-Rodriguez *et al.*, 2012) as well in understanding mechanism involved in disease development and treatment executed. In the present study, cardiac enzymes profile and proteomic analysis of the rat serum were investigated to explore the possible mechanisms of *F. deltoidea* extracts in cardioprotection. The cardioprotective property of *F. deltoidea* leaves extract against cardiac damage via CCl_4 induction was revealed by monitoring alteration in protein expression. Ten protein spots respectively from CCl_4-induced group showed significant ($p < 0.05$) differential expression with statins and *F. deltoidea* extracts pre-treatments. By comparing their PMF to the SwissProt databases, seven known proteins were identified.

The administration of *F. deltoidea* leaves extract with carbon tetrachloride (CCl_4) revealed the potential cardioprotective property against CCl_4 intoxication by augmenting the lipid peroxidation as well its cardiac functions and antioxidant enzymes in our animal study. CCl_4 is a toxic chemical compound that damage tissues via generation of hazardous free radicals (Adaramoye, 2009). Excessive formation of this free radicals exaggerated production of lipid peroxides resulting in irreversible damage to the cardiac and aorta in animal model subjected to CCl_4 induction (Golikov *et al.*, 1989). Therefore, CCl_4 not only possesses hepatotoxicity, but also induced cardiotoxicity (Jayakumar *et al.*, 2008; Nikolas A Botsoglou *et al.*, 2009). Thus, MI caused by CCl_4 intoxication to the rats would notably change the protein expressions. The putative roles of these cardioprotective proteins are discussed in the next section.

5.2.3 Potential cardioprotective putative proteins

Among the identified differentially expressed proteins, focus was emphasized on TPM2, MYH6, SUMO3, Apo A1 and Apo E, which are involved in cytoskeleton/contractile and structural function, cholesterol metabolism and transport, inflammation and immune response.

5.2.3.1 Apolipoproteins (Apo E and Apo A1)

High density lipoprotein (HDL) and low density lipoprotein (LDL) are major cholesterol carriers in blood plasma. High levels of HDL however integrally demonstrated cardioprotective effect (Heinecke, 2009). Intriguingly, Apo E decreased in abundance in CCl_4 induced-myocardial infarct rats. Our proteomic finding coherent with previous study carried out by Huang *et al.* (2011). They discovered that the marked reduction of Apo E abundancy occurred upon injury of the vessels and neointimal thickening in their animal subjects. Earlier report by Wientgen and friends (2004) (Wientgen *et al.*, 2004) subsequently revealed reduction of Apo E in abundance after 3 weeks' induction with CCl_4 to induce MI that enhanced progressive physiosclerosis.

The alteration of Apo A1 was observed to be slightly decreased in abundance in comparison to control rats whereas Apo E was elevated in HC rats. However, defective Apo A1 and increased Apo E expressions in serum proteome following *F. deltoidea* extract treatments were in agreement with our positive control using Atorvastatin, the lipid lowering medicine drug. Indeed, *F. deltoidea* treatments leading to the elevation of HDL blood that consequently inhibited cholesterol buildup and clearance of cholesterol-rich proteins from plasma respectively. Due to their biological process in lipid metabolism, the expressions demonstrated could favor the appearance of the progression of cardiovascular disease and prothrombotic circumstances (Mahley & Rall, 2000; Májek *et al.*, 2011).

5.2.3.2 Small Ubiquitin-Related Modifier 3 (SUMO3)

Small ubiquitin-like modifiers (SUMOs) belong to the ubiquitin like proteins (Ubls) superfamily that are covalently conjugated to their substrates via enzymatic cascade reactions (Wang, 2009). The SUMOylation pathway catalyzed the conjugated SUMO homolog proteins (SUMO1 - 3) to lysine protein residues. This ubiquitin-related transient post translational modification pathway indicating a critical role in cellular homeostasis and balanced de-/SUMOylation is mandatory for appropriate cardiac development and metabolism (Mendler *et al.*, 2016). These SUMO homolog proteins have been observed in vertebrates (Wilson & Heaton, 2008) in which SUMO-1 is the most interesting isoform for investigation under physiological conditions (Saitoh & Hinchey, 2000). Both SUMO-2 and -3 showed amino acid level of 95% similarity and only 50% match with SUMO-1. However, SUMO-4 was at first found to influence the pathogenesis of diabetes Type 1 but later discovered that it was incovalently conjugated due to the existence of proline-90 amino acid residue that obstruct its maturity process (Owerbach *et al.*, 2005).

In the recent proteomic investigation on myocardial infarct (MI)-induced rats, several proteins exhibited high abundancy in SUMO3 level after CCl_4 intoxication (Spot 2415, 3014 and 3135). Hence, it can be speculated that SUMO3 is detrimental when elevated in MI states. Post treatments with cholesterol lowering drug and *F. deltoidea* extracts (Ato, E250 and E50) lowered SUMO3 abundancy. However, *F. deltoidea* extracts at higher dose (E250) overall demonstrated near normalization when compared to control rats group. Hence, this study focused on the underlying features of SUMO conjugation system and its possible implication in cardiovascular progression either pathologically or physiologically.

5.2.3.3 Tropomyosin beta chain (TPM2)

Tropomyosin actively binds to actin filaments in both muscle and non-muscle cells. Tropomyosin isoforms (α-tropomyosin, β-tropomyosin and κ-tropomyosin) are markedly known with their functional roles in stabilizing actin filaments and regulate muscle contraction (Peng *et al.*, 2013). The cardiac troponin complex (troponin C, troponin I, and troponin T) bind with the myofilament protein, tropomyosin beta chain (β-tropomyosin or TPM2), were significantly raised in the cytosolic fragment after myocardial infarction. Hence, the calcium-binding to troponin regulate the mobility of TPM2 and thus accelerate muscle contraction (Bacchiocchi & Lehrer, 2002) the physiological role of tropomyosin in both muscle and non-muscle cells is not fully understood.

Based on the animal model study in CCl_4-induced myocardial infarction state, TPM2 is three times greater (3.4 fold) compared to control rats group. Treatments induced with Ato and E250 intriguingly bring TPM2 to normal level, but not lower dose extract (E50). With respect to this, proteome studies performed on the MI induced rats had identified differential expression of TPM2 that is associated with regulating the myocardial contraction and destression. Thus, this may explain the cardioprotection effects of *F. deltoidea* extracts.

5.2.3.4 Cardiac Alpha-Myosin (MYH6)

Cardiac myosin heavy chain is isoforms inclusive of α-myosin (MHY6) and β-myosin (MHY7). These isoforms are articulated in smooth muscle skeletal and cardiac where contractile activity of the muscles was triggered. During infarct episode, many living cardiac cell died due to necrosis in the human myocardium (Malliaras *et al.*, 2013). Hence, we speculated that decrease of abundancy in MYH6 may associate with the cardiac recovery observed in *F. deltoidea* extracts treated group.

