CARDIOPROTECTIVE EFFICACY OF *FICUS DELTOIDEA* JACK AGAINST CARBON TETRACHLORIDE-INDUCED DAMAGE AND HYPERLIPIDEMIA IN RATS

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2015

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DISSERTATION SUBMITTED IN PARTIAL
FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF MASTER OF BIOTECHNOLOGY

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

2015

UNIVERSITI MALAYA

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ABSTRACT

The purpose of this study was to evaluate the effects of *Ficus deltoidea* leaf extracts on carbon tetrachloride (CCl_4)-induced myocardial infarction (MI) and diet-induced hyperlipidemia in rats. Water fraction, ethanolic, and water extracts were prepared, and their *in-vitro* antioxidant capacity was tested using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Cupric ion Reducing Antioxidant Capacity (CUPRAC) assays. The total phenolic content (TPC) was measured using Folin–Ciocalteau assay, and the ability of the extract to inhibit the lipid oxidation was tested using lipid peroxidation assay. Finally, hydroxymethylglutaryl-coenzymeA (HMG-CoA) reductase assay was conducted *in-vitro* to evaluate the inhibition of cholesterol biosynthesis. The three extracts exhibited a significant antioxidant and cholesterol lowering activity *in-vitro*, with the ethanol extract showing comparatively better activity. In a study to determine the efficacy of *F. deltoidea* ethanol extract as a cardioprotective agent, rats were pre-treated with ethanol extract followed by CCl_4 to induce MI. Meanwhile, to evaluate the capability of *F. deltoidea* extracts as anti-atherogenic agent, rats were given daily treatment of the extract along with high-fat diet to induce hyperlipidemia. Cardiac tissue marker enzymes, lipid profile, inflammation rate and NADP concentration (as an indicator of oxidative stress) assays were conducted, both in tissue and serum samples. Finally, histopathology of the heart tissue was also carried out. *In-vivo* assays indicated a higher value of cardiac tissue marker enzymes and lipid profile components in CCl_4- and high fat diet-induced rat groups compared to rats treated with the ethanol extract. The inflammation rate and oxidative stress (NADP concentration) were found to be higher in induced rats as compared to treated rats. Finally, the histopathology of induced rats showed visible necrosis to the heart tissue as compared to little or no damage to treated rat's tissue. Thus, *F. deltoidea* extracts can be further researched as a potential cardioprotective natural compound.

ABSTRAK

Tujuan kajian ini adalah untuk menilai kesan-kesan ekstrak daun *Ficus deltoidea* pada tikus-tikus yang menerima aruhan infarksi-miokardial (MI) oleh carbon-tetraklorida (CCl4) dan tikus-tikus hiperlipidemia aruhan-diet. Fraksi air, ekstrak etanol, dan ekstrak air telah disediakan, dan kapasiti anti-oksida diuji secara *in-vitro* menggunakan 2.2-diphenyl-1-picrylhydrazyl (DPPH) dan ujian penurunang kapasiti anti-oksida ion kuprik (Cupric Ion Reducing Antioxidant Capacity-CUPRAC). Jumlah kandungan fenolik diukur dengan menggunakan ujian Folin-Ciocalteau, dan keupayaan ekstrak untuk menghalang pengoksidaan lipid telah diuji menggunakan ujian perencatan peroksidasi lipid. Ujian menggunakan enzim hydroxymethylglutaryl-coenzymeA (HMG-CoA) reductase telah dijalankan *in-vitro* sebagai ujian untuk melihat keupayaan ekstrak menghalang sintesis kolesterol. Keputusan kajian ini menunjukkan bahawa ketiga-tiga ekstrak telah mempamerkan aktiviti anti-oksida dan perencatan sintesis kolesterol *in-vitro* yang signifikan dengan ekstrak etanol menunjukkan aktiviti yang lebih baik. Dalam kajian untuk menilai keberkesanan ekstrak *F. deltoidea* sebagai agen pelindung-kardio, tikus-tikus dirawat terlebih dahulu dengan ekstrak etanol diikuti oleh suntikan aruhan CCl_4. Manakala dalam kajian kebolehan ekstrak *F. deltoidea* sebagai agen anti-aterogenik, tikus-tikus dirawat dengan ekstrak etanol dan pada masa yang sama diberi makanan tinggi lipid untuk menjadikannya hiperlipidemik. Ujian-ujian *in-vivo* untuk menguji enzim penanda tisu jantung, profil lipid, kadar keradangan dan kepekatan NADP (sebagai penunjuk tekanan oksidatif) telah dijalankan pada sampel-sampel tisu dan serum. Kajian histopatologi tisu jantung juga telah dijalankan. Ujian-ujian *in-vivo* menunjukkan nilai enzim penanda tisu jantung dan komponen profil lipid yang lebih tinggi di dalam kumpulan tikus yang diaruh oleh CCl_4 dan diet tinggi lipid jika dibandingkan dengan tikus-tikus yang dirawat dengan ekstrak etanol. Kadar inflamasi dan tekanan oksidatif (kepekatan NADP) lebih tinggi pada tikus-tikus yang diaruh

berbanding dengan tikus-tikus yang dirawat. Akhir sekali, kajian histopatologi tikus-tikus yang diaruh-CCl_4 menunjukkan nekrosis pada tisu manakala tikus-tikus diaruh-CCl_4 yang dirawat hanya mengalami sedikit atau tiada kerosakan. Sebagai kesimpulan, ekstrak *F. deltoidea* boleh dikaji dengan lebih lanjut sebagai agen semulajadi yang berpotensi digunakan untuk penjagaan jantung.

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ACKNOWLEDGEMENTS

Firstly, I would like to acknowledge my gratitude to God the Almighty for blessing me with the strength and patience to fulfill my goals. I would like to express my deepest appreciation to both my supervisors, Dr. Norhaniza Aminuddin and Prof. Noorlidah Abdullah for continually guiding and helping me with my work, for being patient with my shortcomings, and for tirelessly providing me with the knowledge and skill base, without which, I would have never completed this research.

I would also like to thank all my colleagues and the staff at the Faculty of Science, and the Department of Biological Sciences for their help and guidance. I would like to show my endless gratitude to my family and friends for their continued, love, support, and encouragement. Last but not least, I would like to specifically thank my parents for their moral and financial support throughout the duration of my master's program.

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LIST OF ABBREVIATIONS AND SYMBOLS

ALP – Alkaline phosphatase

ALT – Alanine aminotransferase

AMI – Acute myocardial infarction

ANOVA – One-way analysis of variance

AST – Aspartate aminotransferase

CAM – Complementary and alternative medicine

CCL_4 – Carbon tetrachloride

CHD – Coronary heart disease

CHF – Congestive heart failure

CK – Creatine kinase

CUPRAC – Copper reducing capacity assay

CVD – Cardiovascular disease

DMRT – Duncan's multiple range test

DPPH – 2,2 – diphenyl – 1 – picryl – hydrazyl

EIA – Enzyme immunoassay

ECG – Electrocardiogram

ET – Electron transfer

FC – Folin - Ciocalteu

FRAP – Ferric reducing antioxidant potential

GAE – Gallic acid equivalents

HAT – Hydrogen atom transfer

HDL – High density lipoproteins

HMG-CoA – 3 – hydroxy – 3 methylglutaryl Coenzyme A

HPLCMS – High performance liquid chromatography mass spectrometry

LDH – Lactate dehydrogenase

LDL – Low density lipoprotein

LSD – Least significant difference

NADPH – Nicotinamide adenine dinucleotide phosphate

ORAC –Oxygen radical absorbance capacity

PBS – Phosphate buffered saline

PPUM – Pusat perubatan universiti malaya

PUFA – Polyunsaturated fatty acids

SET – Single electron transfer

SOD – Superoxide dimutase

STAR – Statistical tool for agricultural research

TAS – Total antioxidant status

TBARS – Thiobarbituric acid reactive substance

TC – Total cholesterol

TG – Triglycerides

TNF – Tumor necrosis factor

TPC – Total phenolic content

TRAP – Total Radical-tapping antioxidant parameter

VLDL – Very low density lipoproteins

CHAPTER 1.0

INTRODUCTION

1.1: Background

Cardiovascular diseases (CVDs) are defined as a category of diseases that are related to heart and blood vessels, and are also widely known as heart diseases. According to the World Health Organization, CVDs remain as the leading cause of death and disability throughout the world. Their statistics state that about 17.3 million people have died from CVDs in 2008, and predict that an approximate of 23.6 million people will die from CVDs by 2030. The American Heart Association states that atherosclerosis may be the main cause to most heart and vessel related diseases. Atherosclerosis is a frequently occurring disorder where fat, cholesterol, and other substances build up in the walls of the arteries leading to the formation of plaques. These plaques can block the arteries and cause problems throughout the body, as well as numerous types of heart diseases such as strokes, heart attacks, and arrhythmia. (A.D.A.M. Medical Encyclopedia, 2012). The World Health Organization reports CVDs account for 32% of overall mortalities in Malaysia, as of 2011. As can be seen in the annual reports for years 2002-2010 and strategic health plans for years 2006-2015, the Malaysian Ministry of Health has dedicated enormous funds to address the issue of health and safety throughout the country.

CVDs can be addressed through various ways. Initially, prevention is the most preferable way to deal with cases that may potentially arise. Prevention may be carried out at the population level or at the individual level. Moreover, it is possible to manage heart failures and resulting conditions and preventing their effect (Ortegón et.al, 2012). The treatment and management plans are mainly associated with focusing on various risk factors. Those are classified into major categories, namely modifiable and non-modifiable risk factors (World Heart Federation). Some of the modifiable factors are high blood

pressure, cholesterol, obesity, and tobacco use. The non-modifiable factors could be due to age, gender or family history.

This study focuses on *Ficus deltoidea* species, also known as mistletoe fig, which is originally found in Southeast Asia, Borneo and Philippines (Brickell and Zuk, 1997). The bioactivity of this plant was studied previously to analyze its effect against diabetes (Choo et.al, 2012). The results showed a significant inhibition of α-glucosidase enzyme to aid in the reduction of blood glucose level in diabetic mice. Other studies were also conducted to prove the antidiabetic activity of this plant by reducing hyperglycemia (Adam et.al, 2009, 2010 and 2011). This plant also exhibits protective activity against the side effects of the conventional medicines used by acting as an antioxidant. This particular activity of the plant was also analyzed and confirmed by various studies (Aris, 2009; Norra 2011). The aim of this study is summarized as furthering the investigations on this species to study its effect on cardioprotective potential and CVD prevention and treatment, based on the available promising data from previous studies. The methodology followed will be focusing on the pre-treatment with ethanol extract (EE), water extract (WE), and aqueous extract (WF) of *F. deltoidea*, looking at its ability in reducing the excess lipids; alteration of mitochondrial and lysosomal enzymes, membrane bound enzymes and cardiac tissue marker enzymes.

1.2: Objectives

- To evaluate the *Ficus deltoidea* leaves extract for *in-vitro* for anti-oxidant and anti-cholesterol activities.
- To investigate the most potent extract of *Ficus deltoidea in-vivo* for cardioprotective and anti-cholesterol activities.

CHAPTER 2.0

LITERATURE REVIEW

2.1: Cardiovascular Diseases

2.1.1: Background

In 1825, the French lawyer and gourmand, Brillant-Savarin published the very famous book "The physiology of taste" where he states that the cure to obesity is abstaining from consumption of everything that is starchy and floury. Since then, much attention has been drawn towards diabetes and heart-related disease research. It was in the early 1900's that advances were made in this area, starting with the formation of American Heart Association (AHA) in 1924. Much of the research then was focused on the American population, and great breakthroughs followed upon worldwide dietary comparisons. In 1951, Ancel Keys, professor in University of Minnesota, realized that lower fat diet in many Mediterranean countries and in Japan led to lower occurrences of heart diseases and published his seven country analysis (Blackburn, 1995). Wide range of researches were conducted after this discovery by both, the AHA and the National Institute of Health (NIH).

According to many epidemological studies, Cardiovascular Diseases (CVDs) were responsible for less than 10% of global deaths in the 20th century. According to more recent findings, this disease accounts for approximately 30% of deaths worldwide (Gaziano, 2005; British Nutrition Foundation, 2005; Sliwa et al., 2005; Sliwa et al., 2008). Now, CVD's have become one of the leading causes of death in most developing countries with about 80% related cases.

CVDs encompass a wide variety of conditions that lead to the malfunction of the heart. These conditions may be due to genetic variations or environmental effects. According to the World Health Organization (WHO, 2004), CVDs can be categorized into coronary heart disease, stroke, inflammatory heart disease, rheumatic heart disease,

hypertensive heart disease, and tumors of the heart or vessels. AHA (2004) similarly includes the same categories as WHO to define CVD. However, the Heart and Stroke Foundation of Canada (HSFC, 2003) states that cardiovascular diseases must be defined as all diseases of the circulatory system, including acute myocardial infarction, ischemic heart disease, valvular heart disease, peripheral vascular disease, arrhythmias, high blood pressure, and stroke.

The various categories of CVDs are caused by different factors, thus it is important to select the category of heart disease that is mostly related to the factor most suitable to the scope of this research. As such, it is beneficial to focus on two aspects which are oxidative damage to the heart and atherosclerosis leading to coronary heart disease. Atherosclerosis is a frequently occurring disorder where fat, cholesterol, and other substances build up in the walls of the arteries leading to the formation of plaques. These plaques can block the arteries and cause problems throughout the body, as well as numerous types of heart diseases such as strokes, heart attacks, and arrhythmia (A.D.A.M. Medical Encyclopedia, 2012; Ross, 1999).

Oxidative damage on the other hand is caused by free radical buildup, which was first proposed as a theory by Denham Harman at the University of Nebraska. This theory suggests that damage caused by free radicals is one factor leading to bodily changes associated with aging (Harman, 1956). Free radicals thus can be defined as chemicals which contain an unpaired electron in their outermost shell making them highly reactive molecules (Halliwell et.al, 1992). Free radicals can be classified into reactive oxygen species, reactive nitrogen species, aromatic compounds, quinonic and semiquinonic compounds, nucleic acids, and thiol radicals (Olinescu, 1994). These free radicals may oxidize cell components such as carbohydrates, proteins, lipids and nucleic acids and cause permanent damage or change to their structure and function (Halliwell, 1996; Diplock et.al, 1998). Normally, the body keeps the level of free radicals at check using

the antioxidants. However, in the presence of excessive free radicals or lack of antioxidants, a condition called oxidative stress may emerge (Maritim et.al, 2003). External factors that may lead to oxidative stress are alcohol drinking, diet, pollution, tobacco use, and metabolic abnormalities (Halliwel and Gutteridge, 1989). The end result of oxidative stress would be oxidation of low density lipoproteins (LDL), which is proposed as a critical step (Jialal and Fuller, 1993), cholesterol and its derivatives, and protein modifications which may lead to formation of foam cells, followed by atherosclerosis (Boullier et.al, 2001).

Researches were conducted to assess the role of oxidative stress in vascular aging and inflammation in animal models (Mármol et al., 2007). Other *in-vivo* studies were conducted on mice to link the increased expression of NADPH oxidase to oxidative stress which leads to the build-up of reactive oxygen species and thus inducing atherosclerosis in parts of the arteries (Haidari et al., 2010). Imai and colleagues (1980) have found through research that arterial damage may occur through oxidation of cholesterol to various oxygenated sterols, leading to the development of atherosclerosis.

Sources of free radicals have been identified since decades ago and used as a preventive measure against diseases caused by free radicals. It is also accessible to public for education purposes and as well as for researchers to study the effects and more specific correlations of these sources to the mechanism of free radical formation. The identification of the sources and mechanisms also aids in developing appropriate methods to assay the presence of free radicals in different test models. Maxwell concluded in his research in 1995, a comprehensive source of free radicals, which is presented in Table 2.1 below.

Table 2.1: Sources of free radicals (Maxwell, 1995)

Source	Mechanism
Mitochondrial electron transport	Leakage of superoxide due to inefficient oxygen reduction
Transition metal ions	Hydroxyl radical formation due to iron and copper
Inflammation	Free radical formation due to activated phagocytes
Enzymes (e.g. xanthine oxidase)	Release of superoxide due to reperfusion of ischemic tissue
Drug metabolism (e.g. paraquat acetaminophen)	Production of free radical metabolites
Cigarette smoking	Concentrated free radicals present in gas phase
Radiation	X-rays and ultraviolet light

2.1.2: Treatment Methods

Treatment methods are adopted according to the risk factors and the management of these factors. The World Health Federation (WHF) classifies risk factors as being modifiable and non-modifiable. Some of the modifiable risk factors include hypertension, tobacco use, increased blood glucose levels, physical inactivity, unhealthy diets, cholesterol, and obesity. Non-modifiable risk factors on the other hand include age, gender, and family history (WHF). Non-modifiable risk factors, as the term suggests, are fixed and cannot be altered. However, majority of risk factors are modifiable to some extent (Berger et.al, 2010; O'Donnell et.al, 2010).

Cholesterol is one of the most sought after risk factor when considering treatment methods. Cholesterol in the body is mainly carried in vesicles known as low density lipoproteins (LDL). LDLs have a major correlation to the development of CVDs as can be seen from pathophysiological (Steinberg, 1997; Tabas, 2002), observational (Neaton et.al, 1992; Castelli et.al, 1992; Wong et.al, 1991) and early genetic studies (Brown and

Goldstein, 1986). Moreover, according to some interventional studies, there is a clear link between the lowering of LDL by statins and the decreased risk of acquiring CVD (Baigent et.al, 2005; Mihaylova et.al, 2012). Finally, genetic studies have been conducted to estimate the level of LDLs using genetic variations to confirm the role of LDLs as a leading cause of CVD (Ference et.al, 2012; Cohen et,al, 2006). The treatment plan that focuses on lowering LDL is attained by using statins. Statins are a class of drugs that act by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase). HMG-CoA reductase is a critical enzyme in cholesterol production, and its inhibition would lead to a loss in regulation of LDL receptors, thus lowering the levels of LDL circulating in the blood (Babelova et.al, 2013).