5.3 General discussions

5.3.1 Summarized Venn diagram of the protein identified (High fat diet induced hypercholesterolemia versus CCl_4-induced MI)

In the present "omics" era, complex and huge analytical datas e.g. from proteomics, genomics and metabolomics disciplines acquire Venn diagram to analyze the interconnection datasets visually; and separation between the overlaps represent the coherent function between the datasets (Cai *et al.*, 2013; Chen & Boutros, 2011; Hulsen *et al.*, 2008). The Venn diagram as shown in Figure 5.1 represents the summary of the number of spots significantly altered in abundancy (as figured beside abbreviated identified protein) either in the group alone or overlap in both groups. Ten protein spots identified in cholesterol lowering group particularly compared to five significant identified protein spots from cardioprotective group demonstrated only two protein spots showed significant expressions in both groups. The two proteins that overlapped in the subsets were Apo A1 and Apo E. The other four proteins (Spectrin, PCYOX, ICal1, ENC2) identified found to be not relevant in the study. All these identified protein spots which comprised of 55% were checked against MASCOT database and online accessible ProFound software (see Section 4.1.4 and 4.2.4). However, out of total 40 significant protein spots excised for MS analysis, 17 protein spots could not be identified (14 spots from cholesterol lowering group and three spots from cardioprotective group). This may due to the relatively low abundance of the protein spots of interest that further lost during pooling and limited detection level of mass spectrophotometer. Thus, all these identified proteins can be grouped into their functional classifications: inflammation and immune response, lipid transport and metabolism, cholesterol homeostasis and cholesterol binding, cytoskeletal and structural proteins (see Section 4.1.5 and 4.2.5).

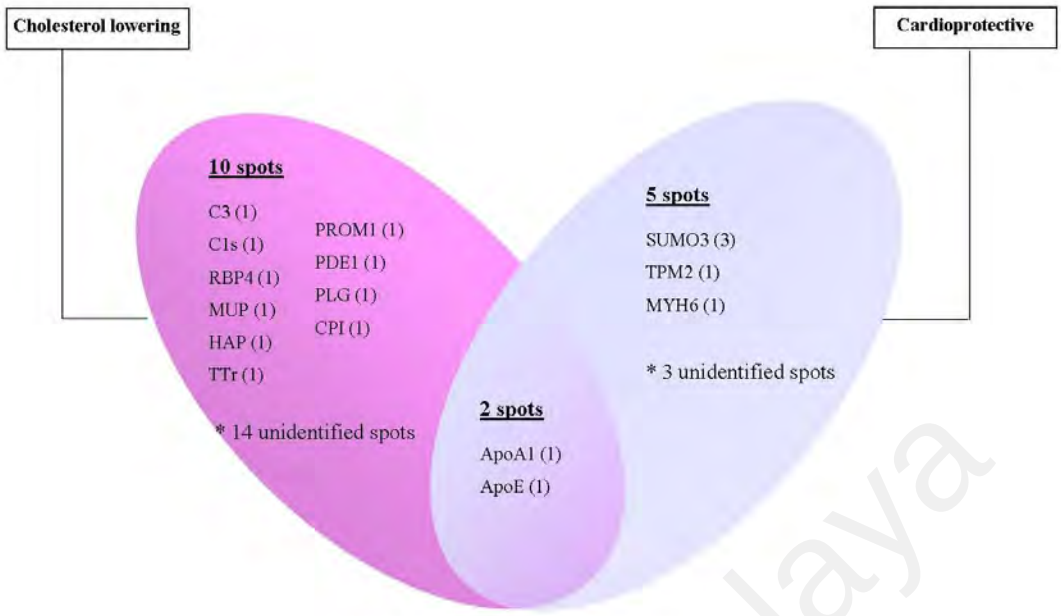

Figure 5.1: Venn diagrams demonstrated comparison between hypercholesterolemia induced groups (cholesterol lowering) with MI-induced group (cardioprotective).

Both sets depict the identified protein spots that significantly altered in protein abundance with their respective common abbreviated identified protein and unidentified protein spots; producing the subset of interest in between the two compared sets.

CHAPTER 6: CONCLUSION

In conclusion, this study demonstrated the suppression of hypercholesterolemia following *F. deltoidea* treatment with differential expression of serum proteins involved in lipid metabolism and transport, inflammation and immune response, anti oxidant protein, acute phase protein, cholesterol homeostasis, fatty acid catabolism and cholesterol binding as well in cytoskeletal and structural effects. These hypercholesterolemic related proteins (Apo A1, Apo E, RBP4, HAP, PDE1, C3, C1s, PROM1, TTR, CPI, MUP and PLG) are the key proteins responsible for the suppression of hypercholesterolemia following treatment with *F. deltoidea*. Consequently, TPM2, MYH6, SUMO3, Apo A1 and Apo E are associated with cardioprotective effects. Interestingly, Apo E and Apo A1 are the two profound putative proteins that exhibited both cholesterol lowering and ameliorate cardio protection effects. The variations of these proteins may provide valuable new molecular insights into the mechanism of cholesterol lowering effect of *F. deltoidea*. It could be speculated that long-term *F. deltoidea* consumption would setback further extent of cardio atherosclerosis and MI, as well subsequent cardiac function deterioration and mortality in HC subjects. Thus, further development of this study will enable the finding of alternative treatments as well as preventions for CVD.

REFERENCES

Abdullah, Z., Hussain, K., Ismail, Z., & Ali, R. M. (2009). Anti-inflammatory activity of standardised extracts of leaves of three varieties of *Ficus deltoidea*. *International Journal of Pharmaceutical and Clinical Research 1*(3), 100-105.

Adaramoye, O. A. (2009). Comparative effects of vitamin E and kolaviron (a biflavonoid from *Garcinia kola*) on carbon tetrachloride-induced renal oxidative damage in mice. *Pakistan Journal of Biological Sciences, 12*(16), 1146.

Adnan, N., & Othman, N. (2012). The relationship between plants and the Malay culture. *Procedia - Social and Behavioral Sciences, 42*, 231-241.

Aguilar, D., & Fernandez, M. L. (2014). Hypercholesterolemia induces adipose dysfunction in conditions of obesity and nonobesity. *Advances in Nutrition, 5*(5), 497-502.

Ahern, D. K., Kreslake, J. M., & Phalen, J. M. (2006). What is eHealth: Perspectives on the evolution of eHealth research. *Journal of Medical Internet Research, 8*(1), e4.

Ahmata, N., Basilona, S., Zakariab, Z. A., & Zainc, W. Z. W. (2008). Antinociceptive activity of the leaves of *Ficus deltoidea* aqueous extract. In Safri, I. and Haryono (Eds), *Proceedings of the International Seminar on Chemistry 2008* (pp. 247-251). Jatinangor, Indonesia: Universitas Padjajaran.

Alonso-Orgaz, S., Moreno, L., Macaya, C., Rico, L., Mateos-Cáceres, P. J., Sacristán, D., . . . López-Farré, A. (2006). Proteomic study of plasma from moderate hypercholesterolemic patients. *Journal of Proteome Research, 5*(9), 2301-2308.

Alwaili, K., Bailey, D., Awan, Z., Bailey, S. D., Ruel, I., Hafiane, A., . . . Genest, J. (2012). The HDL proteome in acute coronary syndromes shifts to an inflammatory profile. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 1821*(3), 405-415.

Alwan, A., Armstrong, T., Cowan, M., & Riley, L. (2011). *Noncommunicable diseases country profiles 2011*. Retrieved from http://www.who.int/nmh/publications/ncd_profiles_report.pdf (Accessed on 14 Aug 2014)

Amiera, Z. U. R., Nihayah, M., Wahida, I. F., & Rajab, N. F. (2014). Phytochemical characteristic and uterotonic effect of aqueous extract of *Ficus deltoidea* leaves in rats uterus. *Pakistan Journal of Biological Sciences, 17*(9), 1046-1051.

Aminudin, N., Chung, Y. S., Chee, E. S., Kang, I.N. & Lee, R. (2007). Blood glucose lowering effect of *Ficus deltoidea* aqueous extract. *Malaysian Journal of Science, 26*(1), 73-78.

Anderson, N. G., Matheson, A., & Anderson, N. L. (2001). Back to the future: The human protein index (HPI) and the agenda for post-proteomic biology. *Proteomics, 1*(1), 3-12.

Aris, T., Muhammad, F. M. Y., Abdul, A. A. G., Noor Ani, A., Mohd, A. O., Tee, G. H., . . . Nurul, A. K. (2015). *National Health and Morbidity Survey 2015: Non-Communicable Diseases, Risk Factors & Other Health Problems*. Kuala Lumpur: Ministry of Health Malaysia.

Aronson, D. (2003). Cross-linking of glycated collagen in the pathogenesis of arterial and myocardial stiffening of aging and diabetes. *Journal of Hypertension, 21*(1), 3-12.

Bacchiocchi, C., & Lehrer, S. S. (2002). Ca^{2+}-induced movement of tropomyosin in skeletal muscle thin filaments observed by multi-site FRET. *Biophysical Journal, 82*(3), 1524-1536.

Barter, P. J., Nicholls, S., Rye, K. A., Anantharamaiah, G. M., Navab, M., & Fogelman, A. M. (2004). Antiinflammatory properties of HDL. *Circulation Research, 95*(8), 764-772.

Baynes, J. W. (1991). Role of oxidative stress in development of complications in diabetes. *Diabetes, 40*(4), 405-412.

Beavis, R. C., Chaudhary, T., & Chait, B. T. (1992). α-Cyano-4-hydroxycinnamic acid as a matrix for matrix assisted laser desorption mass spectromtry. *Organic Mass Spectrometry, 27*(2), 156-158.

Bergamini, S., Bellei, E., Reggiani Bonetti, L., Monari, E., Cuoghi, A., Borelli, F., . . . Tomasi, A. (2014). Inflammation: An important parameter in the search of prostate cancer biomarkers. *Proteome Science, 12*(1), 32.

Bjellqvist, B., Ek, K., Giorgio Righetti, P., Gianazza, E., Görg, A., Westermeier, R., & Postel, W. (1982). Isoelectric focusing in immobilized pH gradients: Principle, methodology and some applications. *Journal of Biochemical and Biophysical Methods, 6*(4), 317-339.

Bolooki, H. M., & Askari, A. (2010). Disease management project in acute myocardial infarction. *Journal of the American College of Cardiology, 50*, 917-929.

Borish, L., King, M. S., Mascali, J. J., Johnson, S., Coll, B., & Rosenwasser, L. J. (1992). Transthyretin is an inhibitor of monocyte and endothelial cell interleukin-1 production. *Inflammation, 16*(5), 471-484.

Brickell, C., & Zuk, J. D. (1997). *The American Horticultural Society A-Z Encyclopedia of Garden Plants*. New York: DK Publishing Inc.

Brugts, J. J., Yetgin, T., Hoeks, S. E., Gotto, A. M., Shepherd, J., Westendorp, R. G. J., . . . Deckers, J. W. (2009). The benefits of statins in people without established cardiovascular disease but with cardiovascular risk factors: Meta-analysis of randomised controlled trials. *BMJ, 338*, 1-8.

Burke, L. E., Ma, J., Azar, K. M. J., Bennett, G. G., Peterson, E. D., Zheng, Y., . . . Quinn, C. C. (2015). Current science on consumer use of mobile health for cardiovascular disease prevention. *A Scientific Statement From the American Heart Association, 132*(12), 1157-1213.

Burnette, W. N. (1981). “Western Blotting”: Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Analytical Biochemistry, 112*(2), 195-203.

Cai, H., Chen, H., Yi, T., Daimon, C. M., Boyle, J. P., Peers, C., . . . Martin, B. (2013). VennPlex–A novel Venn diagram program for comparing and visualizing datasets with differentially regulated datapoints. *PLoS ONE, 8*(1), e53388.

Calò, L. A., Maiolino, G., Pagnin, E., Vertolli, U., & Davis, P. A. (2014). Increased RBP4 in a human model of activated anti-atherosclerotic and antiremodelling defences. *European Journal of Clinical Investigation, 44*(6), 567-572.

Carbone, F., Mach, F., & Montecucco, F. (2015). The role of adipocytokines in atherogenesis and atheroprogression. *Current Drug Targets, 16*(4), 295-320.

Carbone, F., & Montecucco, F. (2015). Inflammation in arterial diseases. *IUBMB Life, 67*(1), 18-28.

Carmeliet, P., & Collen, D. (1995). Role of the plasminogen/plasmin system in thrombosis, hemostasis, restenosis and atherosclerosis evaluation in transgenic animals. *Trends in Cardiovascular Medicine, 5*(4), 117-122.

Chauvet, J., Dostal, J. P., & Acher, R. (1976). Isolation of a trypsin-like enzyme from *Streptomyces paromomycinus* (paromotrypsin) by affinity aborption through kunitz inhibitor-sepharose. *International Journal of Peptide and Protein Research, 8*(1), 45-55.

Chen, H., & Boutros, P. C. (2011). VennDiagram: A package for the generation of highly-customizable Venn and Euler diagrams in R. *BMC Bioinformatics, 12*(1), 35.

Cheung, F. (2011). TCM: Made in China. *Nature, 480*(7378), S82-S83.

Chiaradia, E., Avellini, L., Tartaglia, M., Gaiti, A., Just, I., Scoppetta, F., . . . Pich, A. (2012). Proteomic evaluation of sheep serum proteins. *BMC Veterinary Research, 8*(1), 66.

Christou, G. A., Tselepis, A. D., & Kiortsis, D. N. (2012). The metabolic role of retinol binding protein 4: An update. *Hormone and Metabolic Research, 44*(01), 6-14.

Conn, P. M. (2013). *Animal models for the study of human disease*. London, UK: Academic Press.

Contois, J. H., McConnell, J. P., Sethi, A. A., Csako, G., Devaraj, S., Hoefner, D. M., & Warnick, G. R. (2009). Apolipoprotein B and cardiovascular disease risk: Position statement from the AACC lipoproteins and vascular diseases division working group on best practices. *Clinical Chemistry, 55*(3), 407-419.

Cubedo, J., Padró, T., Alonso, R., Cinca, J., Mata, P., & Badimon, L. (2012). Differential proteomic distribution of TTR (pre-albumin) forms in serum and HDL of patients with high cardiovascular risk. *Atherosclerosis, 222*(1), 263-269.

Dahlbäck, B., & Nielsen, L. B. (2006). Apolipoprotein M–a novel player in high-density lipoprotein metabolism and atherosclerosis. *Current Opinion in Lipidology, 17*(3), 291-295.

Deitcher, S. R., & Jaff, M. R. (2002). Pharmacologic and clinical characteristics of thrombolytic agents. *Reviews in Cardiovascular Medicine, 3*(2), 25-33.