Statins are thus used as the primary medicine of choice for treating high LDL levels (Stone et.al, 2013) and has been shown to have a potential positive effect in treating patients with low risk of CVD (Taylor et.al, 2013). The main observable drawback of this drug class is that only about half of the patients have shown promising results by reaching the set treatment goals (Kotseva et.al, 2009; 2010). Moreover statin therapy may lead to a decrease in the level of natural antioxidants in the body, resulting in an increase in free radical damage to cells in various parts of body such as liver, nerves, and various muscles (Fernández et.al, 2008; Golomb et.al, 2009; Venero et.al, 2009). Finally, clinical studies on patients have shown that other side effects might occur, such as myopathy (defined by NIH as "a neuromuscular disorders in which the primary symptom is muscle weakness due to dysfunction of muscle fiber"), rhabdomyolysis (defined by NIH as "the breakdown of muscle tissue that leads to the release of muscle fiber contents into the blood"), myocardial contractility, and autoimmune disease (Radcliff and Campbell, 2008; Venero et.al, 2009). Some examples of most commonly used statins are atorvastatin, lovastatin, and pravastatin.

Aside from cholesterol levels, lifestyle also plays an important role in the development of CVDs. One major factor is cigarette smoking (WHO, 2009), leading to strokes (Marmot and Poulter, 1992), and myocardial infarction (Yusuf et.al, 2004). Cigarette smoke contains compounds that result in oxidative stress caused by an increase in free radical concentrations. Moreover, it results in modification of LDLs by oxidation, inflammation, and an increased level of catecholamine which leads to high blood pressure and glucose levels (Ambrose and Barua, 2004). Cigarette smoke is only one example of an environmental source of free radicals. Other examples include ozone from air pollution, pesticides, automobile exhaust emissions, deep fried foods, excessive radiation, and inhalation or ingestion of toxic metals (Pryor et al,1995; Diplock et al, 1998; Nojiri et al., 2004; Patel et al., 2007).

The best preventive measure is to reduce or eliminate smoking as well as the other free radical sources all together, which should reduce the free radical build-up in the body, in turn reducing oxidative stress to most vital organs. However, in extreme cases, it is important to increase the body's antioxidant levels, which will break down the free radicals and reduce oxidative stress. The body has its own cascade of enzymes and molecules that are called endogenous antioxidants. Studies have been conducted to analyze enzymes such as superoxide dismutase (SOD), glutathione peroxidases, catalases and thioredoxin reductases (Forsberg et.al, 2001). Furthermore, it is possible to obtain the necessary antioxidants from the diet or medicines and in which case they are called exogenous antioxidants. Some of these exogenous antioxidants act as glutathione donors or superoxide dismutase and catalase mimics, as well as different xanthine oxidase inhibitors and lipid-soluble chain breaking compounds (Halliwell and Gutteridge, 1990). Quercetin (3,3',4',5,7-pentahydroxyflavone), for example is a widely known polyphenolic compound and can be obtained from plant sources such as apples, broccoli,

onions and other medicinal plants like *Dorema aitchisonii*, *Doplotaxis harra*, *Asparagus cochinchinensis*, and *Lysimachia clethroides*.

Most exogenous antioxidants are ingested as dietary supplements which contain vitamins, mineral, fatty acids, amino acids and fibers. These supplements also act as antioxidants such as vitamin A (carotenes), vitamins C and E, ubiquinone, glutathione and polyphenols (flavonoids) (Raskin et.al, 2002). These dietary supplements usually undergo many quality control and safety checks, and are approved only after the appropriate clinical trials are conducted (FDA). However, there are still many risks of using these dietary supplements, the biggest of which is called antioxidative stress or antioxidant-induced stress, and defined as a negative side effect of antioxidants (Dundar and Aslan, 2000; Poljsak and Milisav, 2012). While lack of antioxidant defense mechanisms lead to oxidative stress, the excess of antioxidants would lead to antioxidative stress, both of which may lead to development of cancer and aging (Halliwel, 2007). Several clinical studies were conducted on individuals' responses to intake of synthetic antioxidants, and the studies concluded that the antioxidant supplementation showed no beneficial effects, while some showed no effect at all (Hercberg et.al, 2007; Bjelakovic et.al, 2008; Myung et.al, 2010; Klein et.al, 2011).

Table 2.2 below shows the various researches conducted on antioxidants, both as dietary supplements and natural antioxidants, to assess their effect on protecting the heart against cardiovascular diseases.

Table 2.2: Summary of the effects of natural antioxidants from plant sources related to CVD

Author(s)	Antioxidant	Findings	Limitations
Hertog et.al (1995)	Flavonoid intake	Average flavonoid intake associated with differences in mortality due to coronary heart disease	Accuracy of results is questionable as food composites that were equivalent to average diet in the different countries were used
Dugas et.al (1999)	β carotene	The oxidation of carotene enriched LDL was greatly inhibited	Enrichment is method dependent as *in-vivo* enrichment is more effective than *in-vitro* enrichment
Bobak et.al (1999)	β carotene and lycopene	Dietary intake of β carotene and lycopene reduces the rate of coronary heart disease (CHD), blood pressure, and mortality	
Arab and Steck (2000)	Lycopene	Preventive activity more likely due its cholesterol synthesis inhibiting effect rather than its antioxidant efficacy	Trials yet to be conducted to confirm its preventive activity

Table 2.2, continued

Author(s)	Antioxidant	Findings	Limitations
Anderson et.al (2001)	English walnuts	Inhibits *in-vitro* plasma and LDL oxidation	
Noijiri et.al (2001)	Total Antioxidant status (TAS)	Antioxidant parameters are related to progression of atherosclerosis	Antioxidants are not an independent risk factor in the development of coronary heart disease
Feldman (2002)	Walnuts	Concluded to be a heart healthy diet with ability to lower blood cholesterol	The duration of the studies was not long enough to confirm the sustainability of the results
Dragland et.al (2003)	Several herbs	High concentration of antioxidant and better dietary antioxidant as compared to fruits, berries, and vegetables	
Leelarungrayub et.al (2006)	Garlic and Shallot	Extracts of garlic and shallot bulbs had significant antioxidant activity as measured by a decreases in free radicals and an ability to inhibit lipid oxidation	No visible effect on protein oxidation

Table 2.2, continued

Author(s)	Antioxidant	Findings	Limitations
Koca and Karadeniz (2009)	Blackberries and blue berries	High antioxidant activity in both fruits due to total phenolic and anthocyanins	
Oviasogie et.al (2009)	Various fruits and vegetables	Ducanuts had highest total phenolics, while grape fruit had the lowest	The values were lower than other similar studies
Sumazian et.al (2010)	Nine Malaysian vegetables	Most vegetables showed high antioxidant activity of boiled aqueous extracts	
Awah et.al (2012)	Seven widely edible Nigerian medicinal plants	Significant antioxidant activities due to high phenolics contents	-Two plants were toxic to humans. -Further *in-vitro* and *in-vivo* studies need to be conducted

The use of herbal products falls under the category of Complementary and Alternative medicine (CAM). Many researches were carried out to investigate the various effects of different bioactive agents, derived from a variety of plants, on CVDs as shown in Table 2.2 above (Li et.al, 2012; Wang et.al, 2012; Jiang et.al, 2012).

The initial aim was to study various fruits and vegetables as a source of antioxidants for the purpose of cardio protection. One of the plants that were studied for antioxidant properties is called *Ficus deltoidea* (Ao et.al, 2008; Manian et.al, 2008; Al-Fatimi et.al, 2007). Phenolic contents of this plant pose the highest potential cause of the plant's antioxidant activity (Cai et.al, 2004; Djeridane et.al, 2006).

Natural products also play a key role in cholesterol inhibition, which is one of the main factors leading to atherosclerosis. Researchers have established that many natural products, some of which are soy proteins and tea extracts, can significantly lower cholesterol levels (Davidson and Geohas, 2003; Chagan et al., 2002). Furthermore, many clinical trials were conducted to conclude that red yeast rice and black tea flavins could also lower LDL (Becker et al., 2009; Davies et al., 2003). Triglyceride levels were also related to levels of marine omega-3 fatty acids (McGowan and Proulx, 2009). Finally, phytosterols, which are active compounds found in almost all vegetables, play an essential role in inhibiting the absorption of intestinal cholesterol, together with a serum-cholesterol inhibition effect (Escurriol et al., 2009).

2.1.3: Parameters measured

The type of CVD to be analyzed in this thesis is as a result of the two factors mentioned earlier. One is due to the free radical build up and the other is caused by atherosclerosis. Thus it is important to analyze all the parameters to be measured in order to accurately detect the presence of CVD or any onsets of the disease. The parameters to be measured were divided into two based on the type of sample to be assayed.

2.1.3.1: In-vitro tests

In-vitro tests are mostly dependent on measuring a substrate, oxidant, initiator, intermediate or final product (Antolovich et.al, 2002). Commonly, antioxidant assays measure the capacity of an antioxidant to inhibit oxidative damage through prevention of the production of oxidative intermediates and final products. Most antioxidant assays are divided into two main categories namely, hydrogen atom transfer (HAT) reaction based and electron transfer (ET) reaction based assays. Both of these categories measure the radical scavenging ability of the antioxidant being tested (Huang et.al, 2005).

HAT based assays measure the ability of an antioxidant to quench free radicals via the donation of hydrogen atoms (Prior et.al, 2005). These types of assays require an

oxidizable probe, and antioxidant, and a free radical generator (Wright et.al, 2001). The first step is adding a transition metal or an azo compound to initiate the oxidation, followed by addition of the antioxidant, and finally measurement of the reaction kinetics using UV spectrophotometer or gas chromatography (Huang et.al, 2005). Examples are oxygen radical absorbance capacity (ORAC) and total radical-trapping antioxidant parameter (TRAP) assays. ET based assays measure the ability of an antioxidant to transfer one electron to reduce the tested compound. Examples are copper reducing capacity assay (CUPRAC) and total phenolics assay (TPC). Thus the kinetics are not considered in these assays, rather the reduction in product formation is calculated, as ET based reactions are slow, and may require a long time to reach completion (Wright et.al, 2001).

In-vitro tests will be conducted on the extracts obtained and before administering the extracts on any healthy tissues or cells. The main aim of this research is to determine the antioxidant status of the extracts used and as an addition also to determine the cholesterol synthesis inhibiting ability of the extracts, if any. The following assays were conducted in this research:

1. <u>DPPH (2,2-diphenyl-1-picryl-hydrazyl) free radical scavenging assay:</u>

   Developed by Blois in 1958, this assay is based on 1,1-diphenyl-2-picrylhydrazyl (DPPH), which is a violet colored free radical with a nitrogen centre (Huang et.al, 2005). The mechanism involves the transfer of a hydrogen atom to DPPH, resulting in a reduction of the free radical, with a color change from violet to yellow/brown. Figure 2.1 below shows the DPPH free radical with its nitrogen centre ($N^{\cdot}$), and the DPPH molecule after the transfer of a hydrogen atom to the nitrogen centre.

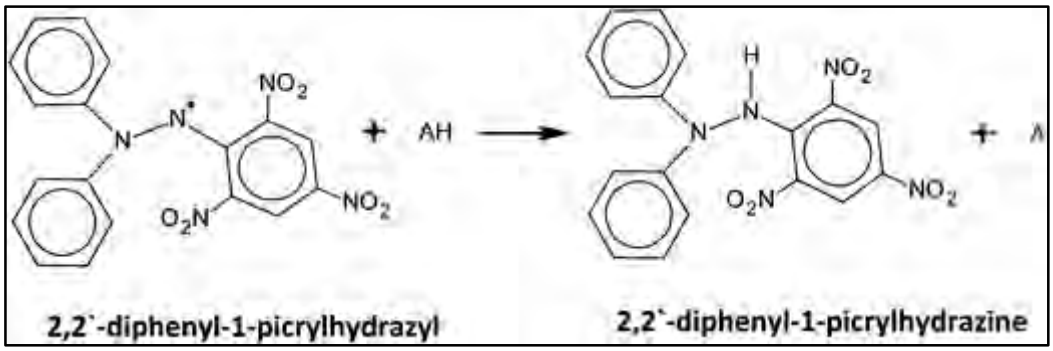


Figure 2.1: DPPH radical and its stable form

(Pyrzynska and Pekal, 2013)

This method is quick, simple and cost effective, thus it is widely used to measure the capacity of a potential antioxidant compound to act as a free radical quencher or hydrogen donor. Aside from food samples, it can also be used to test biological samples, both liquid and solid (Prakash, 2001). Some examples of samples that were tested include wheat grain, vegetables, herbs, and edible seed oils (Yu, 2001; Parry et.al, 2005). Moreover, it can be used as an antioxidant assay for cysteine, glutathione, ascorbic acid, and tocopherol (Masahiro et.al, 2005). The main advantge of this method is the long duration of the reaction, which is sufficient enough for DPPH to react with the whole sample, rendering itself a very efficient assay in testing weak antioxidants (Prakash 2001). Moreover, DPPH assay can be used in both aqueous and non polar organic solvents, as well as to examine both hydrophilic and lipophilic antioxidants (Prior et.al, 2005). On the other hand, this method is limited due to its sensitivity to light, oxygen, some lewis bases and solvents (Ancerewicz et.al, 1998; Min, 1998). Moreover, since the DPPH radical interacts with other radicals, the time response curve is not linear (Brand-Williams et.al, 1995; Sanchez-Moreno et.al, 1998). While carrying out this assay, the most commonly sought after paramter is called EC_{50} or IC_{50} which was introduced by Brand-Williams and his colleagues in 1995. It is defined as the concentration of substrate which causes 50% loss of the DPPH activity.

Table 2.3 below shows some of the researches conducted to test the DPPH free radical scavenging ability of various potential natural antioxidants from plants.

Table 2.3: Previous research on DPPH radical scavenging activity assay of various plant extracts

Author(s)	Antioxidant sample	Findings
Saha et.al (2008)	Methanol extract of *Mimusops elengi linn* leaves	IC_{50} = 43.26 µg/ml
Aris et.al (2009)	Fruits of *F. deltoidea*	IC_{50} of methanolic extract=250 µg/ml and chloroform extract=125 µg/ml
Hakiman and Maizah (2009)	Leaf aqueous extracts of 13 different accessions of *F. deltoidea*	Percentage inhibition ranged from 32.86 to 99.87%
Dzolin et.al (2010)	4 different varieties of *F. deltoidea* leaf and fruit aqueous extracs	Percentage inhbition at 31.3 µg/ml ranged from 72 to 82 % for leaf extracts and 51 to 71% for fruit extracts
Wahid et.al (2010)	Hot water extracts of leaves of different accessions of *F. deltoidea*	Percentage inhibition ranged from 43 to 88%
Ding et.al (2011)	Ethanolic extract, ethyl acetate and *n*-butanol fractions of *Rubus chingii*	IC_{50} values of 17.9, 3.4 and 4.0 µg/mL respectively
Norra (2011)	Leaves of different accessions of *F. deltoidea*	Percentage inhinition was 60% for MFD4 and 49% for MFD6
Valyova et.al (2012)	*Tagetes erecta* L	IC_{50} of 4.3 µg/mL

Table 2.3, continued

Author(s)	Antioxidant sample	Findings
Zhao et.al (201)	*Nigella glandulifera* seeds	IC_{50} of about 1.6 mg/ml
Bothon et.al (2013)	Hydro-alchoholic extracts of *Polygonum senegalensis* and *Pseudocedrela kotschyi*	IC_{50} of *Pseudocedrela kotschyi*=6.7 µg/ml and IC_{50} of *Polygonum senegalensis*=6.8 µg/ml
Muruhan et.al (2013)	Ethanolic extract of *Solanum surattense*	IC_{50} of 60 µg/ml

The positive control used in this experiment was quercetin which has been used in DPPH assay for the testing of various plants. Some of the researches conducted were on *Dorema aitchisonii* with a reported IC_{50} of 5.28 µg/ml (Seyed et.al, 2012) and *Lysimachia clethroides* with a reported IC_{50} of 6.94 µg/ml (Jin-feng et.al, 2012). Further researches established that the IC_{50} of quercetin ranged from 10.25 to 60 µg/ml (Vasagam et.al, 2011; Nimmi and George, 2012).

2. CUPRAC (cupric reducing antioxidant capacity) assay

This assay is a type of total antioxidant capacity assay that is based on single electron transfer (SET) and measures the change in the amount of $^{+}[Cu_2(Nc)]$ complex formed during the conversion of Cu(II) to Cu(I) upon changing concentrations of antioxidant added to the mixture, which is later oxidized by copper(II) (Özyürek et.al, 2011). According to Bioxytech AOP-490 assay, bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline) forms a 2:1 complexes with Cu(I), resulting in a chromophore with absorbance at 490 nm. Similarly the CUPRAC assay uses another compound called neocuprine (2,9-dimethyl-1,10-phenanthroline) and the Cu(I) complex with absorbance at 450 nm. This assay has some advantages over FRAP assay which utilizes iron instead of copper as it can detect almost all antioxidants with little interference and has faster

reaction kinetics when compared to iron. However, the assay may take 30-60 minutes for detection of complex molecules, thus an appopriate reaction time must be selected (Prior et.al, 2005). Güçlü and colleagues conducted CUPRAC assay to measure the total antioxidant capacity of Malatya apricots (*Prunus armeniaca*) and noted down that some of the advantages of this assay are the stability, low cost and accessibility of the reagent. Moreover, it is an easy procedure and does not get affected by air, sunlight, humidity, and pH. It is a precise method that has a working pH of 7, which is close to the physiological pH making it more accurate.

One such study was conducted by Öztürk and colleagues on the stem and root extract of *Rheum ribes* (Rhubarb) and the results of CUPRAC assay was plotted as absorbance versus concentration in (µg/ml). The absorbance of all extracts increased with increasing concentration but was still lower than that of the control (Öztürk et.al, 2007). Another similar research was carried out by Sabudak and colleagues to assess the antioxidant properties of *Trifolium echinatum* Bieb. The results were reported in terms of absorbance and specifically the concentration of sample that gives 0.5 nm of absorbance ($A_{0.50}$). As such, the absorbance of the different bioactive compounds isolated from this plant ranged from 6 to 400 µM (Sabudak et.al, 2013). Further similar research was conducted on different natural product extracts by other researches, utilizing the same method and similar methods of data presentation (Gülçin, 2009; Sghaier et.al, 2011; Öztürk, 2012).

3. Total phenolic content (TPC):

Upon certain stressful conditions such as infections or exposure to ultraviolet radiation, or during normal growth, the plant produces secondary metabolites knows as phenolics (Uppu, Murthy and Parinandi, 2010). Phenolics content of a sample determines its strength as an antioxidant (Eberhardt et.al,

2000; Liu et.al, 2002). Most vegetables and fruits may contain polyphenols which are considered to be a stronger antioxidant than vitamin C, E, and β-carotene within the sample plant (Chu et.al, 2002). Flavonoids are the most commonly occurring polyphenols and can be divided into flavanols, flavanones, flavones, anthocyanidins and flavonols, as shown in Figure 2.2.