Dominguez-Rodriguez, A., Abreu-Gonzalez, P., & Avanzas, P. (2012). The role of melatonin in acute myocardial infarction. *Frontiers in Bioscience, 17*, 2433-2441.

Dousa, T. P. (1999). Cyclic-3′,5′-nucleotide phosphodiesterase isozymes in cell biology and pathophysiology of the kidney. *Kidney International, 55*(1), 29-62.

Dreisewerd, K. (2014). Recent methodological advances in MALDI mass spectrometry. *Analytical and Bioanalytical Chemistry, 406*(9), 2261-2278.

Eddouks, M., Chattopadhyay, D., De Feo, V., & Cho, W. C. (2014). Medicinal plants in the prevention and treatment of chronic diseases 2013. *Evidence-based Complementary and Alternative Medicine, 2014*, 1-3.

Edwards, J. J., Anderson, N. G., Nance, S. L., & Anderson, N. L. (1979). Red cell proteins. I. Two-dimensional mapping of human erythrocyte lysate proteins. *Blood, 53*(6), 1121-1132.

Ellulu, M. S., Khaza'ai, H., Abed, Y., Rahmat, A., Ismail, P., & Ranneh, Y. (2015). Role of fish oil in human health and possible mechanism to reduce the inflammation. *Inflammopharmacology, 23*(2), 79-89.

Eng, T. R. (2001). The eHealth landscape: A terrain map of emerging information and communication technologies in health and health care. *Canadian Women's Health Network.* Retrieved from http://www.informatics-review.com/thoughts/rwjf.html (Accessed on 2 May 2014)

Engström, G., Hedblad, B., Berglund, G., Janzon, L., & Lindgärde, F. (2006). Plasma levels of complement C3 is associated with development of hypertension: A longitudinal cohort study. *Journal of Human Hypertension, 21*, 276-282.

Fliser, D., Novak, J., Thongboonkerd, V., Argilés, À., Jankowski, V., Girolami, M. A., . . . Mischak, H. (2007). Advances in urinary proteome analysis and biomarker discovery. *Journal of the American Society of Nephrology, 18*(4), 1057-1071.

Fredrickson, D. S. (1971). An international classification of hyperlipidemias and hyperlipoproteinemias. *Annals of Internal Medicine, 75*(3), 471-472.

Friedewald, W. T., Levy, R. I., & Fredrickson, D. S. (1972). Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clinical Chemistry, 18*(6), 499-502.

Fugh-Berman, A. (2000). Herbs and dietary supplements in the prevention and treatment of cardiovascular disease. *Preventive Cardiology, 3*(1), 24-32.

Ghazali, S. M., Seman, Z., Cheong, K. C., Hock, L. K., Manickam, M., Kuay, L. K., . . . Mustafa, A. N. (2015). Sociodemographic factors associated with multiple cardiovascular risk factors among Malaysian adults. *BMC Public Health, 15*(1), 68.

Girod, W. G., Jones, S. P., Sieber, N., Aw, T. Y., & Lefer, D. J. (1999). Effects of hypercholesterolemia on myocardial ischemia-reperfusion injury in LDL receptor–deficient mice. *Arteriosclerosis, Thrombosis, and Vascular Biology, 19*(11), 2776-2781.

Golikov, A. P., Polumiskov, V., Davydov, B. V., Karev, V. A., Bashkatova, V. G., Belozerov, G. E., . . . Berestov, A. A. (1989). Lipid peroxidation and the major factors of its activation in patients with myocardial infarction. *Kardiologiia, 29*(7), 53-59.

Gong, F. M., Liang, S. F., He, C. M., Shen, G. B., Xu, Y. H., Xu, Z. Z., . . . Wei, Y. Q. (2008). The optimized conditions of two dimensional polyacrylamide gel electrophoresis for serum proteomics. *Journal of Proteomics & Bioinformatics, 1*, 250-257.

Görg, A., Weiss, W., & Dunn, M. J. (2004). Current two-dimensional electrophoresis technology for proteomics. *Proteomics, 4*(12), 3665-3685.

Goufman, E. I., Moshkovskii, S. A., Tikhonova, O. V., Lokhov, P. G., Zgoda, V. G., Serebryakova, M. V.,…Archakov, A. I. (2006). Two-dimensional electrophoretic proteome study of serum thermostable fraction from patients with various tumor conditions. *Biochemistry (Moscow), 71*(4), 354-360.

Graham, T. E., Wason, C. J., Blüher, M., & Kahn, B. B. (2007). Shortcomings in methodology complicate measurements of serum retinol binding protein (RBP4) in insulin-resistant human subjects. *Diabetologia, 50*(4), 814-823.

Grundy, S. M., Becker, D., Clark, L. T., Cooper, R. S., Denke, M. A., Howard, J., . . . McBride, P. (2002). Detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III). *Circulation, 106*(25), 3143-3421.

Gustafson, D. H., Hawkins, R. P., Boberg, E. W., McTavish, F., Owens, B., Wise, M., . . . Pingree, S. (2002). CHESS: 10 years of research and development in consumer health informatics for broad populations, including the underserved. *International Journal of Medical Informatics, 65*(3), 169-177.

Haas, B., Serchi, T., Wagner, D. R., Gilson, G., Planchon, S., Renaut, J., . . . Devaux, Y. (2011). Proteomic analysis of plasma samples from patients with acute myocardial infarction identifies haptoglobin as a potential prognostic biomarker. *Journal of Proteomics, 75*(1), 229-236.

Hadijah, H., Normah, A., Ahmad Tarmizi, S., & Abidin, H., Musa, Y. (2004). *Safety evaluation of mas cotek tea: Effect on organ weight and blood biochemistry*. Paper presented at the Seminar on medicinal and aromatic plants: Current trends and perspective, Kepong, Malaysia.

Hakiman, M., & Maziah, M. (2009). Non enzymatic and enzymatic antioxidant activities in aqueous extract of different *Ficus deltoidea* accessions. *Journal of Medicinal Plants Research, 3*(3), 120-131.

Hansson, G. K., & Libby, P. (2006). The immune response in atherosclerosis: A double-edged sword. *Nature Reviews Immunology, 6*, 508-519.

Harrison, D., Griendling, K. K., Landmesser, U., Hornig, B., & Drexler, H. (2003). Role of oxidative stress in atherosclerosis. *American Journal of Cardiology, 91*(3), 7-11.

Hasham, R., Choi, H.-K., Sarmidi, M. R., & Park, C.-S. (2013). Protective effects of a *Ficus deltoidea* (Mas cotek) extract against UVB-induced photoageing in skin cells. *Biotechnology and Bioprocess Engineering, 18*(1), 185-193.

Hayek, T., Ito, Y., Azrolan, N., Verdery, R. B., Aalto-Setälä, K., Walsh, A., & Breslow, J. L. (1993). Dietary fat increases high density lipoprotein (HDL) levels both by increasing the transport rates and decreasing the fractional catabolic rates of HDL cholesterol ester and apolipoprotein (Apo) A-I. Presentation of a new animal model and mechanistic studies in human Apo A-I transgenic and control mice. *The Journal of Clinical Investigation, 91*(4), 1665-1671.

Heinecke, J. W. (2009). The HDL proteome: a marker–and perhaps mediator - of coronary artery disease. *Journal of Lipid Research, 50*(Supplement), S167-S171.

Heiss, G., Tamir, I., Davis, C. E., Tyroler, H. A., Rifkand, B., Schonfeld, G., . . . Frantz, I. D. (1980). Lipoprotein-cholesterol distributions in selected North American populations: the lipid research clinics program prevalence study. *Circulation, 61*(2), 302-315.

Hidaka, H., Hanyu, N., Sugano, M., Kawasaki, K., Yamauchi, K., & Katsuyama, T. (2007). Analysis of human serum lipoprotein lipid composition using MALDI-ToF mass spectrometry. *Annals of Clinical & Laboratory Science, 37*(3), 213-221.

Hill, R. E., Shaw, P. H., Boyd, P. A., Baumann, H., & Hastie, N. D. (1984). Plasma protease inhibitors in mouse and man: Divergence within the reactive centre regions. *Nature, 311*, 175-177.

Hulsen, T., de Vlieg, J., & Alkema, W. (2008). BioVenn - a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams. *BMC Genomics, 9*(1), 488.

Ingelsson, E., Sundström, J., Melhus, H., Michaëlsson, K., Berne, C., Vasan, R. S., . . . Ärnlöv, J. (2009). Circulating retinol-binding protein 4, cardiovascular risk factors and prevalent cardiovascular disease in elderly. *Atherosclerosis, 206*(1), 239-244.

Irshad, M., & Dubey, R. (2005). Apolipoproteins and their role in different clinical conditions: an overview. *Indian Journal of Biochemistry & Biophysics, 42*(2), 73-80.

Ivano, E., Davide, A., Ingrid, M., Pia, V., Maddalena, F., Pietro, G., . . . Elisabetta, G. (2000). Proteins of rat serum V: Adjuvant arthritis and its modulation by nonsteroidal antiinflammatory drugs. *Electrophoresis, 21*(11), 2170-2180.

Jabit, M. L., Husin, N., Mat, M., Wahab, A., & Musa, Y. (2005, September). *Antioxidant and cytotoxic activities of seven accessions of Ficus deltoidea Jack.* Paper presented at the Seminar on medicinal and aromatic plants, FRIM, Kepong, Malaysia.

Jayakumar, T., Sakthivel, M., Thomas, P. A., & Geraldine, P. (2008). *Pleurotus ostreatus*, an oyster mushroom, decreases the oxidative stress induced by carbon tetrachloride in rat kidneys, heart and brain. *Chemico-Biological Interactions, 176*(2), 108-120.

Johnson, W. B., Katugampola, S., Able, S., Napier, C., & Harding, S. E. (2012). Profiling of cAMP and cGMP phosphodiesterases in isolated ventricular cardiomyocytes from human hearts: Comparison with rat and guinea pig. *Life Sciences, 90*(9), 328-336.

Kalman, D. S., Schwartz, H. I., Feldman, S., & Krieger, D. R. (2013). Efficacy and safety of *Elaeis guineensis* and *Ficus deltoidea* leaf extracts in adults with pre-diabetes. *Nutrition Journal, 12*(1), 36.

Karas, M., & Hillenkamp, F. (1988). Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Analytical Chemistry, 60*(20), 2299-2301.

Khan, T. A., Shah, T., Prieto, D., Zhang, W., Price, J., Fowkes, G. R., . . . Casas, J. P. (2013). Apolipoprotein E genotype, cardiovascular biomarkers and risk of stroke: Systematic review and meta-analysis of 14 015 stroke cases and pooled analysis of primary biomarker data from up to 60 883 individuals. *International Journal of Epidemiology, 42*(2), 475-492.

Khanna, A. K., Chander, R., & Kapoor, N. K. (1996). *Terminalia arjuna*: An ayurvedic cardiotonic, regulates lipid metabolism in hyperlipaemic rats. *Phytotherapy Research, 10*(8), 663-665.

Kim, H. J., Yoo, H. S., Kim, P. K., Kim, M. R., Lee, H. W., & Kim, C. W. (2011). Comparative analysis of serum proteomes of patients with cardiovascular disease. *Clinical Biochemistry, 44*(2), 178-184.

Klose, J. (1975). Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. *Humangenetik, 26*(3), 231-243.

Kontush, A., & Chapman, M. J. (2006). Functionally defective high-density lipoprotein: A new therapeutic target at the crossroads of dyslipidemia, inflammation, and atherosclerosis. *Pharmacological Reviews, 58*(3), 342-374.

Lane-Donovan, C., & Herz, J. (2014). Is apolipoprotein e required for cognitive function in humans? Implications for Alzheimer drug development. *JAMA Neurology, 71*(10), 1213-1215.

Lefer, A. M., Scalia, R., & Lefer, D. J. (2001). Vascular effects of HMG CoA-reductase inhibitors (statins) unrelated to cholesterol lowering: New concepts for cardiovascular disease. *Cardiovascular Research, 49*(2), 281-287.

Leong, X. F., Ng, C. Y., & Jaarin, K. (2015). Animal models in cardiovascular research: Hypertension and atherosclerosis. *BioMed Research International, 2015*, 11.

Leopold, J. A., & Loscalzo, J. (2009). Oxidative risk for atherothrombotic cardiovascular disease. *Free Radical Biology and Medicine, 47*(12), 1673-1706.

Levy, R. I., Troendle, A. J., & Fattu, J. M. (1993). A quarter century of drug treatment of dyslipoproteinemia, with a focus on the new HMG-CoA reductase inhibitor fluvastatin. *Circulation, 87*(4 Suppl), III45-III53.

Liao, J. K., & Ulrich, L. (2005). Pleiotropic effects of statins. *Annual Review of Pharmacology and Toxicology, 45*(1), 89-118.

Lim, T. O., Ding, L. M., Zaki, M., Suleiman, A. B., Fatimah, S., Siti, S., . . . Maimunah, A. H. (2000). Distribution of body weight, height and body mass index in a national sample of Malaysian adults. *The Medical Journal of Malaysia, 55*(1), 108-128.

Liz, M. A., Gomes, C. M., Saraiva, M. J., & Sousa, M. M. (2007). ApoA-I cleaved by transthyretin has reduced ability to promote cholesterol efflux and increased amyloidogenicity. *Journal of Lipid Research, 48*(11), 2385-2395.

Lopez-Garcia, E., Schulze, M. B., Meigs, J. B., Manson, J. E., Rifai, N., Stampfer, M. J., . . . Hu, F. B. (2005). Consumption of trans fatty acids is related to plasma biomarkers of inflammation and endothelial dysfunction. *The Journal of Nutrition, 135*(3), 562-566.

Ludman, A., Venugopal, V., Yellon, D. M., & Hausenloy, D. J. (2009). Statins and cardioprotection - More than just lipid lowering? *Pharmacology & Therapeutics, 122*(1), 30-43.

Lugnier, C. (2006). Cyclic nucleotide phosphodiesterase (PDE) superfamily: A new target for the development of specific therapeutic agents. *Pharmacology & Therapeutics, 109*(3), 366-398.

Mączewski, M., & Mączewska, J. (2006). Hypercholesterolemia exacerbates ventricular remodeling in the rat model of myocardial infarction. *Journal of Cardiac Failure, 12*(5), 399-405.