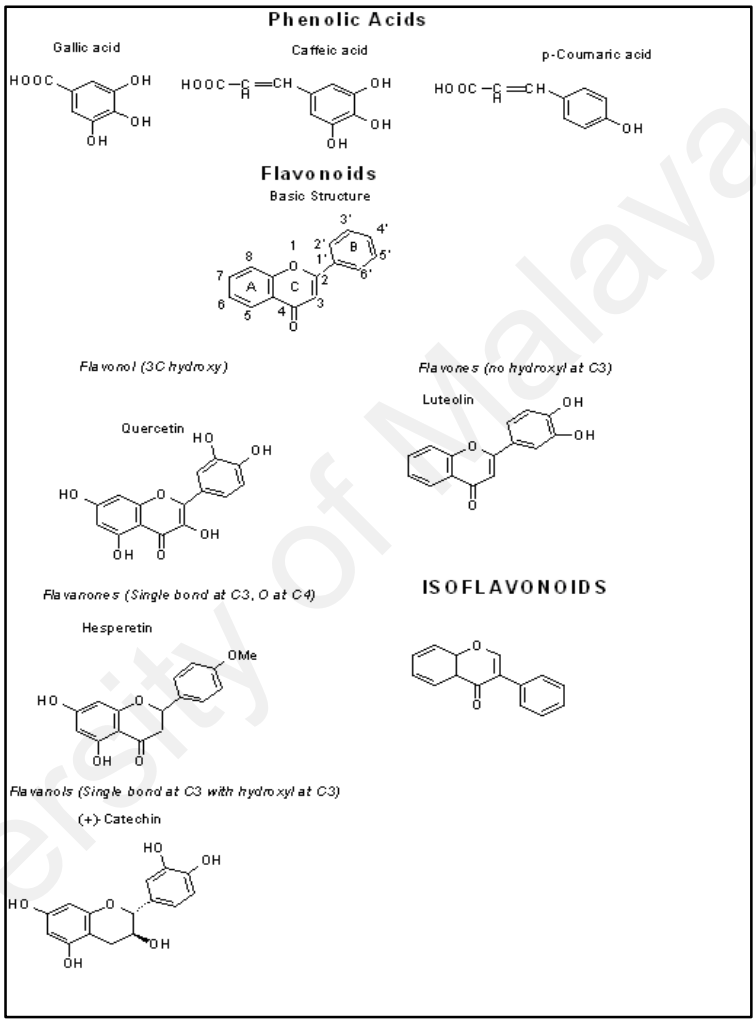


Figure 2.2: Common flavonoids found in plants

The assay method makes use of the Folin-Ciocalteu (FC) reagent which was used to assay the total phenolics content of a sample in this study. Initially, this method was developed to analyze amino acid residues, tyrosine, and tryptophane (Folin and Ciocalteu, 1927). Later, Singleton and Rossi (1965) and Singleton and Slinkard (1977) improved this method to test for total phenolic content of samples. The FC reagent is an oxidizing agent which contains heteropolyphosphotungstane-molybdate, and oxidizes phenolates producing a

blue colored product which is reported as gram gallic acid equivalents per liter of solvent (g GAE/L). Gallic acid, or also known as 3,4,5-trihydroxybenzoic acid, is compound rich in phenolics and can be found in plants and plant-derived foods and beverages (Bravo, 1998; Obreque-Slier et.al, 2010; Landete, 2012). Thus, it has a well-established role as a free radical scavenger and inhibitor of lipid oxidation (Dwibedy et.al, 1999).

Some of the researches conducted to measure the TPC of various natural potential antioxidants are listed in Table 2.4 below.

Table 2.4: Previous researches on total phenolic content (TPC)

Author(s)	Antioxidant	Findings
Adom and Liu (2002)	Various grain extracts	-Corn TPC= 15.55 µmol (GAE)/g of grain -Wheat TPC = 7.99 µmol (GAE)/g of grain -Oats TPC = 6.53 µmol (GAE)/g of grain -Rice TPC = 5.56 µmol (GAE)/g of grain
Hakiman and Maziah (2009)	Leaf aqueous extracts of 13 different accessions of *F. deltoidea*	Range of TPC = 1.60 to 4.27 mg GAE/g FW
Aris et.al (2009)	Fruits of *F. deltoidea*	TPC of hexane, methanol, and choloroform extracts are 259.2 mg GA/g, 245.2 mg GAE/g, and 159.2 mg GAE/g, respectively
Satnkovic (2011)	Extracts of *Marrubium peregrinum* L.	TPC ranges from 27 to 49 mg GAE/g
Bernaert et.al (2012)	Various leek extracts	TPC ranges from 8 to 10 mg GAE/g dry weight

Table 2.4, continued

Author(s)	Antioxidant	Findings
Saeed et.al (2012)	Methanolic extract of *Torilis leptophylla*	TPC = 121.9 mg GAE/g extract
Zilic et.al (2012)	10 maize genotypes	Highest TPC was dark-blue maize = 10528.8 mg GAE/kg, and lowest was multicolored maize = 4491.1 mg GAE/kg
Krishnaiah et.al (2015)	Methanolic extract of *Morinda citrifolia* L.	TPC = 43.18 mg GAE/10 g of sample

The above quoted studies only indicate the general total phenolic content found in this plant. It is worthy to note the results of studies focusing on specific phenolic content of *F.deltoidea* as well. One such study was conducted by Hakiman and colleagues (2012), where different varieties of *F.deltoidea* was tested for the phenolic content using HPLC method. The HPLC results showed two peaks corresponding to two flavonoids, namely rutin and naringin. The variety of *F.deltoidea* used in this study (*var. kunstleri*), contained the highest rutin content when compared with the other two tested varieties of the plant (12.83 µg/g DW). Another similar more comprehensive study was carried out by Omar and colleagues (2011), using HPLC as well to analyze the flavonoids compounds in *F.deltoidea* and the results showed that more than 25 compounds were identified. The compounds can be categorized as proanthocyanidins, flavan-3-ol monomer and flavones glycosides. Choo and colleagues (2012) identified the presence of vitexin and isovitexin in the leaves of *F.deltoidea*.

4. Lipid peroxidation

As a part of a cell's normal function, free radicals, such as oxygen radicals, are constantly produced, which can cause damage to the living tissue. This

damage mainly involves oxidation of lipids, which are the building blocks of the membranes of most cells and organelles. Once the lipids are oxidized, the membranes break down leading to the release of cell components which consequently results in atherogenesis (Matsuoka, 2001; Poli et al., 2004).

The mechanism by which lipids are oxidized can be divided into initiation, propagation, and termination. Figure 2.3 below represents these three steps as applied to a polyunsaturated fatty acid (PUFA). Initiation basically involves the formation of the unstable fatty acid radical due to the attack of the hydroxyl free radical which shifts the double bond. Following this is propagation where the stabilization of this fatty acid radical is established via the addition of a hydrogen or oxygen atom to generate another radical. Finally, termination step transforms this radical into a more stable compound such as hydrocarbons, alcohols, volatile ketones, and polymers, some of which may be harmful (Gordon, 1989; Laguerre et.al, 2007).

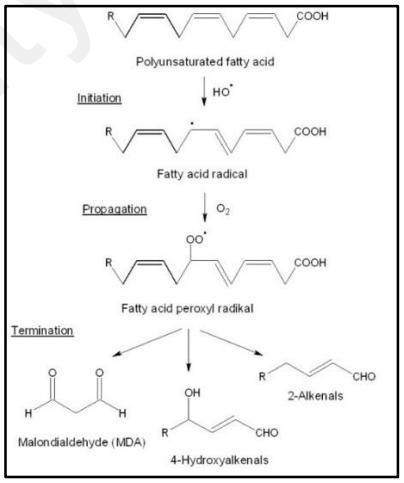


Figure 2.3: Steps in lipid peroxidation
(Mimica-Dukić et.al, 2012)

Many different assays can be conducted to measure the degree of lipid peroxidation *in-vitro*. Some of which are conjugated diene assay, Thiobarbituric acid reactive substances (TBARS) assay, quantitative measurement of lipid hydroperoxide, and lipid peroxide assay. In this research, TBARS assay was

chosen to test the extracts *in-vitro*, since it has been more frequently and commonly used. The basis of this assay is the reaction between thiobarbituric acid and one of the aldehydes formed due to oxidation of lipids (Halliwell and Chirico, 1993). Some of the studies conducted are summarized in Table 2.5 below

Table 2.5: Previous researches on inhibition of lipid peroxidation assay of various plant extracts

Author(s)	Antioxidant	Findings
Tang et.al (2007)	Polysaccharide extract from *Nostoc sphaeroides*	IC_{50} = 31.8 µg/ml
Aris et.al (2009)	Fruits of *F. deltoidea*	All extracts showed strong antioxidant activities within the range of 91-96 %
Shirwaika et.al (2011)	Defatted ethanolic extract of the seeds of *Lepidium sativum* Linn.	IC_{50} = 71.39 µg/ml
Poorna et.al (2012)	Leaf Extract of *Excoecaria agallocha* Linn Mangrove Plant	IC_{50} = 100 µg/ml
Adithya et.al (2013)	Methanol extracts from bark and stem of *Mahonia leschenaultia*	IC_{50} of bark extract = 80 µg/ml and stem extract = 100 µg/ml
Parimala and Shoba (2013)	Hydroalcoholic seed extract of *Nymphaea nouchali* Burm. F	IC_{50} = 54.65 µg/ml
Soumaya et.al (2014)	*Cyperus rotundus*	IC_{50} of methanol extract was 40 µg/ml IC_{50} of aqueous extract was >800 µg/ml

Quercetin was used as a positive control in this assay as well. According to Parimala and Shoba, the IC_{50} of quercetin was around 20 μg/ml (2013).

5. HMG-CoA Reductase Assay

HMG-CoA reductase which stands for hydroxymethylglutaryl-coenzymeA reductase is an enzyme that catalyzes the conversion of hydroxymethyl-glutaryl-coenzymeA (HMG-CoA) into mevalonate, which in turn is converted into phosphorylated fatty acids. This step is therefore considered to be the rate-determining step in cholesterol biosynthesis (Vonbergmann et.al, 1987; Park et.al, 2001). In 1972, Brown and Goldstein presented some comprehensive work on the regulation of cholesterol biosynthesis and also discovered the low density lipoprotein (LDL)-receptors. Due to this development, compactin, a selective HMG-CoA reductase inhibitor, was also discovered shortly after. This discovery initiated a new potential class of medicines in cardiovascular disease treatment, now more commonly known as statins (Endo et.al, 1976). According to the Pfizer annual review in 2008, Lipitor© (atorvastatin) is selected as the world's best-selling drug used to treat high levels of LDL cholesterol, total glycerides, and to prevent cardiovascular diseases.

In order to determine the cholesterol inhibition ability of the extract, therefore its potential use as a statin, HMG-CoA reductase assay was carried out according to the kit protocol from Sigma-Aldrich. A research was carried out by Xie and colleagues in 2009, which states that *Gingko bilboa* extract has a proven potential to reduce the cholesterol level by inhibiting the HMG-CoA reductase enzyme. One of the oldest studies, conducted by Wang and colleagues in 1997, states that red yeast has a great potential in inhibiting HMG-CoA reductase enzyme, thus effectively lowering the cholesterol levels. Similarly, Lachenmeier and colleagues tested red yeast rice (*Monascuc spp.*) for HMG-CoA reductae

inhibition, and found that there was a dose-dependant inhibition of the HMG-CoA reductase enzyme by the red yeast rice samples (2012). *Ficus palmata* bark aqueous extract was studied for HMG-CoA reductase inhibition activity and the IC_{50} was found to be 9.1 µg/ml (Iqbal et.al, 2014).

2.1.3.2: In-vivo Assays

1. Cardiac tissue marker enzymes

Acute myocardial infarction (AMI) has been known as an important cause of death all over the world. Thus, diagnostic methods have been the focus of most researches for earlier detection, prevention and treatment. The world health organization (1971) states that two out of the three following criteria should be fulfilled for the detection of AMI:

i) Typical anamnestic signs (for example chest pain)
ii) Typical electrocardiogram (ECG) findings
iii) Increase in the serum cardiac enzymes lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and creatine kinase (CK).

Cardiac marker enzymes are released during cardiac injury, and can be measured directly in the blood samples. Aspartate aminotransferase (AST) was one of the first markers to be analyzed by LaDue and colleagues in 1954. This was followed by the development of newer assaying methods in the 1960s, which involved two markers, lactate dehydrogenase (LDH) and creatine kinase (CK) (Amador et.al, 1963; Dunn et.al, 1965). In this research CK, LDH, AST and two other markers namely alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were measured directly from the serum samples. AST and ALT are enzymes used to transfer an amino group from one amino acid to another (Devlin, 2002). They are both found in heart, skeletal and liver muscles, and are used as markers of heart and liver damage, where raised levels of these enzymes may

indicate myocardial infarction, liver necrosis, and cirrhosis (Zilva and Pannall, 1979).

CK specifically catalyzes the transfer of phosphate from adenosine triphosphate (ATP) to creatine, by which phosphocreatine is produced. Thus, the reverse of this reaction serves as a provider of a phosphoryl group to form ATP in times of energy demand due to increased muscle activity (Bhagavan 2002). This enzyme leaks from the injured heart cells, making it a useful marker of myocardial infarction, as it also becomes detectable 4-6 hours after onset of disease, and lasts up to 10 days (Kachmar and Moss 1976). LDH catalyzes the final step in glycolysis in the absence of oxygen to produce more NAD^+ for glycolysis, and in turn, reduce pyruvate to L-lactate. Similarly to the previous three enzymes, this enzyme is found in skeletal and heart muscle, as well as liver and kidneys (Devlin, 2002). High LDH levels indicate various disorders such as myocardial infarction, hemolysis, renal cortical, liver disease and skeletal muscle damage (Bhagavan, 2002).

Finally, ALP belongs to a group of enzymes that work at an alkaline pH and catalyze the production of a phosphate group from an organic molecule. This role was first proposed by Poelstra and colleagues in 1997 as a detoxifying agent through dephosphorylation of lipopolysaccharides and adenosine triphosphate (Bentala et.al, 2002; Eckle et.al, 2007). This enzyme also has a role in bone mineralization, and its elevated expression in heart arterioles may indicate vascular hardening and cardiovascular disease (Hui and Tenenbaum, 1998; Van Hoof and De Broe, 1994).

Recent researches such as the one conducted by Palanivel and colleagues in 2011, and Kumar and colleagues in 2012 make use of these cardiac tissue marker enzymes to detect necrosis to the heart muscles. The levels of cardiac

tissue marker enzymes such as AST, ALT, and ALP were highest in rats that were induced with necrosis using isoproterenol when compared to normal rats and rats that were pre-treated with cardioprotective agents. Similarly, Evran and colleagues conducted a study recently of the effect of carnosine on antioxidant status of rats with isoproterenol-induced myocardial infarction. The levels of CK, LDH, and AST in the plasma were higher in isoproterenol induced rats as compared to rats pre-treated with carnosine (2014).

2. Lipid profile

The lipid profile that was presented in this research consists of the levels of total cholesterol (TC), low density lipoproteins (LDL), high density lipoproteins (HDL), and triglycerides (TG), which are essential parameters evaluated in the development of atherosclerosis. Total cholesterol as suggested by the name is the sum of the cholesterol that circulates in the blood. Cholesterol is a compound that is insoluble in water and biosynthesized by all cells in the body to form a part of the cell membrane and maintain its integrity and spherical shape (Morales et.al, 2006). Cholesterol in the body is transported in small vesicles known as lipoproteins that are made up of both lipids and proteins (LDL, HDL, and VLDL). Thus, when obtaining the lipid profile, the total cholesterol measurement actually indicates the sum of HDL, LDL and VLDL (very low density lipoproteins) in the blood (Dipiro and Talbert, 2005). Triglycerides, which are polar lipids that are hydrophobic in aqueous environments, are included in the lipid profile measurements, as they act as the core of lipoproteins. Most of the triglycerides are carried by VLDLs, whereas 50% of the cholesterol is carried by LDLs (Dipiro and Talbert, 2005).

LDL plays an important role in the progression of atherogenesis, due to its high cholesterol content and its function as transporter of cholesterol from liver to

body cells. Moreover, oxidized LDL has adverse side effects on the vessel walls and aid in the atherogenic process (Breslow et.al, 1997; Kavurma et al., 2005). HDL, on the other hand has been known as good cholesterol, due to its function as transporter of deposited cholesterol from vessel walls to the liver (Gotto and Farmer, 1988). The Framingham Heart Study reported that levels of HDL below 40 mg/dL result in about 44% of coronary complications (Castelli et.al, 1986). Similarly, several studies have established a clear link between high levels of LDL and the development of coronary heart disease (Lipid Research Clinics (LRC), 1984; Stamler et.al, 1986; Wilson et.al, 1998).

Triglyceride level has also been established as one of the factors leading to atherosclerosis (Assmann et.al, 1998; Austin et.al, 1998). Moreover, total cholesterol can be assessed to measure the risk of cardiovascular disease, however, it is not an independent measurement. The total cholesterol, as mentioned earlier is a sum of the HDL, LDL, and VLDL in the blood stream, thus high total cholesterol may sometimes be due to high HDL, which indicates lower risk of CVD, thus it is important to measure the individual lipoprotein levels as well (Dipiro and Talbert, 2005).

This research focuses on two factors of CVD, namely free radical damage and atherogenesis. Numerous researches were conducted concerning the lipid profile in rats with induced myocardial infarction, and those with induced hyperlipidemia. Some of these studies are summarized in Table 2.6 below

Table 2.6: Previous researches on lipid profile

Author(s)	Sample	Findings
Ugochukwu et.al (2003)	*Gongronema latifolium* leaf extracts	Extract decreased TG levels and normalized TC concentration
Radhiga et.al (2012)	Ursolic acid (UA)	High levels of TC, TG and LDL, and low level of HDL in ISO-induced rats. UA administration normalized levels of these parameters in blood plasma
Shaik et.al (2012)	Ethanolic extract of *Terminalia pallida* fruits	High levels of TC, TG and LDL, and low level of HDL in ISO-induced rats. Pretreatment with the extract inhibited the effects of ISO
Upaganlawar and Balaraman (2012)	*Lagenaria siceraria* fruit juice	ISO treated rats showed higher level of TC, TG, and LDL, and lower level of HDL when compared to pretreated rats
Abo-Gresha et.al (2014)	Evening primrose oil (EPO)	Treatment with EPO improved the serum lipid profile

TG – Total glycerides, TC – Total cholesterol, LDL – Low density lipoproteins, HDL – High density lipoproteins, ISO – Isoproterenol

Table 2.6, continued

Author(s)	Sample	Findings
Kim et.al (2014)	*Sasa quelpaertensis* (SQE) leaf extract	TG, LDL and TC were decreased upon treatment with SQE, and HDL levels were restored as compared to untreated plasma samples
Sadjadi et.al (2014)	Caraway extracts	Diabetic rats receiving the extract had lower TC, TG and LDL than diabetic control rats
Kaushik et.al (2013)	*Alpinia galanga* alcoholic extract	The extract lowered TC, TG, and LDL, and increased HDL

TG – Total glycerides, TC – Total cholesterol, LDL – Low density lipoproteins, HDL – High density lipoproteins, ISO – Isoproterenol

3. Inflammation

TNF alpha or tumor necrosis factor is small protein located in the serum, more specifically anchored in the cell membrane, and induced by endotoxins to cause necrosis of tumors (Carswell et.al, 1975). Its production is triggered by infectious or inflammatory stimuli, and although some of the TNF-α is retained in the cell, most of the TNF-α is released into the circulation (Kriegier et.al, 1988). In 1990, Levine and colleagues established that TNF-α may participate in the progression of heart disease, or congestive heart failure (CHF). This was due to the observation of higher levels of circulating TNF-α in patients with heart failure. Eventually, Mann and colleagues reached the conclusion that the failing human heart expresses significantly greater amounts of this protein when compared to the non-failing human heart (1996).

Aside from its connection to CHF, TNF-α has an important role as a contributor to the development of atherosclerosis. According to literature, this role is achieved by its ability to express adhesion molecules on endothelial cells, recruit and activate inflammatory cells, and initiate inflammatory reaction in the arterial wall (Ross et.al, 1999; Skoog et.al, 2002). As a result of this protein's interference in the metabolic pathways of TC and cholesterol, patients with high TNF-α concentrations due to inflammatory disorders show an increased level of TG as well (Suffredini et.al, 1989; Torre-Amione et.al, 1996). According to several studies, the administration of TNF-α to mice and humans results in an increase of total TG in plasma (Eichenholz et.al, 1992; Matsumori et.al, 1994; McTiernan et.al, 1997). The mechanism is via the increase in free fatty acids and substrate for TG synthesis, and the decrease in rate of clearance of TG-rich lipoproteins (VLDLs) from the blood circulation (Gulick et.al, 1989; McTiernan et.al, 1997).

In this study, TNF-α is measured to indicate inflammation and presence of cardiac failure. The selection of this assay is based on other similar researches that measure the level of this protein together with the lipid profile and cardiac marker enzymes. One such research is the one conducted by Abo-Gresha and colleagues to analyze the effects of evening primrose oil on platelet aggregation cardiac recovery (2014). Their results indicate that TNF-α was really high in rats with isoproterenol induced myocardial infarction, whereas the level of this protein was significantly reduced after treatment with evening primrose oil.

4. Oxidative stress – NADPH concentration

During aerobic respiration, cells are in contact with free radicals such as hydrogen peroxide, which would damage the cells and even lead to cell aging, death, and a wide variety of diseases (Gutteridge and Halliwell, 2000). Oxidative phosphorylation, which produces adenosine triphosphate (ATP), is the final step of aerobic respiration whereby most of the free radicals are produced. NADPH comes into play at this stage to provide a reductive environment by scavenging the free radicals (Giro et.al, 2006; Marino et.al, 2007). The NADPH is mostly generated from the pentose phosphate pathway and supports the antioxidant enzymes such as glutathione peroxidase, superoxide dismutase, and catalase (Holmgren, 1989).

$NADP^+$ concentration is also a determinant of oxidative stress. A key enzyme of the pentose phosphate pathway (PPP) is Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49).This enzyme is involved in antioxidant defense as it produces NADPH (Luzzatto, 1967 and Kletzien et al., 1994). The remaining $NADP^+$ concentration is an indicator of the antioxidant capacity of the tested sample. Lower levels of $NADP^+$ is characteristic of a good antioxidant, as it indicates higher levels of NADPH, which acts as a free radical scavenger (Diaz-Flores et.al, 2006). Accordingly, higher levels of $NADP^+$ indicates lower levels of NADPH, thus a build-up for free radicals which is the main component leading to oxidative stress. According to one research conducted by Diaz-Flores and colleagues, the livers of rats with severe hyperglycemia showed a higher concentration of $NADP^+$ when compared to healthy rats and rats with less severe hyperglycemia (2006).

5. Histopathology of cardiac tissue

Histopathology is a word derived from three Greek words namely, histos = tissue, pathos = disease or suffering, and logos = study. Hence, it can be summarized as the study or examination, under a microscope, of diseased or infected cells and tissue. In this research, the heart tissue of the rats were excised and processed as described in the methodology chapter to observe the changes caused by CCl_4, as well the *F. deltoidea* extract.

6. Carbon tetrachloride (CCl_4)

Isoproterenol is classified as a drug used for treatment of heart diseases, and may in excess doses lead to adverse side effects, such as cardiac arrest. It is therefore used in most similar researches as inducer for cardiac damage. As an attempt to adopt a different method, CCl_4 was used in this research as an alternative to the use of isoproterenol. CCl_4 is a clear, volatile, and heavy liquid that causes tissue (liver, kidneys, lungs, and heart) toxicity through the generation of free radicals (Adarmoye, 2009; Öztürk et.al, 2003). The transformation of CCl_4 to trichloromethyl-free radical ($CCl_3\cdot$) leads to the initiation of lipid peroxidation process (Brattin et.al, 1985; Shenoy et.al, 2001; Adewole et.al, 2007). The cardiotoxicity is mainly caused by an increase in the amount of cellular free calcium, damage to ion transport and permeability, and cell destruction (Giordano, 2005). The end result is a loss of structural integrity of myocardial cells and a decrease in cardiac function leading to cardiac failure (Plaa and Witschi, 1976). Eshaghi and colleagues conducted a research based on the use of CCl_4 as the inducer for cardiac toxicity. The results obtained were similar to the researches using isoproterenol, as the levels of cardiac tissue marker enzymes were highest in CCl_4 induced rats as compared to the rats pre-treated with the antioxidant source (2012). Other similar researches were conducted to confirm the

potential of using CCl_4 as an alternative to isoproterenol (Mohamed et.al, 2009; Chang et.al, 2014; Sahreen et.al, 2014; Yang et.al, 2014).

Although CCl_4 was mainly used for hepatotoxicity studies, it has long been known that cirrhosis of the liver leads to cardiac dysfunction in the form of fibrosis and myopathy (Wong, 2009). Timoh and colleagues conducted an extensive review on the cardiomyopathy caused by cirrhosis, or now more commonly known as cirrhotic cardiomyopathy, and concluded the presence of such a condition in majority of cirrhosis patients with about 7-15% deaths after liver transplantation which was related to cardiac dysfunction (Therapondos et.al, 2004). Among the many cellular changes that occur, a major one is inflammation as detected by an increase in the levels of Tumor Necrosis Factor-α (TNF- α) (Moller and Henricksen, 2002). The other parameters measured in this research have not been studied previously, thus, this will be an attempt to see the changes in those parameters under the pathological condition caused by CCl_4.

2.2: *Ficus deltoidea*

2.2.1: Background

Ficus deltoidea, also known as 'mas cotek' and 'mistletoe fig', belongs to the Moraceae or mulberry (fig) family. This plant is a shrub that can reach 15 feet in height, which makes it comparatively smaller than other fig families. Thus, it is mainly used as a houseplant, harbors no flowers, and has large leaves and greenish-white fruits (Lansky et al., 2008). *F. deltoidea* is mainly found in South East Asia, Borneo and the Philippines, and has been long recognized as a traditional remedy for various conditions as well as used for other purposes. Its fruits are dried and eaten and leaves used for tea making (Ao et.al, 2008). Figure 2.4 below shows the general appearance of the plant.

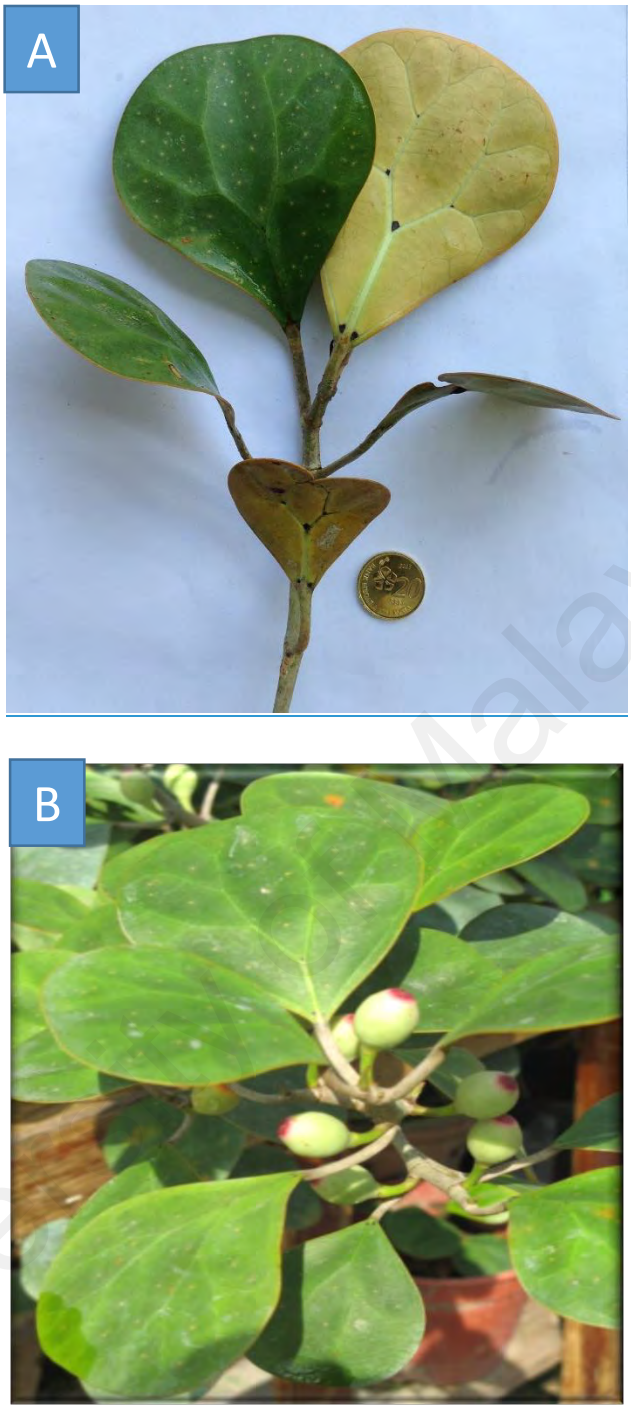


Figure 2.4: *F. deloidea* plant

A. Supervisor's personal photograph – *F.deltoidea* var *kunstleri* (sent to Herbarium, Rimba Ilmu, University of Malaya
B. Supervisor's personal photograph – *F. deltoidea* var *kunstleri* grown in the plantation

This plant has been traditionally used to prevent and cure many diseases such as breast cancer and diabetes. Leaves are the main part of the plant used to prepare herbal medicine, some use other parts of the plants like the roots and the fruits. Its use as a traditional medicine has encouraged scientists to pursue this plant and its bioactive compounds as a new potential compound to treat and protect from various diseases. Several studies regarding this plant have reported that the plant possesses significant antioxidant activity due to its phenolics content (Teixeira et.al, 2006; Çalışkan and Polat, 2011). There exists many products using the extracts of parts of these plants, some of which are sold in the form of herbal tea, capsules, and ointments.

2.2.2: Properties and applications

As mentioned earlier, this plant's most active component is established to be the phenolics compounds. One of these compounds is the flavonoids which leads to its ability to act as an antioxidant, help in pigmentation and protect against harmful microorganisms. Moreover, the flavonoid content gives it the ability to act as an anti-inflammatory and anti-cancer agent (Henderson et.al, 2000). Tannins also contribute to the bioactivity of this plant by acting as an anti-diarrheal and anti-hemorrhoidal agent (Vattem et.al, 2005). The volatile compounds produced by the fruits of this plant, which were present as floral fragrances, was first analyzed by Grison-Pigé and colleagues (2002), and belonged to terpenoid and aliphatic groups. Later moretenol and antibacterial compounds were isolated from the leaves (Lip et.al, 2009; Suryati et.al, 2011). Omar and colleagues (2011) finally managed to isolate about 25 compounds from the aqueous leaf extracts of *F. deltoidea* using HPLCMS-based method, most of which were flavonoids.

Due to its bioactive content, several studies were conducted on this plant to assess its capacity to act as a bioactive agent in various areas. One such study was conducted by Aminudin in 2006 using rats to measure the ability of the plant, in tea and fruit extract form, to affect the glucose levels. Results showed that rats that have been given the plant

showed a reduction of 15.6% in glucose levels as compared to a 14.8% reduction in untreated rats. Similarly, the researcher compared the ability of this plant to reduce glucose with other plants and found that *F. deltoidea* exhibited the highest amount of glucose reduction. Zainah and colleagues (2007) conducted a study to show that the aqueous extract of the plant also had glucose reducing ability in mildly diabetic rats. Another interesting use of this plant was demonstrated by various studies and states that the plant extract can help in strengthening the uterus and regulating the menstrual flow, thus can be used by mothers after giving birth (Fasihuddin and Din, 1995; Anon, 2000).

The anti-inflammatory effect of the plant was studied by Abdullah and colleagues (2009), whereby the leaf extracts were tested using three *in-vitro* assays. The results clearly show the extracts' ability to reduce inflammation, thus its potential role as a pain reliever (Abdullah et.al, 2009). The antioxidant effect of the extract was measured through the total phenolic content and the ferric reducing antioxidant potential (FRAP) assay. The results show that flavan-3-ol monomers and proanthocyandins caused more than 80% of the antioxidant activity (Omar et.al, 2011). Hakiman and Maziah (2009) also conducted a research on different extracts of *F. deltoidea* using various assays, and confirmed the antioxidant potential of the extracts.

Another potential use of *F. deltoidea* was studied by Zahra and colleagues (2009) and assessed its ability to heal against gastric ulcers in rats. Pretreatment with the extract resulted in a considerably less gastric damage due to ulcers. Higher dose of extract (500 mg/kg) showed better results when compared to lower dose (250 mg/kg). The wound healing capacity of the plant was studied by Abdullah and colleagues (2010) whereby a group of rats were treated with the extracts and another group was given only deionized water. The treated rats showed faster wound healing and lower scar width when compared to the control group. As for the safety of use of this plant extract, various studies have been conducted to measure its toxicity, such as the ones conducted by Shafaei and

colleagues (2011), which confirmed that the plant does not contain any toxic elements. Moreover, acute toxicity tests showed that the lethal dosage (LD_{50}) of the extract was higher than 5000 mg/kg (Shafaei et.al, 2011).

The two factors of cardiovascular diseases that were studied in this research are the free radical damage to the heart and atherosclerosis caused by cholesterol buildup. Therefore, due to the bioactive contents that are mentioned above and its efficacy as an antioxidant and anti-diabetic agent, *F. deltoidea* leaf extracts are tested in this study for cardio protective and cholesterol lowering effects.

2.2.3: Extraction Methods

The amount of some specific bioactive compounds are rather low in plants, such as antioxidant compounds. Thus, it is necessary to obtain the desired compound in a more concentrated form for effective result. Usually, the easiest way to achieve this is by drying to remove the water. However, it is also possible and more precise to use the method of extraction, whereby the selection of extraction solvent is the key step. The two main factors studied in the selection of solvents are the nature of the plant material and that of the antioxidant (Pokorný and Korczak, 2001).

Different kinds of extraction processes may be used to obtain various types of extracts with different concentration of bioactive compounds. The most common and easiest to prepare is the water extract, including cold and hot water extracts (Syazwani et.al, 2010). This extract is mainly used to assess the antioxidant and total phenolics content of the plant (Wahid et.al, 2010). Ethanolic and methanolic extracts also yield a compound with higher antioxidant content, and also yields higher concentration of extract per ml of sample when compared to water extracts (Adam et.al, 2012).

Organic solvents such as methanol, ethanol, ethyl acetate, and hexane, are generally used for the extraction of antioxidants (Pokorný and Korczak 2001). The extraction yield and the antioxidant activity are dependent on the type of solvent used.

According to Julkunen-Tiitto (1985), the extraction yield of total phenolics was highest in methanol extract, followed by acetone. Studies conducted on cocoa and buckwheat also stated that methanolic extract contained the highest antioxidant activity (Przybylski, 1998). However, other researches established that different plants require different extraction solvents for optimum antioxidant activity. For instance, lentil seeds had optimum antioxidant activity in aqueous acetone extract, as opposed to optimum activity in chloroform or ethyl acetate extract in the extraction of tea leaf catechins (Pokorný and Korczak 2001; Amarowicz et.al, 2003).

The procedure and type of solvent used in the extraction process significantly effects the yield of bioactive compounds obtained. Antioxidant compounds are of different nature and have varied chemical characteristics and polarities (Julkunen-Tiitto, 1985). Polar solvents are usually used for the extraction of polyphenolic compounds from plants, and are usually in aqueous mixture forms containing methanol, ethanol, and ethyl acetate. The most widely used solvent would be absolute ethanol or ethanol mixed with water at a certain ratio (Gray et.al, 2002; Yu et.al, 2002; Adom et.al, 2003; Liyana-Pathirana and Shahidi, 2006).

Since no one solvent can be regarded as the best solvent for extraction, 70% ethanol and water was used to extract bioactive compounds from *F. deltoidea* leaves. The solvent with higher activity according to *in-vitro* tests was selected. The method selected for ethanolic extraction was using soxhlet apparatus, which is a standardized method used as reference for assessing the performance of other extraction methods. It is a well-established technique with higher performance when compared to other extraction techniques (Luque de Castro and Garcia-Ayuso, 1998). Figure 2.4 below shows the soxhlet extraction apparatus as retrieved from www.chemwiki.ucdavis.edu. The sample, which in this case is the powdered *F. deltoidea* leaves, is placed in the thimble, and the round bottom flask is filled with 70% ethanol. This process is a continuous extraction of

bioactive agents from the sample by recycling of the solvent using the water-cooled condenser. Eventually, the extracted compounds, which dissolve in the solvent, are collected in the flask, and need to be separated from the solvent later on.