Magdeldin, S., Enany, S., Yoshida, Y., Xu, B., Zhang, Y., Zureena, Z., . . . Yamamoto, T. (2014). Basics and recent advances of two dimensional - polyacrylamide gel electrophoresis. *Clinical Proteomics, 11*(1), 16.

Mahley, R. W., & Rall, S. C. (2000). Apolipoprotein E: Far more than a lipid transport protein. *Annual Review of Genomics and Human Genetics, 1*(1), 507-537.

Májek, P., Reicheltová, Z., Suttnar, J., Malý, M., Oravec, M., Pečánková, K., & Dyr, J. E. (2011). Plasma proteome changes in cardiovascular disease patients: Novel isoforms of apolipoprotein A1. *Journal of Translational Medicine, 9*(1), 84.

Malliaras, K., Zhang, Y., Seinfeld, J., Galang, G., Tseliou, E., Cheng, K., . . . Marbán, E. (2013). Cardiomyocyte proliferation and progenitor cell recruitment underlie therapeutic regeneration after myocardial infarction in the adult mouse heart. *EMBO Molecular Medicine, 5*(2), 191-209.

Mathers, C. D., & Loncar, D. (2006). Projections of global mortality and burden of disease from 2002 to 2030. *PLOS Medicine, 3*(11), e442.

Mendis, S., Puska, P., & Norrving, B. (2011). *Global atlas on cardiovascular disease prevention and control*. Geneva: World Health Organization.

Mendler, L., Braun, T., & Müller, S. (2016). The ubiquitin-like SUMO system and heart function. *From Development to Disease, 118*(1), 132-144.

Ministry of Health Malaysia. (2010). *Country Health Plan: 10th Malaysia Plan 2011-2015*. Putrajaya, Malaysia: Bahagian Perancangan dan Pembangunan, Ministry of Health Malaysia. Retrieved from http://www.moh.gov.my/images/gallery/Report/Country_health.pdf (Accessed on 24 November 2015).

Ministry of Health Malaysia, National Heart Association of Malaysia, & Academy of Medicine Universiti Malaysia Kebangsaaan. (2011). *4th Edition Clinical Practice Guideline: Management of Dyslipidemia*. Putrajaya, Malaysia: CPG Secretariat Health Technology Assessment Unit. Retrieved from http://www.moh.gov.my/penerbitan/CPG2017/6632.pdf (Accessed on 24 November 2015).

Misbah, H., Aziz, A. A., & Aminudin, N. (2013). Antidiabetic and antioxidant properties of *Ficus deltoidea* fruit extracts and fractions. *BMC Complementary and Alternative Medicine, 13*(1), 118.

Moore, K. J., & Tabas, I. (2011). Macrophages in the pathogenesis of atherosclerosis. *Cell, 145*(3), 341-355.

Morimatsu, M., Syuto, B., Shimada, N., Fujinaga, T., Yamamoto, S., Saito, M., & Naiki, M. (1991). Isolation and characterization of bovine haptoglobin from acute phase sera. *Journal of Biological Chemistry, 266*(18), 11833-11837.

Musa, Y. (2005). Kepelbagaian morfologi dan agronomi beberapa aksesi emas cotek yang terdapat di Kelantan dan Terengganu. *Buletin Teknologi Tanaman, 2*, 35-48.

Muscari, A., Bastagli, L., Poggiopollini, G., Tomassetti, V., Massarelli, G., Boni, P., & Puddu, P. (2001). Short term effect of atorvastatin and vitamin E on serum levels of C3, a sensitive marker of the risk of myocardial infarction in men. *Cardiovascular Drugs and Therapy, 15*(5), 453-458.

Muscari, A., Bozzoli, C., Puddu, G. M., Sangiorgi, Z., Dormi, A., Rovinetti, C., . . . Puddu, P. (1995). Association of serum C3 levels with the risk of myocardial infarction. *The American Journal of Medicine, 98*(4), 357-364.

Nikolas, A. B., Ioannis, A. T., Evropi, B., Ioannis, Z., Alexandra, K., Efterpi, C., & Efstathios, N. (2009). Effect of long-term dietary administration of oregano and rosemary on the antioxidant status of rat serum, liver, kidney and heart after carbon tetrachloride-induced oxidative stress. *Journal of the Science of Food and Agriculture, 89*(8), 1397-1406.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *The Journal of Biological Chemistry, 250*(10), 4007-4021.

Oh, M. J., Abdul Hamid, M., Ngadiran, S., Seo, Y. K., Sarmidi, M. R., & Park, C. S. (2011). *Ficus deltoidea* (Mas cotek) extract exerted anti-melanogenic activity by preventing tyrosinase activity in vitro and by suppressing tyrosinase gene expression in B16F1 melanoma cells. *Archives of Dermatological Research, 303*(3), 161-170.

Ostadal, P., Alan, D., & Vejvoda, J. (2005). Statins in the first-line therapy of acute coronary syndrome - similar to aspirin? *Experimental & Clinical Cardiology, 10*(1), 9-16.

Owerbach, D., McKay, E. M., Yeh, E. T. H., Gabbay, K. H., & Bohren, K. M. (2005). A proline-90 residue unique to SUMO-4 prevents maturation and sumoylation. *Biochemical and Biophysical Research Communications, 337*(2), 517-520.

Peng, Y., Yu, D., Gregorich, Z., Chen, X., Beyer, A. M., Gutterman, D. D., & Ge, Y. (2013). In-depth proteomic analysis of human tropomyosin by top-down mass spectrometry. *Journal of Muscle Research and Cell Motility, 34*(3-4), 199-210.

Phuong, N. T. M., Binh, D. V., Minh, P. D., Nhi, N. B., & Van Chi, P. (2014). Profiling of thermostable proteins in diabetic cardiovascular rat plasma. *International Journal of Life Science and Medical Research, 4*(1), 9-14.

Plow, E. F., & Hoover-Plow, J. (2004). The functions of plasminogen in cardiovascular disease. *Trends in Cardiovascular Medicine 14*(5), 180-186.

Pushparaj, P., Tan, C. H., & Tan, B. K. H. (2000). Effects of *Averrhoa bilimbi* leaf extract on blood glucose and lipids in streptozotocin-diabetic rats. *Journal of Ethnopharmacology, 72*(1), 69-76.

Quaye, I. K. (2008). Haptoglobin, inflammation and disease. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 102*, 735-742.

Rabilloud, T., Vuillard, L., Gilly, C., & Lawrence, J. J. (1994). Silver-staining of proteins in polyacrylamide gels: A general overview. *Cellular and molecular biology, 40*(1), 57-75.

Ram, H., Jatwa, R., & Purohit, A. (2014). Antiatherosclerotic and cardioprotective potential of *Acacia senegal* seeds in diet-induced atherosclerosis in rabbits. *Biochemistry Research International, 2014*, 6.

Ramamurthy, S., Kumarappan, C., Dharmalingam, S. R., & Sangeh, J. K. (2014). Phytochemical, pharmacological and toxicological properties of *Ficus deltoidea*: A review of a recent research. *Annual Research & Review in Biology, 4*(14), 2357-2371.