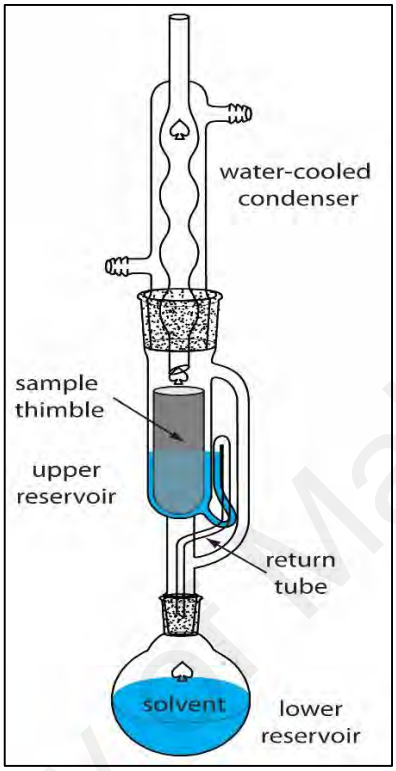


Figure 2.5: Soxhlet apparatus (http://community.asdlib.org/imageandvideoexchangeforum/2013/07/24/soxhlet-extraction/)

Water extraction can be carried out on the same residual sample in order to remove sugars and other undesirable water-soluble, inactive substances (López-Sebastián et.al, 1998). However, the required antioxidants may also be removed in this step (Pokorný and Korczak, 2001), thus, in this research, powdered sample was freshly extracted with water as well so as to compare the end product's bioactivity to that of the re-extracted sample.

CHAPTER 3.0

METHODOLOGY

3.1: Plant preparation and extraction

Ficus deltoidea var *kunstleri* was harvested from the plantation in Rembau, Negri Sembilan, Malaysia. Plant sample was cleaned and sent to the Herbarium, Rimba Ilmu University of Malaya and voucher specimen number was given as KLU 046470.

The fresh leaves of *F. deltoidea* were cut into pieces and dried to constant weight. The dried pieces (500 g) were ground into powder using an electrical blender. The dried powdered plant leaves were extracted with 90% ethanol using soxhlet apparatus (Favorit, Malaysia). The solvent was evaporated under vacuum using rotary evaporator (BÜCHI Rotavapor R-114, Switzerland) which was expected to yield a semisolid mass (23% w/w) with respect to the dried powder. Ethanol extract (EE) was finally stored in tight containers in desiccators until further use in the study.

For water fraction preparation (WF), dried leaf residues from the EE method were boiled with distilled water for 4 hours in a 1 L round bottom conical flask, decanted and centrifuged for 10 minutes at 3500 rpm. The supernatant was then dried using freeze-drier to obtain crude extract powder and was used for the study.

For water extract (WE), *F. deltoidea* leaves were air-dried and ground to form a fine mesh and were extracted using distilled water at boiling temperature for 4 hours in a 1 L round bottom conical flask. WE was then cooled and filtered using cloth sieve. The filtrate was finally subjected to centrifugation for 10 minutes at 3500 rpm, and the supernatant was freeze-dried. The flow chart below summarizes the procedure.

Ficus deltoidea leaves collected, washed, dried and ground.

Sample weight : Ethanol volume (1:20)
Solvent - Ethanol (90%)
Temperature - 80°C
Time - 8 hours

Ground leaves boiled in water for 4 hours.

Residue filtered. Filtrate centrifuged and freeze dried to be used as:
Hot water extract (WE)

Residue washed with water, dried in an oven at 45°C

Supernatant sent for rotary evaporation and dried to be used as:
Ethanolic extract (EE) (Ethanol)

Sample weight : Water volume (1:30)
Temperature - 100°C
Time – 5 hours

Residue filtered, filtrate centrifuged and freeze dried to be used as:
Water fraction (WF)

3.2: *In-vitro tests*

3.2.1: Free radical scavenging assay (DPPH)

1,1-diphenyl-2 picrylhydrazil (DPPH) method was used according to Yamaguchi and colleagues (1998). DPPH was dissolved in methanol (0.395 mg/ml) to form the stock solution and 3 ml of this stock solution was then added to 37 mL of methanol to obtain the working solution. 100 ml of working solution was added to an equal volume of the extracts. The concentrations of the extract selected for testing ranged from 0.25 µg/ml to 10µg/ml. The mixture was shaken vigorously and the decrease in absorbance was measured at 515 nm after 30 minutes. Quercetin was used as positive control and distilled water was used as negative control. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and was calculated using:

Scavenging effect (%) = [1- (A sample / A control)] × 100

3.2.2: Cupric ion Reducing Antioxidant Capacity (CUPRAC)

An amount of 0.2 ml 10 mM $CuCl_2$, 0.2 ml of 7.5 mM Neocuproine and 0.2 ml of 1 M ammonium acetate were added to a test tube. After vortexing the mixture, 100 µl of sample (0.01 – 0.06 mg/ml) and 120 µl of ultra-pure water were added and the absorbance at 450 nm was measured after 30 minutes. Quercetin was used as a positive control and the concentrations measured were same as the extract (0.01 – 0.06 mg/ml).

3.2.3: Estimation of Total Phenolic Compounds (TPC) by Folin-Ciocalteu method

The total phenolic content was determined in all the three extracts using the Folin-Ciocalteu method. The extract (100 µg/ml) was mixed with 5 ml of Folin-Ciocalteu reagent, previously diluted in distilled water (1:10), and 4 ml of sodium carbonate (1M in H_2O). The mixture was incubated at 37ºC for 15 minutes for colour development. The absorbance was measured at 760 nm using UV/Vis spectrophotometer. Samples of the extracts were evaluated at a final concentration of 1 mg/ml. The total phenolic content

was expressed as gallic acid equivalents (GAE) in milligrammes per gram of dry material using the equation obtained from the standard calibration curve:

$y = 0.004x+0.0201$

The standard calibration curve was prepared by using gallic acid instead of extract to obtain the absorbance versus gallic acid concentration.

3.2.4: Lipid peroxidation assay:

This assay was based on the modified thiobarbituric acid reactive substances (TBARS) assay developed by Ohkowa and colleagues in 1979, with some modification. Egg yolk homogenate was used as the lipid source (Dorman et.al, 1995; Ruberto et.al, 2000). 1 ml of buffered egg yolk (1 mg in 20 ml of 0.1 M phosphate buffer saline) was mixed with 100µl of 1000 µM ferrous sulphate and 100µl of extract of concentrations ranging from 2 to 12 mg/ml. The mixture was then incubated at 37°C for 1 hour, followed by addition of 500µl of 15 % TCA and 1000µl of 1% TBA. The final mixture is boiled for 30 minutes and cooled down to room temperature before centrifuging for 10 minutes at 3500 rpm to obtain the supernatant. The absorbance of the supernatant is measured at 532 nm using a spectrophotometer (Janero, 1990). The percentage inhibition of lipid peroxidation was calculated using the formula:

$$\% \, inhibition = \left(\frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \right) X \, 100$$

Where control in this case is distilled water and the positive control is quercetin.

3.2.5: HMG-CoA reductase assay:

This assay was conducted using the kit obtained from Sigma-Aldrich. The reagents were prepared according to the procedure in the kit. 50 µl of extracts, with concentrations ranging from 10 to 80 mg/ml, were added to Eppendorf tubes followed by 25 µl of 0.1M sodium hydroxide and heating at 50 C° for 2 hours. 25 µl of 0.1M HCL was then added and the mixture was incubated for 1 hour at room temperature. Table 3.1 below was used as a guide to the volume of each reagent to be added to the 96-well plate.

The absorbance of each well was measured at 340 nm at an interval of 15 seconds for up to 5 minutes using a plate reader. Pravastatin was used as an inhibitor.

Table 3.1: Reagent volumes according to HMG-CoA reductase assay kit

Sample	1 x assay Buffer	Extract	NADPH	HMG-CoA	HMGR(HMG-CoA Reductase)
Blank	184 µl	-	4 µl	12 µl	-
Extract	182 µl	1 µl	4 µl	12 µl	2 µl

3.3: Animal studies

3.3.1: Animals

Wistar albino rats, weighing 120–150 g, procured from the Animal House, University Science Malaysia (USM) were used. Universiti of Malaya Institutional Animal Ethics Committee (UMIACUC) approved this study (Ethic No: ISB/25/04/2013/NA(R)). Animals were acclimatized under standard lab conditions at 25±2˚C and 50±15 % room humidity and normal photoperiod (12h light: dark cycle) for 7 days. The animals were fed with commercial rat pellet diet and water *ad libitum*.

3.3.2: Animal experiment's setup

The experiment was divided into two sub experiments to test the cardioprotective and cholesterol lowering effects of the chosen extract simultaneously. All animals after acclimatization (6–7 days) in the animal quarters and later were randomly divided into nine groups of six animals each.

3.3.2.1: Animals with CCL_4-induced cardiac damage

For cardioprotective effect, animals were given normal rat pellets along with the following customized treatments:

- **Group I:** Normal Control and received distilled water (1 ml/kg, per oral (p.o)) daily for 30 days and in addition also received distilled water (0.5 ml/kg, sub cutaneous (s.c) injection) on the 31st day.

- **Group II**: Labelled as CCl_4 Control, and received distilled water (1 ml/kg, p.o.) daily for 36 days and in addition also received CCl_4 (1 mg/kg, s.c.) on 37^{th} day.
- **Group III**: Labelled as F50, received extract (50 mg/kg, p.o.) daily for 36 days and in addition also received CCl_4 (1 mg/kg, s.c.) on the 37^{th} day.
- **Group IV**: Labelled as F250, received extract (250 mg/kg, p.o.) daily for 36 days and in addition also received CCl_4 (1 mg/kg, s.c.) on the 37^{th} day.
- **Group V**: Labelled as Q10, received Quercetin (10 mg/kg, p.o.) daily for 36 days and in addition also received CCl_4 (1 mg/kg, s.c.) on the 37^{th} day.

For induction of myocardial infarction, carbon tetrachloride (CCl_4) was dissolved in olive oil (1:1) and injected intraperitoneally to the rats on the 37^{th} day (Eshagi et.al, 2011).

3.3.2.2: Animals with high cholesterol-induced hyperlipidemia

The rats were fed with high cholesterol or atherogenic diet for 25 days to induce hyperlipidemia, except for the normal rats (group I), which received normal diet for 30 days then sacrificed. The composition of atherogenic diet was wheat flour (15 g), roasted Bengal gram flour (47 g), groundnut flour (10 g), milk powder (5 g) health mix (15 g), salt (4 g) and butter (4 g). After 25 days, the rats were treated as follows for another 20 days:

- **Group I**: Control and received distilled water (1 ml/kg, p.o.) daily for 30 days and in addition also received water (1ml/kg, p.o.) on the 31^{st} day.
- **Group II:** Hyperlipidemic control group (HL). Rats were administrated with atherogenic diet for 25 days.
- **Group III**: Labelled as F50, and received extract (50 mg/kg, p.o.) daily for 20 days.

- **Group IV**: Labelled as F250, and received extract (250 mg/kg, p.o.) daily for 20 days.
- **Group V**: Labelled as A10, and received Atorvastatin (10 mg/kg, p.o.) daily for 20 days.

After treatment, animals were deprived of food, but not water, overnight and then euthanized under carbon dioxide asphyxiation. Animals in different groups were sacrificed by cervical dislocation. Blood was collected from an incision made in the jugular vein and serum was separated and used for various assays. Hearts were excised from the experimental animals of each group and were immediately washed with ice-cold normal saline and homogenized in 0.1 mol tris-HCl buffer (pH 7.4). The homogenate was finally centrifuged and the supernatant was obtained for use in various assays.

3.4: *In-vivo* assays

3.4.1: Cardiac tissue marker enzymes and Lipid profile

The collected tissue and serum samples were transferred into small Eppendorf tubes and sent to CDL lab in Pusat Perubatan Universiti Malaya (PPUM) for analysis.

3.4.2: Inflammation –Tumor Necrosis Factor-alpha (TNF-α)

TNF-α (human) EIA (enzyme immunoassay) kit was purchased from Cayman to test the levels of the necrosis factor in the serum samples obtained from animal studies. The kit is based on a double-antibody technique, where two types of monoclonal antibodies, specific to TNF-α, are supplied to the test and bind to it on either side. The complex is then detected by adding Ellman's reagent which reacts with one of the antibodies, forming a yellow color. The darker the color obtained, the higher is the concentration of TNF-α present in the test sample.

The standard is prepared and the curve is plotted according to the instructions in the kit as absorbance versus TNF-α concentration in pg/ml. The serum samples are tested

and the concentration of TNF-α present are calculated from the equation of the line obtained from the standard curve.

3.4.3: Oxidative stress – NADP concentration

This assay was carried out using the kit purchased from AnaSpec Inc., which describes a colorimetric assay to determine the NADP and NADPH concentration. The basis of this assay is the use of an enzyme cycling reaction in order to produce a blue colored product, known as formazan, detected at 565 nm on a microplate reader. NADP or NADPH concentration is directly proportional to the intensity of the final color. The standard curve is prepared by testing the NADP standard provided in the kit, and the blank control well's is measured and subtracted from the absorbance of test and standard samples. The absorbance of NADP standard is plotted against the different concentrations prepared, and the equation of the line is to calculate the concentration of NADP in test samples.

3.4.4: Histopathology

The collected tissue samples were sent to the Medical Faculty (Universiti Malaya) for slide preparation. The preparation involved fixing the tissue in formalin, followed by immersing it in paraffin using an automated tissue processor (Leica TP1050, Leica Instruments GMbH, Nussloch, Germany). The samples were then cut in 4µm lengths, placed on a microscopic slide coated with polysine, and finally incubated at 60°C for 30 minutes and cooled. The paraffin was removed from the tissue surface by incubating the samples in xylene, rehydrating in absolute, 95% and 70% ethanol for 15 seconds each, and finally washing the samples in PBS.

The tissue was stained using two dyes, namely haematoxylin and eosin (H&E), which are the two most common dyes used in animal histology. Haematoxylin produces a purplish-blue stain in the presence of DNA and RNA, while eosine produces a pinkish-red stain in the presence of most cytoplasmic proteins. Staining is carried out by dipping

the deparaffinised tissue samples in distilled water prior to incubation with the dyes, and the final dehydration in absolute alcohol before mounting in Eukitt.

3.5: Statistical analysis

Statistical analysis was carried out on all assay results, *in-vivo* and *in-vitro*. One-way analysis of variance (ANOVA) was performed using the software "Statistical Tool for Agricultural Research (STAR)". The post-hoc tests carried out where Least Significant Difference (LSD) test and Duncan's Multiple Range Test (DMRT). P values less than 0.05 were considered significant.

CHAPTER 4.0

RESULTS AND DISCUSSION

4.1: Antioxidant Capacity of Extracts *In-vitro*

4.1.1: Free radical scavenging activity (DPPH)

Figure 4.1 below shows the scavenging activities of Quercetin as a positive control for DPPH free radical scavenging assay. Figure 4.2 shows the free radical scavenging activities of the various *F. deltoidea* extracts. According to the calculated IC_{50} values, EE exhibits the highest DPPH scavenging activity followed by WF and WE.

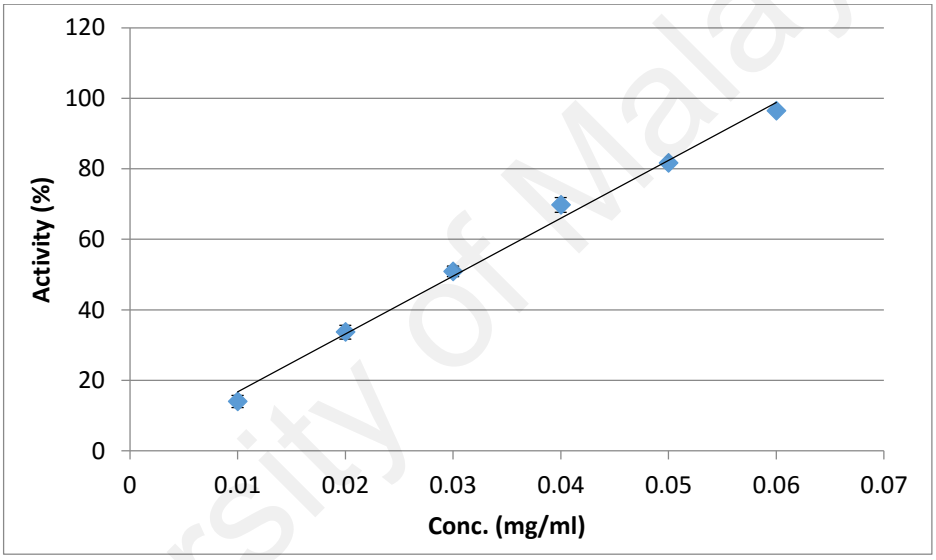


Figure 4.1: Radical scavenging activities of Quercetin as a positive control.

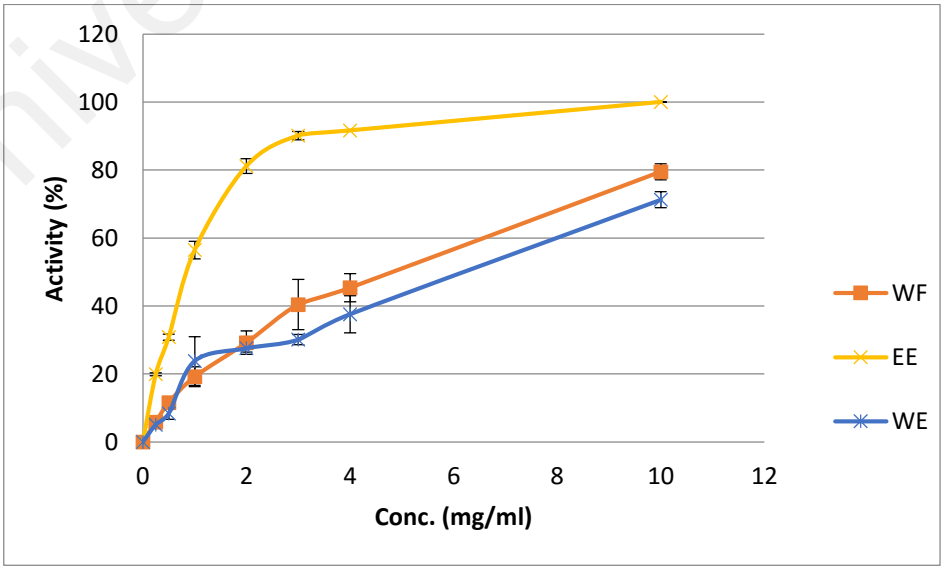


Figure 4.2: Radical scavenging activities of *F. deltoidea* extracts

The IC_{50} values were calculated from the equations of the lines in the graphs above, and were 0.03, 1.5, 6.03, and 6.28 mg/ml for quercetin, EE, WF, and WE respectively. However, due to the nature of curves in Figure 4.2, the equation of the linear gradient to the curves cannot accurately determine the IC_{50} values, thus, it is more effective to approximate these values by directly correlating the y and x values. Using this method, the IC_{50} values are approximately 1 mg/ml for EE, 6 mg/ml for WE, and about 5 mg/ml for WF. Due to the fact that the extracts are complex compounds with various components, it is more accurate to use direct correlation instead of calculation based on the equation of the line.