Ray, K. K., Kastelein, J. J., Matthijs Boekholdt, S., Nicholls, S. J., Khaw, K.-T., Ballantyne, C. M., . . . Lüscher, T. F. (2014). The ACC/AHA 2013 guideline on the treatment of blood cholesterol to reduce atherosclerotic cardiovascular disease risk in adults: the good the bad and the uncertain: a comparison with ESC/EAS guidelines for the management of dyslipidaemias 2011. *European Heart Journal, 35*(15), 960-968.

Ray, S., Kumar, V., Bhave, A., Singh, V., Gogtay, N. J., Thatte, U. M., . . . Srivastava, S. (2015). Proteomic analysis of *Plasmodium falciparum* induced alterations in humans from different endemic regions of India to decipher malaria pathogenesis and identify surrogate markers of severity. *Journal of Proteomics, 127*, 103-113.

Razali, N., Dewa, A., Asmawi, M. Z., Ismail, Z., Manshor, N. M., & Hassan, Z. (2013). Vascular reactivity on aortic rings of spontaneously hypertensive rats treated with methanolic and water extracts of *Ficus deltoidea*. *Journal of Experimental & Integrative Medicine, 3*(2), 93-102.

Rehm, S., & Espig, G. (1991). *The cultivated plants of the tropics*. Weikersheim, Germany:Verlag Josesf Margraf and CTA.

Reiner, Z., & Tedeschi-Reiner, E. (2006). Th-W47: 2 Atherosclerosis - A paradox of Eastern European countries. *Atherosclerosis Supplements, 7*(3), 461.

Robinson, T. W., & Freedman, B. I. (2016). The Apolipoprotein L1 gene and cardiovascular disease. *Methodist DeBakey Cardiovascular Journal, 12*(4 Suppl), 2-5.

Roguin, A., Hochberg, I., Nikolsky, E., Markiewicz, W., Meisel, S. R., Hir, J., . . . Levy, A. P. (2001). Haptoglobin phenotype as a predictor of restenosis after percutaneous transluminal coronary angioplasty. *The American Journal of Cardiology, 87*(3), 330-332.

Roy, A. K., & Neuhaus, O. W. (1966). Identification of rat urinary proteins by zone and immunoelectrophoresis. *Proceedings of the Society for Experimental Biology and Medicine, 121*(3), 894-899.

Rybalkin, S. D., Rybalkina, I., Beavo, J. A., & Bornfeldt, K. E. (2002). Cyclic nucleotide phosphodiesterase 1C promotes human arterial smooth muscle cell proliferation. *Circulation Research, 90*(2), 151-157.

Rye, K. A., & Barter, P. J. (2014). Regulation of high-density lipoprotein metabolism. *Circulation Research, 114*(1), 143-156.

Sachdewa, A., & Khemani, L. D. (2003). Effect of hibiscus *Rosa sinensis Linn.* ethanol flower extract on blood glucose and lipid profile in streptozotocin induced diabetes in rats. *Journal of Ethnopharmacology, 89*(1), 61-66.

Saitoh, H., & Hinchey, J. (2000). Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. *Journal of Biological Chemistry, 275*(9), 6252-6258.

Salleh, N., & Ahmad, V. N. (2013). In-vitro effect of *Ficus deltoidea* on the contraction of isolated rat's uteri is mediated via multiple receptors binding and is dependent on extracellular calcium. *BMC Complementary and Alternative Medicine, 13*(1), 359.

Sehestedt, T., Lyngbaek, S., Eugen-Olsen, J., Jeppesen, J., Andersen, O., Hansen, T. W., . . . Olsen, M. H. (2011). Soluble urokinase plasminogen activator receptor is associated with subclinical organ damage and cardiovascular events. *Atherosclerosis, 216*(1), 237-243.

Seibert, V., Wiesner, A., Buschmann, T., & Meuer, J. (2004). Surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI ToF-MS) and ProteinChip® technology in proteomics research. *Pathology-Research and Practice, 200*(2), 83-94.

Sekar, M., Zulhilmi, M., Hamdi, A., Nabila, N., Zahida, Z., & Shafiq, M. (2014). Ten commonly available medicinal plants in Malaysia used for the treatment of diabetes - A review. *Asian Journal of Pharmaceutical and Clinical Research, 7*(1), 1-5.

Shen, X., Young, R., Canty, J. M., & Qu, J. (2014). Quantitative proteomics in cardiovascular research: Global and targeted strategies. *Proteomics-Clinical Applications, 8*(7-8), 488-505.

Shevchenko, A., Tomas, H., Havli, J., Olsen, J. V., & Mann, M. (2007). In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nature Protocols, 1*, 2856.

Sousa, M. M., Berglund, L., & Saraiva, M. J. (2000). Transthyretin in high density lipoproteins: Association with apolipoprotein A-1. *Journal of Lipid Research, 41*(1), 58-65.

Steiner, S., Gatlin, C. L., Lennon, J. J., McGrath, A. M., Aponte, A. M., Makusky, A. J., . . . Anderson, N. L. (2000). Proteomics to display lovastatin-induced protein and pathway regulation in rat liver. *Electrophoresis, 21*(11), 2129-2137.

Sulaiman, M. R., Hussain, M. K., Zakaria, Z. A., Somchit, M. N., Moin, S., Mohamad, A. S., & Israf, D. A. (2008). Evaluation of the antinociceptive activity of *Ficus deltoidea* aqueous extract. *Fitoterapia, 79*(7), 557-561.

Tachjian, A., Maria, V., & Jahangir, A. (2010). Use of herbal products and potential interactions in patients with cardiovascular diseases. *Journal of the American College of Cardiology, 55*(6), 515-525.

Takahara, H., & Sinohara, H. (1982). Mouse plasma trypsin inhibitors. Isolation and characterization of alpha-1-antitrypsin and contrapsin, a novel trypsin inhibitor. *Journal of Biological Chemistry, 257*(5), 2438-2446.

Tall, A. R., & Yvan-Charvet, L. (2015). Cholesterol, inflammation and innate immunity. *Nature Reviews Immunology, 15*, 104.

Tappia, P. S., Xu, Y. J., & Dhalla, N. S. (2013). Reduction of cholesterol and other cardiovascular disease risk factors by alternative therapies. *Clinical Lipidology, 8*(3), 345-359.

Taufik, H. M., Nurul Khalbi, A., & Zulkhairi, A. (2005). *Effect of Ficus deltoidea on cholesterol level and its antioxidant properties on lipid peroxidation in cholesterol-fed rats*. Paper presented at the seminar on medicinal and aromatic plants, Kepong, Malaysia.

Thadikkaran, L., Siegenthaler, M. A., Crettaz, D., Queloz, P. A., Schneider, P., & Tissot, J. D. (2005). Recent advances in blood-related proteomics. *Proteomics, 5*(12), 3019-3034.

Tonelli, M., Lloyd, A., Clement, F., Conly, J., Husereau, D., Hemmelgarn, B., . . . Manns, B. (2011). Efficacy of statins for primary prevention in people at low cardiovascular risk: A meta-analysis. *Canadian Medical Association Journal, 183*(16), 1189-1202.

Towbin, H., Staehelin, T., & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences, 76*(9), 4350-4354.

Trigatti, B., Rayburn, H., Viñals, M., Braun, A., Miettinen, H., Penman, M., . . . Rigotti, A. (1999). Influence of the high density lipoprotein receptor SR-BI on reproductive and cardiovascular pathophysiology. *Proceedings of the National Academy of Sciences, 96*(16), 9322-9327.