Generally, when DPPH absorption has a higher percentage decrease, the reactivity with DPPH is higher, indicating a greater hydrogen atom donating ability, thus a higher antioxidant capacity. As for the IC_{50} values, the lower the value the better is the DPPH scavenging ability of the sample. Quercetin, which is a known antioxidant, exhibits an IC_{50} value of 30 µg/ml, meaning that 50% of the DPPH free radicals were scavenged at a concentration of 30 µg/ml. This value is more or less consistent with the previous literature (Vasagam et.al, 2011; Nimmi and George, 2012), which aids in validating the procedure used.

As for the *F. deltoidea* extracts, The IC_{50} values were comparatively higher than the previous researches. For instance, Aris and colleagues (2009) obtained an IC_{50} value of about 125 and 250 µg/ml for fruits of *F.* deltoidea, whereas Hakiman and Maizah (2009) presented their results in terms of percentage inhibition (32.86-99.87%). Although the IC_{50} value is quite high for this particular research, the targeted percentage inhibition is reached, and at higher concentrations, the extracts reach up to 80-100 percent of inhibition. Thus, it can be said that the values obtained in this research are unique to the operating conditions and parameters used in this assay, and aids in confirming the presence of antioxidant activity due to the scavenging of DPPH free radicals.

4.1.2: Antioxidant capacity (CUPRAC) assay

The CUPRAC assay utilizes copper (II)-neocuproine (Cu (II)-Nc) reagent as the chromogenic oxidizing agent. It is based on the measurement of absorbance at 450 nm by the formation of a stable complex between neocuproine and copper (I). The cupric ion (Cu^{2+}) reducing ability of various concentrations of quercetin is shown in Figure 4.3 and that of *F. deltoidea* leaves is shown in Figure 4.4. Cu^{2+} reducing capability measured by this method was found to be concentration-dependent. Cu^{2+} ions reducing power of *F. deltoidea* extracts are highest in EE followed by WF and WE almost equally.

The presence of bioactive compounds such as antioxidants, which in this case is the phenolic content of the extract, leads to the reduction of copper (II) in the presence of neocuproine. From Figures 4.3 and 4.4 below, the steady increase in absorbance indicates the formation of the final reduced product, copper (II)-neocuproine, in turn demonstrating the presence of antioxidants in the tested samples. The graphs are consistent to those presented by previous studies discussed in chapter 2. Öztürk and colleagues (2007) tested the ability of their plant extract to reduce copper (II), and obtained a similar graph with a slight difference in the absorbance values. The absorbance of the control used in this experiment reaches a maximum of about 1, while the extracts reach an absorbance of about 0.25 for EE and 0.2 for both, WE and WF. These findings are in line with previous researches, whereby the control always exhibits higher absorbance than the tested samples, and the absorbance increases with increasing concentration of the tested sample (Öztürk et.al, 2007, Sabudak et.al, 2013).

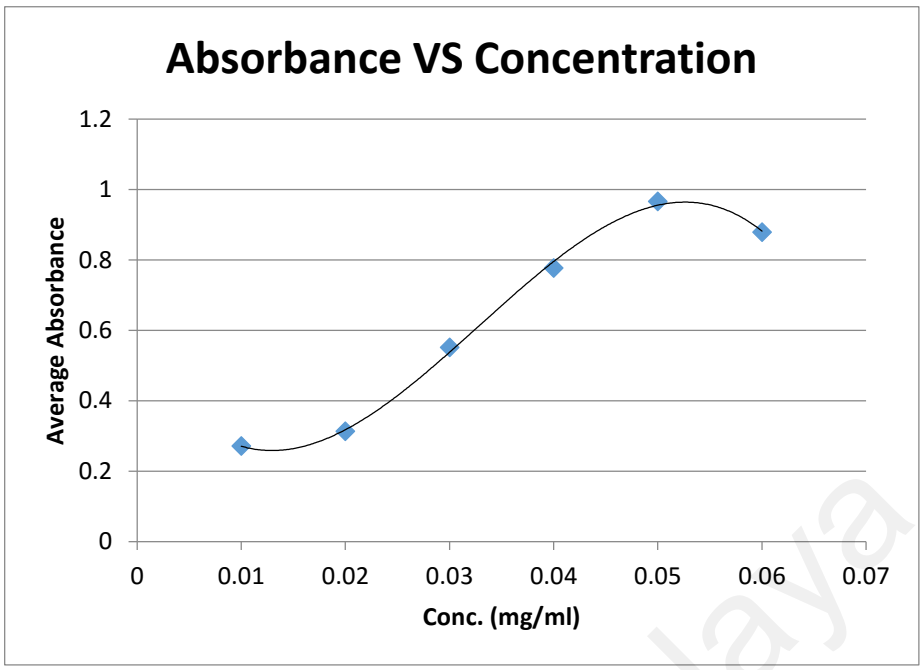


Figure 4.3**:** Cupric ion reducing ability of quercetin

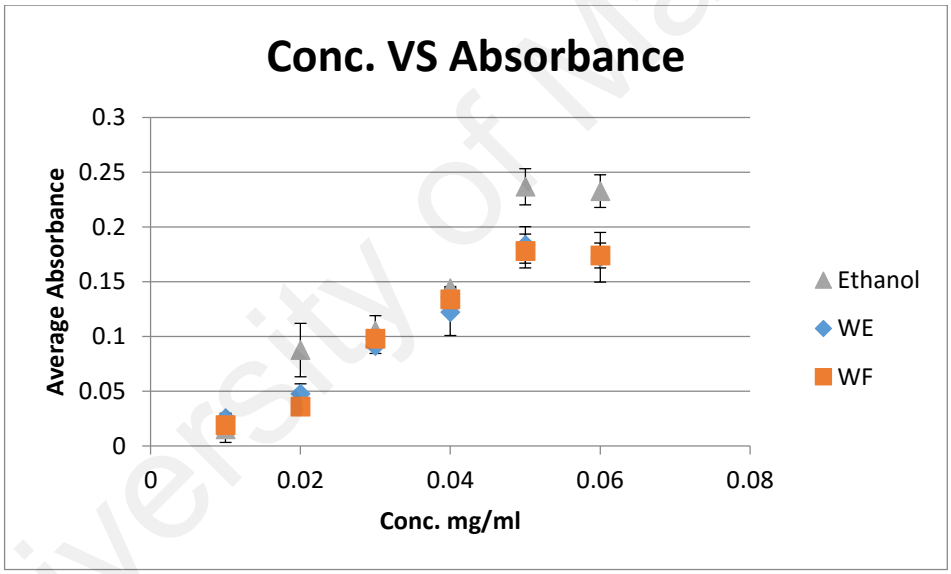


Figure 4.4**:** Cupric ion reducing ability of various extracts

It must be added to the discussion that the absorbance pattern of Quercetin is slightly inconsistent with previous research when the concentration increases above 0.05 mg/ml. This kind of pattern was not observed in any similar research. As the reaction is completed when the color change stops, the reduction in absorbance could be due to the completion of the reaction and the absorbance value could indicate the excess amount of quercetin present in the sample measured. This similar pattern is observed towards the higher concentrations of the extract as well, as shown in figure 4.4. There is also the

possibility that quercetin may start acting as a pro-oxidant at higher concentrations (0.06 mg/ml) as indicated by the drop in absorbance towards the end of the experiment in figure 4.3 above.

4.1.3: Total phenolic content (TPC) assay

A linear calibration curve of gallic acid (GAE) with an r^2 value of 0.998 was obtained as shown in Figure 4.5 below.

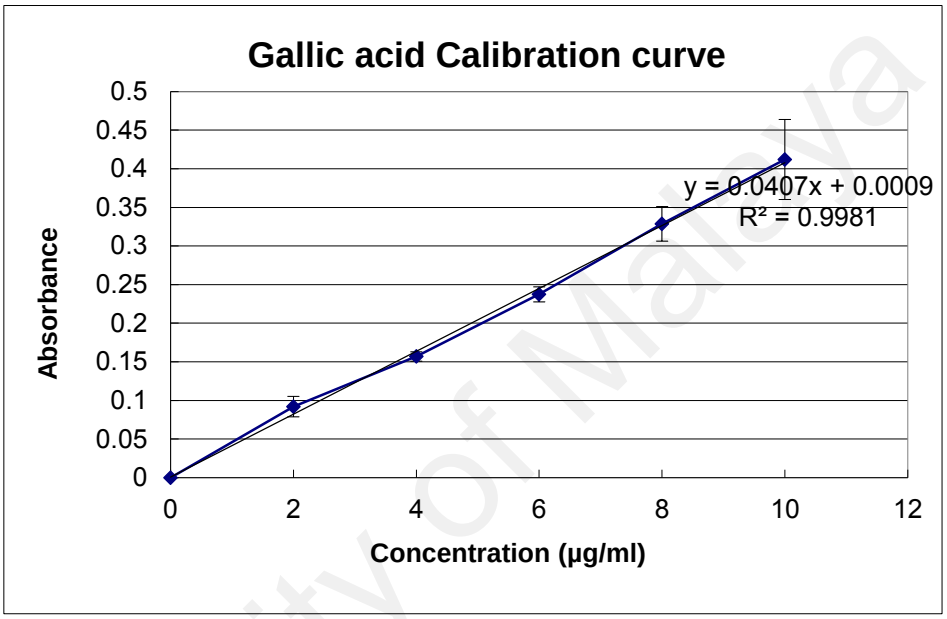


Figure 4.5**:** A linear calibration curve of gallic acid.

Figure 4.6 shows mean total phenolics content of the leaf extracts calculated using the GAE equation of y = 0.0407x + 0.0009 (R^2 = 0.998), whereby y=absorbance at 765nm and x=concentration of total phenolic compounds in mg/mL of the extract. The TPC of the extracts are finally presented as mg of gallic acid equivalents (GAE) per gram of extract. Among the *F. deltoidea* extracts, EE had the highest TPC (100 mg GAE/g), followed by WF (96 mg GAE/g) and lastly WE (90 mg GAE/g). The results indicate the presence of bioactive compounds in the extracts in the form of phenolic compounds acting as antioxidants as they are consistent with the previous research. Hakiman and Maizah calculated a TPC content of 1.60 to 4.70 mg/g in *F. deltoidea* leaf extracts (2009), while Aris and colleagues calculated 160 to 260 mg/g in fruit extracts of *F. deltoidea* (2009). The results obtained here are more in line with the latter research.

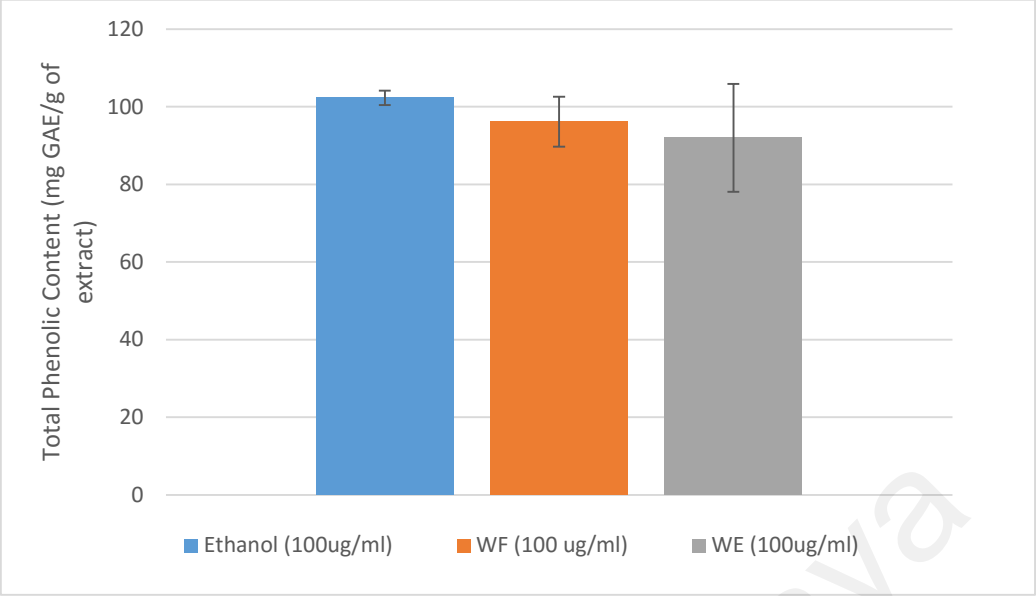


Figure 4.6**:** Total phenolic content of the *F. deltoidea* extracts.

4.1.4: Inhibition of lipid peroxidation assay

Figure 4.7 below shows the plot of the percentage lipid peroxidation inhibition achieved by different concentrations of the flavonoid rich compound, quercetin. The flavonoid content enhances the ability of quercetin to scavenge free radicals, thus preventing the oxidation of lipids. As can be observed in the graph, the highest concentration of quercetin used was 0.4 mg/ml which results in an inhibition of about 60%. The IC_{50} as obtained from the equation of the straight line is 0.25 mg/ml.

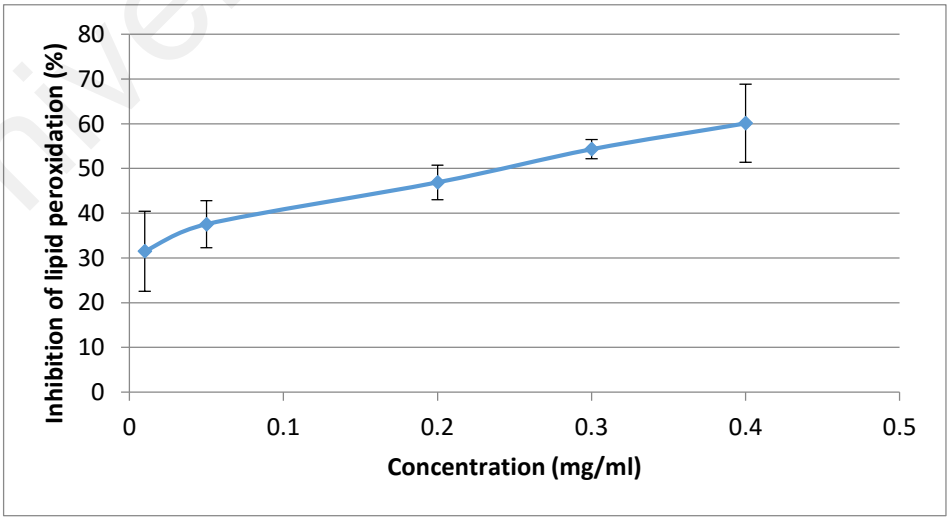


Figure 4.7: Inhibition of lipid peroxidation by quercetin

The three extracts' ability to inhibit lipid peroxidation is also studied and compared to that of the positive control, which in this case is quercetin. Figure 4.8 below is a presentation of the results obtained for EE, WE, and WF.

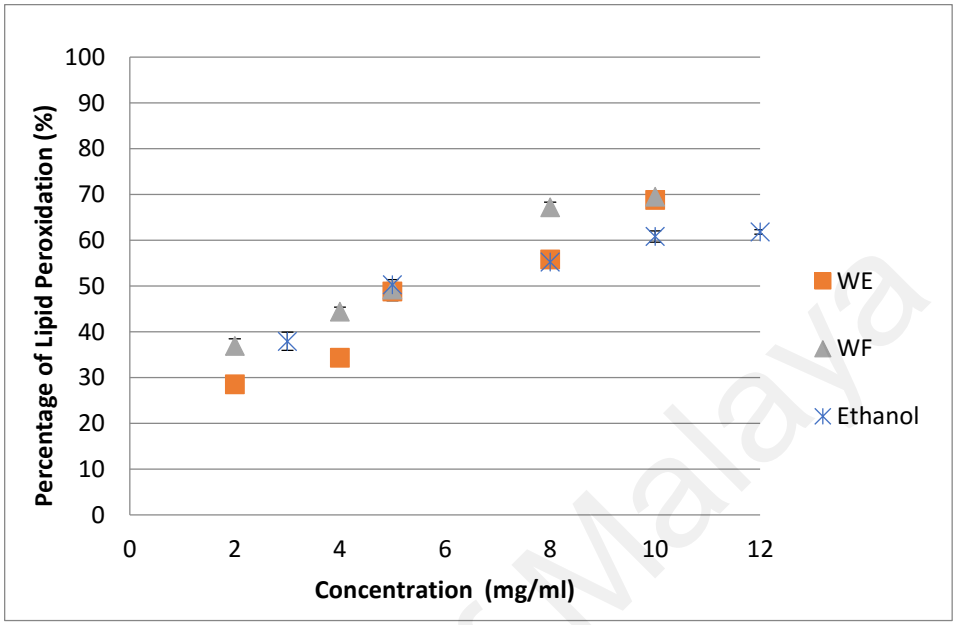


Figure 4.8: Inhibition of lipid peroxidation by the extracts

The graph above, when compared to Figure 4.7, shows a similar pattern of a concentration dependant increase in inhibition of lipid peroxidation by all three extracts. The highest inhibition is reached by WF and WE at approximately 70% followed by EE at about 60%. The IC_{50} values were calculated as 6.33, 6.33, and 5.01 mg/ml for EE, WE, and WF respectively. However, when direct correlation is done from the graph, all three extracts' IC_{50} values appear to be around 5 mg/ml. These values are lower than those obtained in the flavanoid rich quercetin and those obtained in previous researches. For instance, Aris and colleagues obtained an inhibition of about 90% at a concentration of 1 mg/ml for fruit extracts of *F. delotidea* (2009). Similarly, Poorna and colleagues obtained an IC_{50} value of about 0.1 mg/ml for *Excoecaria agallocha* extracts. According to the IC_{50} values and maximum inhibition reached by extracts and quercetin, the extracts exhibit some flavanoid content, but need further method and extraction solvent optimization in order to achieve higher flavanoid content, thus, higher inhibition of lipid peroxidation.

4.1.5: HMG-CoA reductase assay

Table 4.1 below shows the results obtained for HMG-CoA reductase inhibition assay. As seen in the table, four concentrations of the extracts were tested, and the concentration was increased accordingly until an inhibition of about 80% percent was obtained. The positive controls used were lovastatin and pravastatin, which are commonly used drugs that belong to the statin category. This means that they act as cholesterol reducers by inhibiting the HMG-CoA reductase enzyme. The pravastatin was obtained directly from the kit, and the inhibition was measured as 88.6%. The lovastatin was prepared as three different concentrations, 50, 100, and 200 µg/ml, and the inhibitions obtained were 91.3, 91.7, and 92.5%, respectively.

As for the extracts, WF inhibits the HMG-CoA reductase enzyme by 88% at 80 mg/ml, while EE and WE exhibit an inhibition of about 80% at 80 mg/ml. This indicates that WF contains higher amount of statins or statin-like compounds which act as HMG-CoA inhibitors, consecutively aiding in cardioprotection by lowering cholesterol (Babelova et.al, 2013).

Table 4.1: HMG-CoA reductase inhibition of *F. deltoidea* extracts

	Percentage of inhibition (%)		
Concentration	**EE**	**WE**	**WF**
10 mg/ml	14.4 ± 9.56	11.67 ± 4.19	30.1 ± 4.45
20 mg/ml	17.39 ± 5.92	40.83 ± 0.82	53.81 ± 2.37
40 mg/ml	57.4 ± 1.89	44.58 ± 0.08	80.85 ± 1.46
80 mg/ml	79.7± 11.59	80 ± 7.26	88.8 ± 6.77

Each value was expressed as mean ± SD (n=3)

The antioxidant content of this plant was tested using DPPH, CUPRAC, lipid peroxidation, and TPC assays. In another similar research by Muruhan and colleagues (2013), the antioxidant activity of *Solanum surattense* leaf extract was analyzed. The

researchers used DPPH, TPC and CUPRAC to determine the potential of this plant as an antioxidant. The results indicated that the extract reaches a maximum DPPH inhibition at about 0.1 mg/ml, a maximum absorption of 0.6 nm at 0.3 mg/ml in CUPRAC assay, and about 46.7 mg of gallic acid equivalents. Similarly, Valyova and colleagues (2012) conducted a research on *Tagetes erecta L.* flowers growing in Bulgaria, where they tested the antioxidant potential using various assays. Their DPPH assay resulted in and IC_{50} of about 7.6 μg/ml and the TPC assay concluded the presence of 154 mg GAE/ g of extract for the ethanolic extract. When compared with these and a couple of other researches, the *F. deltoidea* extract tested here resulted in a higher IC_{50} value for the DPPH assay, but shows a linear increase which eventually reaches close to 80-100 percent of inhibition, indicating the presence of an antioxidant potential. The validity of the assay is also confirmed by the colour change observed. As for CUPRAC, the shape of the graph obtained is more or less similar to the previous reseach conducted, especially the one obtained by Muruhan and colleagues (2013), where the maximum absorbance of the flavanoid rich sample reached about 0.6 at 300 μg/ml of the sample. The maximum absorbance reached by *F. Deltoidea* extracts were around 0.25 at 600 μg/ml of extract concentrations, and although this value is much lower than the one obtaiend by Muruhan and colleagues (2013), it can be attributed to the usage of different plant samples, and the relevance is established due to te similar shape of the graph. The TPC results obtained for *F. deltoidea* range from 80-100 mg GAE/ g of extract which is higher than the one obtained by Valyova and colleagues (517.8 mg GAE/g) (2012), but still lower than the one obtained by Muruhan and colleagues (46.7 mg GAE/g) (2013). Finally, the ability of the extracts to inhibit lipid peroxidation can be compared to those results obtained by Aris and colleagues (2009) where they managed to reach a maximum inhibition of about 96%, whereas in this research the maximum inhibition reached was about 70%. However, Aris and colleagues (2009) used linoleic acid as a source of lipids, rather than egg yolk, as was

used in this research, which accounts for the difference in maximum inhibitions reached. Overall, these assay results indicate the compatibility and the potential use of *F. deltoidea* extracts as an antioxidant source as there was comparable respone to the four antioxidant assays.

The HMG-CoA assay was used to determine the ability of the extract to reduce cholesterol levels by inhibiting HMG-CoA reductase enzyme, an important enzyme in the cholesterol synthesis pathway. Xie and colleagues (2009) tested the *Ginkgo biloba* extract and found that the highest inhibition of 71% was obtained at a concentration of about 3 mg/ml. As for Iqbal and colleagues (2014), the results were expressed as IC_{50} values for extracts of *Ficus palmata*. The lowest IC_{50} obtained was 9.1 µg/ml. When compared with the *F. deltoidea* extract, the IC_{50} values could be approximated as 20-40 mg/ml for the extratcs which is significantly higher than that obtained by Iqbal and colleagues (2014). However, the maximum inhibition reached by all extracts was approximately 80% and higher, which indicates a linear increase of inhibition with an increase in extract concentration.

According to these *in-vitro* tests conducted, EE exhibits a significantly higher antioxidant capacity as compared to the other two extracts. However, the cholesterol lowering activity of the WF seems to be higher than that of the EE. Regardless of this outcome, EE was selected for use in animal studies *In-vivo* as it can provide both antioxidant and cholesterol lowering effect.

4.2: *In-vivo* Assays

4.2.1: Cardiac tissue marker enzymes

4.2.1.1: Rats with CCL_4-induced cardiac damage

Tables 4.2 and 4.3 below summarize the results obtained for the cardiac tissue marker enzymes (ALP, AST, ALT, CK and LDH) for rats receiving normal diet. N refers to the normal untreated rats as described in chapter 3. Q 10 is the quercetin pretreated

rats, while CCl_4 refers to the carbon tetrachloride induced rats. F50 and F250 are the rats pretreated with low and high doses of EE respectively.

Table 4.2: Cardiac tissue marker enzymes in 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)
N	290 ± 2.43	104 ± 1.57	69.2 ± 1.05	1149 ±21.38	321.5 ± 4.97
Q10	247 ± 1.25	3923 ± 65	3920 ± 59	1195 ±11.23	6200 ± 118.78
CCl_4	272 ± 2.65	1708 ± 58	1199 ± 54	1524 ± 20.1	895.8 ± 28.24
F50	223 ± 5.54	1615 ± 39	1255 ± 32.6	1448 ± 26.51	474.2 ± 15.05
F250	310 ± 6.21	3982 ± 69	2995 ±61.25	1333 ±5.66	861.3 ± 19.86

Values are mean ± SEM. ALP – Alkaline phosphatase, AST – Aspartate aminotransferase, ALT – Alanine aminotransferase, CK – Creatine kinase, LDH – Lactate dehydrogenase. Units are International Units per liter (IU/L)

Table 4.2 above indicates an increased level of AST, ALT, CK and LDH in serum of CCl_4 induced rats when compared to the normal rats. These elevated levels of the marker enzymes indicates damage to the heart tissue induced by high dose injection of CCl_4, which leads to leakage of cardiac tissue marker enzymes into the serum (Nigam et.al, 2007). The group of rats pre-treated with quercetin show a decrease in ALP and CK only, while exhibiting no positive effect in the other parameters (AST, ALT, LDH). In the F50 extract pre-treated group, the levels of ALP, AST, CK and LDH have been reduced after the damage caused by CCl_4 induction, thus demonstrating the desired protective effect on the heart tissue, which in turn prevents the leakage of the marker enzymes into the serum. However, the group treated with higher dose of extract (F250) only show a reduction in the levels of CK and LDH.

CCl_4-generated free radicals aid in the peroxidation of the fatty acids in the membranes of tissues, which leads to considerable damage to the structure and function of the heart. Although this is an indirect damage, as CCl_4 is more commonly known to affect the liver, the damage is still visible from the pattern obtained in the cardiac tissue marker enzyme levels. This indirect damage, therefore, leads to the release of marker

enzymes into the blood stream. As shown in Table 4.3 below, the levels of ALP, AST, ALT, CK, and LDH are lower in tissue of CCl_4 induced rats when compared to normal and quercetin pre-treated rats. These results indicate strong heart muscle protection by quercetin. As for the extract pre-treated groups (F50 and F250), the levels of AST, ALT and LDH are higher when compared to CCl_4-induced rats, indicating heart protection to only some degree, as the other two markers (ALP and CK) do not increase significantly. The results are analyzed using one way ANOVA, and show a significance between the marker values of the different groups presented by the letters a to c.

Table 4.3: Cardiac tissue marker enzymes in heart tissue 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)
N	96.25 ± 2.39^{b}	22716.25 ± 15.52^{b}	659.25 ± 2.89^{ab}	2572.25 ± 4.09^{a}	23429.75 ± 33.65
Q10	145.67 ± 1.88^{a}	28765 ± 27.42^{a}	856.67 ± 0.94^{a}	2321.67 ± 7.25^{a}	31008 ± 32.4
CCl_4	79.25 ± 0.62^{bc}	17346.75 ± 15.49^{c}	602 ± 5.18^{b}	1103.25 ± 10.22^{b}	22976.5 ± 51.23
F50	61.67 ± 0.59^{c}	22617 ± 4.36^{b}	730.33 ± 76^{ab}	1018 ± 4.18^{b}	24744 ± 6.61
F250	75.33 ± 0.66^{bc}	24632 ± 21.11^{ab}	759.67 ± 4.44^{ab}	1322 ± 6.61^{b}	23126 ± 43.48

Values are mean ± SEM. ^{a,b,c}Means in the same column not sharing a common superscript are significantly different (p<0.05). ALP – Alkaline phosphatase, AST – Aspartate aminotransferase, ALT – Alanine aminotransferase, CK – Creatine kinase, LDH – Lactate dehydrogenase. Units are International Units per liter (IU/L)

4.2.1.2: Rats with high cholesterol diet-induced hyperlipidemia

Tables 4.4 to 4.5 below summarize the results obtained for the cardiac tissue marker enzymes (ALP, AST, ALT, CK and LDH) for atherogenic diet rats. N refers to the normal rats as described in chapter 3. A10 is the atorvastatin pretreated rats, while AT refers to group induced with hyperlipidemia due to the high cholesterol diet. F50 and F250 are the rats pretreated by low and high doses of ethanolic extracts respectively.

Table 4.4: Cardiac tissue marker enzymes in serum of atherogenic diet-induced rats

Group	Cardiac tissue marker enzymes				
	ALP (IU/L)	AST (IU/L)	ALT (IU/L)	CK (IU/L)	LDH (IU/L)
N	290.17 ± 2.43^{b}	104.17 ± 1.57^{b}	69.17 ± 1.05	1148.67 ± 21.38^{b}	321.5 ± 4.97^{b}
AT	398.67 ± 1.37^{a}	170.67 ± 3.23^{a}	84 ± 2.23	2912 ± 26.32^{a}	693.67 ± 15.3^{a}
A10	301.83 ± 1.94^{b}	118 ± 1.28^{b}	70.67 ± 0.97	1235.17 ± 17.36^{b}	354.17 ± 6.79^{b}
F50	258.5 ± 2.33^{b}	109.5 ± 1.26^{b}	72.5 ± 1.1	1389.5 ± 12.21^{b}	329.5 ± 4.86^{b}
F250	270 ± 2.41^{b}	110.75 ± 1.06^{b}	67.25 ± 1.46	1116.25 ± 9.55^{b}	367.25 ± 4.98^{b}

Values are mean ± SEM. ^{a,b}Means in the same column not sharing a common superscript are significantly different (p<0.05). ALP – Alkaline phosphatase, AST – Aspartate aminotransferase, ALT – Alanine aminotransferase, CK – Creatine kinase, LDH – Lactate dehydrogenase. Units are International Units per liter (IU/L)

Table 4.4 above shows that the group given the atherogenic diet (AT) similarly causes the most damage to the heart and also leads to lipogenesis which releases the cardiac tissue marker enzymes into the serum, thus the levels of the marker enzymes are highest in the AT group (represented by the superscript a), and certainly higher than those rats in the control group (N). Atorvastatin aids in reducing the levels of all marker enzymes. Compared to the values in AT, atorvastatin managed to significantly reduce the marker enzymes to near normal values with high significance. The rats pre-treated with the lower dose of the extract (F50) protect the heart as leakage of all marker enzymes are prevented. This can be seen from the results in Table 4.4 and how the levels of the marker enzymes were reduced back near normal levels as compared to the AT group. Rats pre-treated with higher dose of the extract (F250) display similarly acceptable results due to the clear reduction pattern in all of the markers when compared to the negative control group (AT), which indicates strong heart tissue protection.

Table 4.5 below displays the results for the same marker enzymes measured in the heart tissue. Since necrosis to heart tissue occurs due to the high cholesterol diet, the marker enzymes are released into the blood stream. Therefore, the levels of ALP, AST,

and ALT in the negative control group (AT) are lower than those in the normal group (N). Similarly, atorvastatin used in the positive control group (A10) helps to protect the heart and keep the marker enzyme levels at a high value, which is the case for ALP, AST, and ALT. The tissue of the rats treated with high dose of the exthanolic extract (F250) exhibit a strong heart tissue protection similar to the rats pre-treated with the lower dose (F50). This is due to the higher level of marker enzymes (ALP, AST, ALT, and CK) in the tissue of the extract pre-treated groups when compared to the tissue of the negative control group (AT).

Table 4.5: Cardiac tissue marker enzymes in heart tissue of atherogenic diet-induced rats

Group	Cardiac tissue marker enzymes				
	ALP (IU/L)	AST (IU/L)	ALT (IU/L)	CK (IU/L)	LDH (IU/L)
N	96.25 ± 2.4^{ab}	22716.25 ± 15.52	659.25 ± 2.89	1870.25 ± 29	17422.25 ± 81.7^{a}
AT	77.25 ± 1.97^{b}	20386.75 ± 20.48	590.25 ± 5.25	2389.5 ± 13.73	21542 ± 37.65^{ab}
A10	106.75 ± 2.51^{ab}	24742.25 ± 85.77	780.25 ± 5.07	2027 ± 11.52	12199.75 ± 58^{c}
F50	141.75 ± 1.45^{a}	22680.5 ± 19.42	827.75 ± 4.43	2456.25 ± 13.54	15276.25 ± 44.71^{bc}
F250	115.5 ± 3.7^{ab}	25484.25 ± 63.5	781 ± 8.77	2541.75 ± 21.93	13998.75 ± 49.74^{bc}

Values are mean ± SEM. ^{a,b,c}Means in the same column not sharing a common superscript are significantly different (p<0.05). ALP – Alkaline phosphatase, AST – Aspartate aminotransferase, ALT – Alanine aminotransferase, CK – Creatine kinase, LDH – Lactate dehydrogenase. Units are International Units per liter (IU/L)

4.2.2: Lipid profile

4.2.2.1: Rats with CCL_4-induced cardiac damage

Lipid profiles in serum and tissue of rats treated with normal diet, induced treated, and untreated are presented in Tables 4.6 and 4.7 below. Since the diet given to the rats is pellet which was not rich in cholesterol, the changes are not that significant, however in the serum of the rats shown in Table 4.6, TG and TC have a higher value in the CCl_4 group as compared to the control group (N), indicating lipogenesis in the CCl_4 induced group. Values shown by the Quercetin treated rats are inconsistent as the lipid profile

parameters are higher than the rats which supposedly have damage to their heart muscle. The extract pre-treated rats (F50 and F250) show a lower value of HDL than the CCl_4 induced rats which is inconsistent with the expected results, as HDL should be higher in rats that have less hyperlipidemie, the changes were also not as noticeable with the other two parameters (TG and TC). TC and HDL measured in the tissue do not show any change in values (Table 4.7), indicating no fluctuation in the lipid content of the heart tissue. However, the changes were detected by the fluctuating levels of TG in the tissue. TG was higher in the CCl_4-induced group as compared to the normal indicating slight lipid deposition in the heart tissue. TG was also slightly higher in the CCL_4-induced group when compared to the group treated with lower dose of the extract.

Table 4.6: Lipid profile in serum of CCl_4-induced rats

Group	**Lipid profile**		
	TG (mg/dL)	TC (mg/dL)	HDL (mg/dL)
N	0.5 ± 0.13^{b}	1.43 ± 0.11	1.3 ± 0.21^{ab}
Q10	1.4 ± 0.47^{a}	2.15 ± 0.43	1.1 ± 0.41^{abc}
CCl_4	0.7 ± 0.74^{ab}	1.7 ± 0.32	1.4 ± 0.42^{a}
F50	0.96 ± 0.81^{ab}	1.7 ± 0.61	0.90 ± 0.24^{bc}
F250	0.75 ± 0.6^{b}	1.5 ± 0.19	0.76 ± 0.33^{c}

Values are mean ± SEM. ^{a,b,c}Means in the same column not sharing a common superscript are significantly different ($p<0.05$). TG – Triglycerides, TC – Total cholesterol, HDL – High density lipoproteins. Units are in milligrams per deciliters (mg/dL)

Table 4.7: Lipid profile in heart tissue of CCL_4-induced rats

Group	Lipid profile		
	TG (mg/dL)	TC (mg/dL)	HDL (mg/dL)
N	0.275 ± 0.1^{c}	1.3 ± 0	0.08 ± 0
Q10	0.45 ± 0.86^{a}	1.3 ± 0	0.08 ± 0
CCl_4	0.4 ± 0.013^{ab}	1.3 ± 0	0.08 ± 0
F50	0.3 ± 0^{bc}	1.3 ± 0	0.08 ± 0
F250	0.4 ± 0.16^{ab}	1.3 ± 0	0.08 ± 0

Values are mean ± SEM. [a,b,c]Means in the same column not sharing a common superscript are significantly different (p<0.05). TG – Triglycerides, TC – Total cholesterol, HDL – High density lipoproteins. Units are in milligrams per deciliters (mg/dL)

4.2.2.2: Rats with high cholesterol diet-induced hyperlipidemia

Tables 4.8 below shows the lipid profile in the serum and tissue of rats fed with atherogenic diet. The serum lipid profile indicates highest triglycerides (TG), total cholesterol (TC) and low density lipoproteins (LDL) in the negative control group due to the high cholesterol diet treatment. When the rats are treated with the extract following the atherogenic diet treatment, the three lipid profile parameters (TG, TC, LDL) decrease due to the ability of the extract to reduce cholesterol as confirmed by the HMG-CoA assay results presented in Table 4.1 in previous section.