Ünlü, M., Morgan, M. E., & Minden, J. S. (1997). Difference gel electrophoresis. A single gel method for detecting changes in protein extracts. *Electrophoresis, 18*(11), 2071-2077.

Upadhyay, R. K. (2015). Emerging risk biomarkers in cardiovascular diseases and disorders. *Journal of Lipids, 2015*, 50.

Vaisar, T., Pennathur, S., Green, P. S., Gharib, S. A., Hoofnagle, A. N., Cheung, M. C., . . . Heinecke, J. W. (2007). Shotgun proteomics implicates protease inhibition and complement activation in the antiinflammatory properties of HDL. *Journal of Clinical Investigation, 117*(3), 746-756.

van Bennekum, A. M., Wei, S., Gamble, M. V., Vogel, S., Piantedosi, R., Gottesman, M., . . . Blaner, W. S. (2001). Biochemical basis for depressed serum retinol levels in transthyretin-deficient mice. *Journal of Biological Chemistry, 276*(2), 1107-1113.

Van Eyk, J. E. (2011). Overview the maturing of proteomics in cardiovascular research. *Circulation Research, 108*(4), 490-498.

Vandeput, F., Wolda, S. L., Krall, J., Hambleton, R., Uher, L., McCaw, K. N., . . . Movsesian, M. A. (2007). Cyclic nucleotide phosphodiesterase PDE1C1 in human cardiac myocytes. *Journal of Biological Chemistry, 282*(45), 32749-32757.

Velayudhan, L., Killick, R., Hye, A., Kinsey, A., Güntert, A., Lynham, S., . . . Lovestone, S. (2012). Plasma transthyretin as a candidate marker for Alzheimer's disease. *Journal of Alzheimer's Disease, 28*(2), 369-375.

Vestal, M. L. (2014). U.S. Patent No. 8,674,292. D. P. T. O. Washington.

von Hagen, J. (2008). *Proteomics sample preparation*: John Wiley & Sons.

Wait, R., Chiesa, G., Parolini, C., Miller, I., Begum, S., Brambilla, D., . . . Gianazza, E. (2005). Reference maps of mouse serum acute-phase proteins: Changes with LPS-induced inflammation and apolipoprotein A-I and A-II transgenes. *Proteomics, 5*(16), 4245-4253.

Wang, J. (2009). SUMO conjugation and cardiovascular development. *Frontiers in Bioscience, 14*, 1219-1229.

Wang, T. D., Wu, C. C., Chen, W. J., Lee, C. M., Chen, M. F., Liau, C. S., . . . Lee, Y. T. (1998). Dyslipidemias have a detrimental effect on left ventricular systolic function in patients with a first acute myocardial infarction. *American Journal of Cardiology, 81*(5), 531-537.

Weisgraber, K. H. (1994). Apolipoprotein E: Structure-function relationships. *Advances in Protein Chemistry, 45*, 249-302.

Weng, X., Roederer, G., Beaulieu, R., & Cloutier, G. (1998). Contribution of acute-phase proteins and cardiovascular risk factors to erythrocyte aggregation in normal and hyperlipidemic individuals. *Thrombosis and Haemostasis, 80*(12), 903–908.

Wiart, C. (2002). *Medicinal plants of Southeast Asia*. Petaling Jaya: Prentice Hall.

Wientgen, H., Thorngate, F. E., Omerhodzic, S., Rolnitzky, L., Fallon, J. T., Williams, D. L., & Fisher, E. A. (2004). Subphysiologic Apolipoprotein E (ApoE) plasma levels inhibit neointimal formation after arterial injury in ApoE-deficient mice. *Arteriosclerosis, Thrombosis and Vascular Biology, 24*(8), 1460-1465.

Wilson, V. G., & Heaton, P. R. (2008). Ubiquitin proteolytic system: Focus on SUMO. *Expert Review of Proteomics, 5*(1), 121-135.

World Health Organization. (1998). *Guidelines for the Appropriate Use of Herbal Medicines*. Manila, Philipines: World Health Organization

World Health Organization. (2013). *Global action plan for the prevention and control of noncommunicable diseases 2013-2020.* Retrieved from http://apps.who.int/iris/bitstream/handle/10665/94384/9789241506236_eng.pdf;jsessionid=935F26A2638234DA726DB052A54F5937?sequence=1 (Accessed on 25 September 2014)

Xiao, H., & Wong, D. T. (2010). Proteomics and its applications for biomarker discovery in human saliva. *Bioinformation, 5*(7), 294-296.

Yang, C., Yang, Y., Gupta, N., Liu, X., He, A., Liu, L., . . . Fang, F. (2007). Pentaspan membrane glycoprotein, prominin-1, is involved in glucose metabolism and cytoskeleton alteration. *Biochemistry (Moscow), 72*(8), 854-862.

Yoshida, A., Matsutani, Y., Fukuchi, Y., Saito, K., & Naito, M. (2006). Analysis of the factors contributing to serum retinol binding protein and transthyretin levels in Japanese adults. *Journal of Atherosclerosis and Thrombosis, 13*(4), 209-215.

Yu, B.L., Wu, C.L., & Zhao, S.P. (2012). Plasma apolipoprotein O level increased in the patients with acute coronary syndrome. *Journal of Lipid Research, 53*(9), 1952-1957.

Zanotti, I., Pedrelli, M., Potì, F., Stomeo, G., Gomaraschi, M., Calabresi, L., & Bernini, F. (2011). Macrophage, but not systemic, Apolipoprotein E Is necessary for macrophage reverse cholesterol transport in vivo. *Arteriosclerosis, Thrombosis and Vascular Biology, 31*(1), 74-80.

Zar, C. T., Teoh, S. L., Das, S., Zaiton, Z., & Farihah, H. S. (2012). Use *Piper sarmentosum* as an effective antidiabetic supplement in South East Asia: A review. *La Clinica Terapeutica, 163*(6), 505-510.

Zaragoza, C., Gomez-Guerrero, C., Martin-Ventura, J. L., Blanco-Colio, L., Lavin, B., Mallavia, B., . . . Egido, J. (2011). Animal models of cardiovascular diseases. *Proteins, 20*, 22.

Zhang, Q., Fillmore, T. L., Schepmoes, A. A., Clauss, T. R., Gritsenko, M. A., Mueller, P. W., . . . Metz, T. O. (2013). Serum proteomics reveals systemic dysregulation of innate immunity in type 1 diabetes. *The Journal of Experimental Medicine, 210*(1), 191-203.

Zhang, X. F., & Tan, B. K. (2000). Effects of an ethanolic extract of *Gynura procumbens* on serum glucose, cholesterol and triglyceride levels in normal and streptozotocin-induced diabetic rats. *Singapore Medical Journal, 41*(1), 9-13.

Zhou, Y., Jiang, L., & Rui, L. (2009). Identification of MUP1 as a regulator for glucose and lipid metabolism in mice. *Journal of Biological Chemistry, 284*(17), 11152-11159.

Zhukova, N. V., Novgorodtseva, T. P., & Denisenko, Y. K. (2014). Effect of the prolonged high-fat diet on the fatty acid metabolism in rat blood and liver. *Lipids in Health and Disease, 13*, 49.