Table 4.8: Lipid profile in serum of atherogenic diet-induced rats

Group	Lipid profile			
	TG (mg/dL)	TC (mg/dL)	HDL (mg/dL)	LDL (mg/dL)
N	0.5 ± 0.13	1.43 ± 0.11^{c}	1.27 ± 0.21^{c}	ND
AT	0.63 ± 0.26	2.43 ± 0.39^{a}	2.02 ± 0.3^{a}	0.12 ± 0.29^{a}
A10	0.57 ± 0.23	1.7 ± 0.3^{bc}	1.56 ± 0.26^{bc}	ND
F50	0.5 ± 0.12	1.7 ± 0.28^{bc}	1.46 ± 0.2^{bc}	0.03 ± 0.34^{b}
F250	0.72 ± 0.2	2.18 ± 0.19^{ab}	1.86 ± 0.17^{ab}	0.04 ± 0.39^{b}

Values are mean ± SEM. [a,b,c]Means in the same column not sharing a common superscript are significantly different (p<0.05). TG – Triglycerides, TC – Total cholesterol, HDL – High density lipoproteins, LDL – Low density lipoproteins, ND – Not detected. Units are in milligrams per deciliters (mg/dL)

The results, although some are inconsistent with the previous literature review, indicate that CCl_4 induces cardiac toxicity in the heart tissue of rats (equivalent to isoproterenol induced rats) and thus lead to an elevated level of cardiac tissue marker enzymes in the serum when compared to the tissue. The normal rats exhibited lowest increase in the cardiac tissue marker enzymes overall which was consistent with the previous researches conducted (Mohamed et.al, 2009; Palanivel et.al, 2012; Eshagi et.al, 2012; Sharmila and Rajadurai, 2012). The lipid profile parameters (TC, HDL and LDL) showed observable differences in some of the groups indicating the presence of changes in the lipid content of the tissue and serum brought about by the extract pre and post treatment. The mode of protection of the extract can be attributed to the phenolic content of the plant as confirmed by the total phenolic content (TPC) assay and the inihibition of lipid peroxidation which in turn qualifies the extract as a potential antioxidant. The prevention of free radical build-up leads to heart muscle protection by preventing the muscle fibres from being damaged which would lead to necrosis as seen by the CCl_4 induced rats, which lack any kind of pre-treatment.

Palanivel and colleagues (2011) conducted a similar research on esculetin, which is a commonly known coumarin with a high phenolic content. Esculetin helped protect the heart muscles by reducing the cardiac tissue marker enzyme levels in the serum when compared to the isoproterenol-induced rats that were lacking any pre-treatment. This is due to the high phenolic content of the compound. In this research, the cardiac marker enzymes are also lower than those measured in the CCl_4-induced rats that were lacking any pre-treatment. Although the difference in the levels of the marker enzymes were not as significant as those observed by Palanivel and colleagues (2011) (as well as other studies), the reduction was still significant enough to deduce a pattern that is caused by the phenolic content of the extract. In another study that uses CCl_4 as an inducer for heart necrosis, Eshagi and colleagues (2012) used *Cornus mas* fruit extract to study its ability

to protect the heart against necrosis by CCl_4 induction. LDH and CK were measured in both, pre-treated and non-pre-treated rats, and the results indicate a significant amount of heart protection. For example LDH in the CCl_4-induced rats reaches about 1000 U/L whereas the pre-treated rats were able to display a level of about 600 U/L. For this study, the CCl_4-induced rats have an LDH level of 895.8 IU/L, whereas the extract pre-treated rats were able to normalize the levels of LDH to about 500 IU/L in lower dose of extract, but the reduction was less significant in the higher dose of extract (861.3 IU/L).

When the second group of rats were given the fat-rich diet instead of a normal diet, the main cause of damage to heart can be explained by the build-up of fatty acids in the arteries of the heart, leading to formation of plaques and eventually atherosclerosis. Since oxidative damage eventually leads to atherosclerosis, it can be said that atherosclerosis also causes some damage to the heart muscles due to the limited blood flow to the heart. The levels of marker enzymes would thus be lowered in an untreated rat as compared to a treated rat. This can be seen in table 4.4 above, where the levels of all marker enzymes are higher in the untreated group (AT) as compared to normal rats, atorvastatin, and extract pre-treated rats.

Abo-Gresha and colleagues (2014) tested evening primrose oil (EPO) for its ability to reduce risk of CVD by lowering cholesterol in rats given a high fat diet. Their results indicate an overall increase in TC, TG, and LDL and a decrease in HDL in untreated rats. The rats treated with EPO managed to display lower levels of TC, TG and LDL, and a higher amount of HDL. They also tested for the levels of the two cardiac marker enzymes, LDH and CK, and found that the untreated rats had higher levels of these marker enzymes in the serum due to the leakage of the enzymes from the heart tissue to the serum. The treated and normal rats had lower levels of LDH and CK indicating a level of protection of the heart by preventing oxidative damage and build-up of cholesterol.

Another study conducted by Sadjadi and colleagues (2014) tested the ability of caraway extracts to reduce risk of CVD in diabetic rats. Similar to the research conducted by Abo-Gresha and colleagues (2014), caraway extracts were able to lower TC and LDL when compared to untreated rats. In our research, the rats given a high fat diet and no treatment displayed higher levels of TG, TC, and LDL as compared to the extract pre-treated rats (Table 4.8). The LDL values in normal and atorvastatin pre-treated rats were not detected, meaning they were too low to be detected, indication the absence of lipid build-up. The levels of HDL were supposed to be lower in atherogenic diet-induced rats as compared to normal and treated rats, as HDL is considered as the good cholesterol. However, the level of HDL is highest in atherogenic diet-induced rats, and this result is quite unexpected especially due to the fact that the other two parameters (TC and TG) displayed reasonable patterns and results. There was no feasible explanation found to this inconsistency, except for the need to optimize the animal studies beforehand, and optimize the period of animal studies, as well as the chemicals and samples used. More troubleshooting is necessary to pinpoint the reason for this result. This indicates the antioxidative potential of *F. deltoidea* extract due to its effect on the cardiac marker enzymes, as well as it lowers the levels of cholesterol and prevent atherosclerosis to some degree. However, due to the extract not being fully optimized and still lacking its main bioactive agents, some optimization and further research is needed to achieve higher levels of heart protection. Moreover, rats are established as cholesterol resistant (Ritskes-Hoitinga and Beynen, 1988) which poses a big disadvantage when it comes to experiments aiming to develop atherosclerosis, such as this one. The research conducted by Kovar and colleagues (2009) attempted the use of a new breed of rats for hypercholesterolemia studies. The reasoning was that rats normally have very low serum cholesterol (below 2 mmol/l). According to Mahley and Holocombe (1977), rats are resistant to dietary cholesterol and drugs affecting thyroid function need to be added to the diet. Moreover,

even if the rat model is responsive to dietary cholesterol, the hypercholesterolemia will not exceed 5 mmol/l, which is considered a normal level in humans (Ouguerram et.al, 1996). Thus, it would be beneficial to explore areas of research aiming to optimize the breed of rats that will be most responsive to cholesterol diets in order to set an accurate base for such experiments.

4.2.2: Inflammation – TNF-α

This assay was conducted to confirm the presence of cardiac failure due to inflammation and possibly indicate the progression of atherosclerosis (Mann et.al, 1996; Eichenholz et.al, 1992). TNF-α standard was used directly as supplied from the kit, and the standard curve was plotted as shown in Figure 4.9 below.

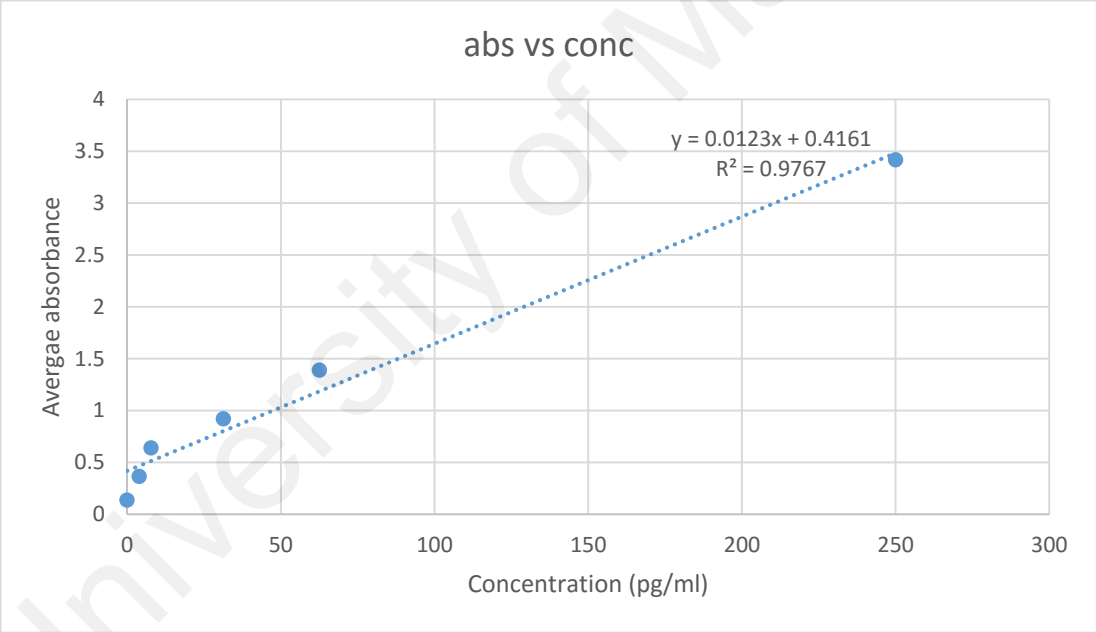


Figure 4.9: TNF-α standard curve

The purpose of plotting the standard curve is to obtain the equation of the straight line for further calculations. The equation of the line and R^2 values are shown in Figure 4.9 above. From this equation, the TNF-α concentrations in the serum samples of rats treated with normal diet are calculated and presented in Figure 4.10 below. The results indicate a high concentration of TNF-α in CCl_4 induced rats' serum. The result is in agreement with the fact that CCl_4 damages the heart tissue and leads to inflammation,

thereby releasing TNF-α into the blood stream (Kriegier et.al, 1988). Normal rats (N) and quercetin pre-treated rats (Q10) have lower inflammation rates due to the absence of an infarction inducing chemical in the N group and the protective effect of quercetin in the Q10 group. As for the extracts, rats treated with higher dose of EE (250 mg/ml – F250) have lower concentration of TNF-α in their serum as compared to the CCl_4 induced rats. Rats treated with lower dose of the EE (50 mg/ml – F50) have TNF-α concentrations that are slightly higher than the F-250 group, but are still lower than the CCl_4 induced rats.

Since the extract was able to normalize the levels of cardiac marker enzymes and lipid profile parameters, the heart muscle protection is present, even if it is in a comparatively smaller extent than previously conducted researches. To confirm this ability of the extract, this test was carried out and it concludes that the inflammation is present in CCl_4-induced rats, while it is minimized in the extract pre-treated rats. Thus, the extract is able to protect the heart against chemical induced inflammation. The results were tested for significance using one way ANOVA, and for p values lower than 0.05, considerable significance was observed as presented in Appendix B and in graph below. The results for CCL_4-induced rats is significantly different from the results of other groups marked with a small a next to the value in the graph.

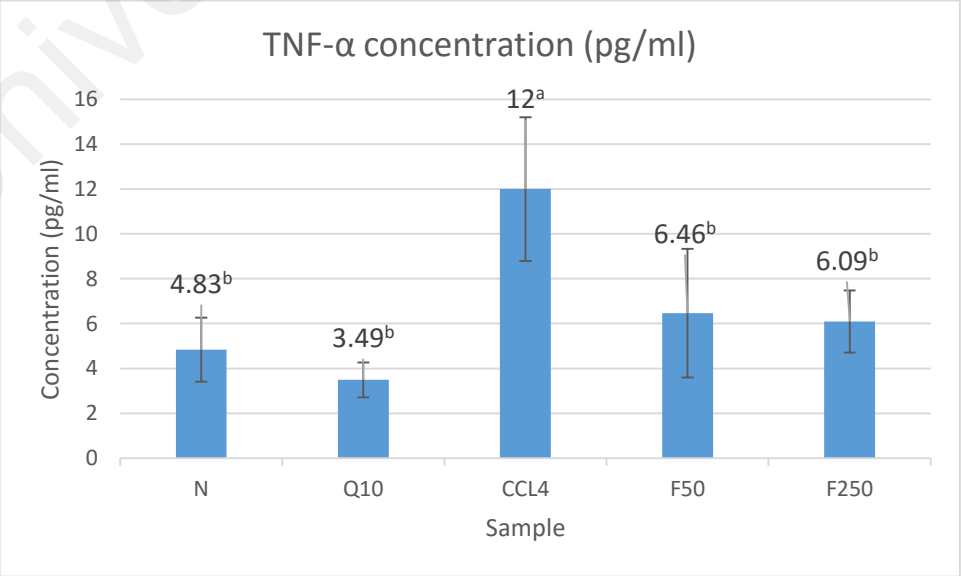


Figure 4.10: TNF-α concentrations

4.2.3: Oxidative stress - NADP concentration

The NADP concentration in normal diet rats were as shown in Figure 4.11 below. Similar to above tables, N represents the normal rats, while Q represents the quercetin pre-treated rats. CCl_4 group induced rats are also shown in Figure 4.11 below.

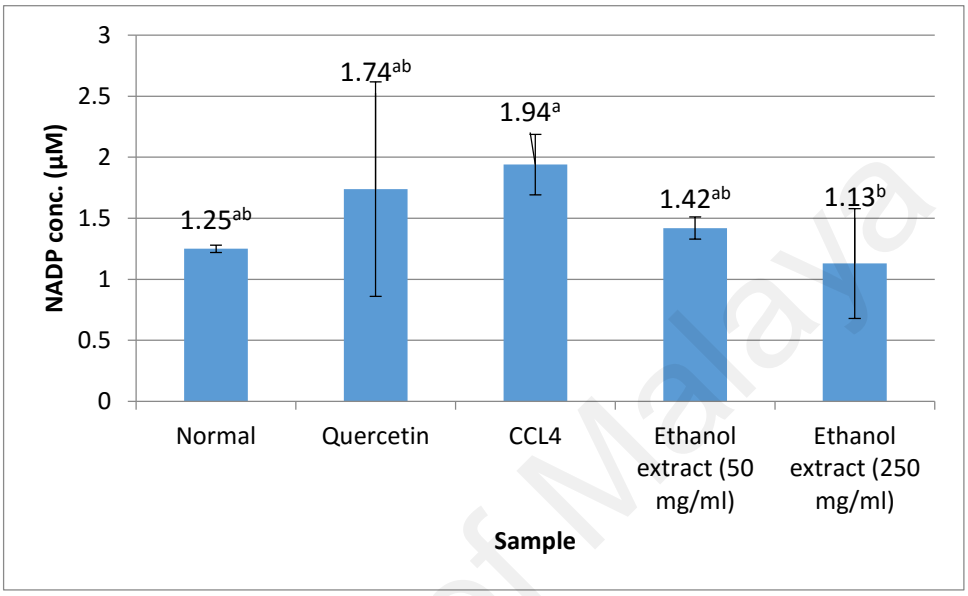


Figure 4.11: NADP concentration in normal diet rats

As shown in Figure 4.11 above, the concentration of NADP was lowest in normal rats that do not exhihibit any oxidative stress. CCl_4 induced rats which have high oxidative stress will also exhibit the highest amount of NADP (Diaz-Flores et.al, 2006). The rats that were pretreated with highest concentration of extract (F250) had lower levels of NADP indicating presence of NADPH which is characteristic of a compound displaying antioxidant properties.

Figure 4.12 below shows the NADP concentration for the atherogenic diet rats. As can be seen, the normal and extract pre-treated rats show the lowest concentration of NADP followed by atorvastatin pre-treated rats. The NADP concentration was highest in rats with high cholesterol diet-induced hyperlipidemia, which leads to oxidative stress and the increase in NADP concentration. The group of rats pre-treated with atorvastatin did not show a very significant decrease in NADP concentration, and the value was close to that of the AT group. This indicates that atorvastatin may not be a suitable positive

control for this mechanism of protection, as it is a commonly used drug that aims to reduce cholesterol through the cholesterol synthesis pathway. The results were analyzed using one way ANOVA and significant differences were detected between the different groups as presented in Appendix B and next to the values in the graphs with small letters (a,b,c).

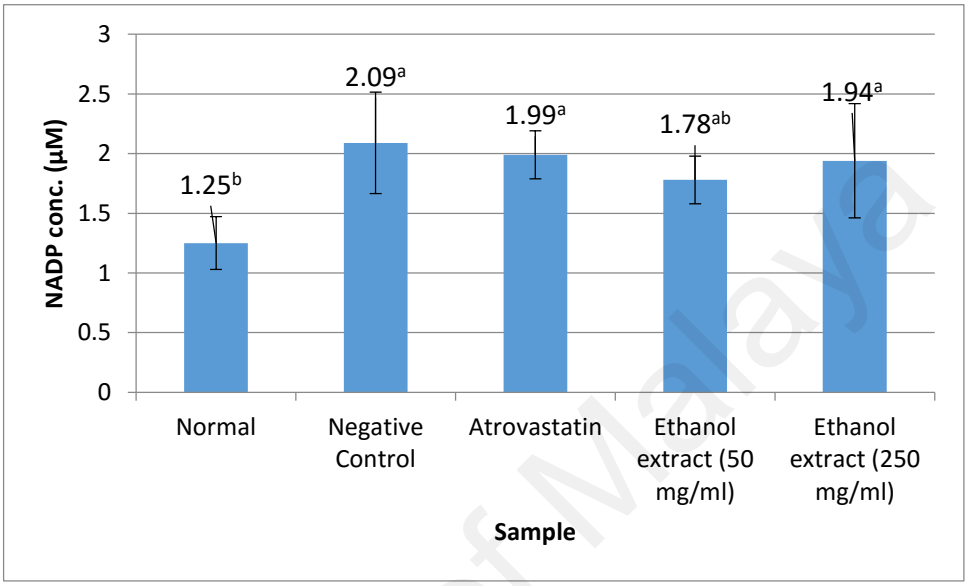
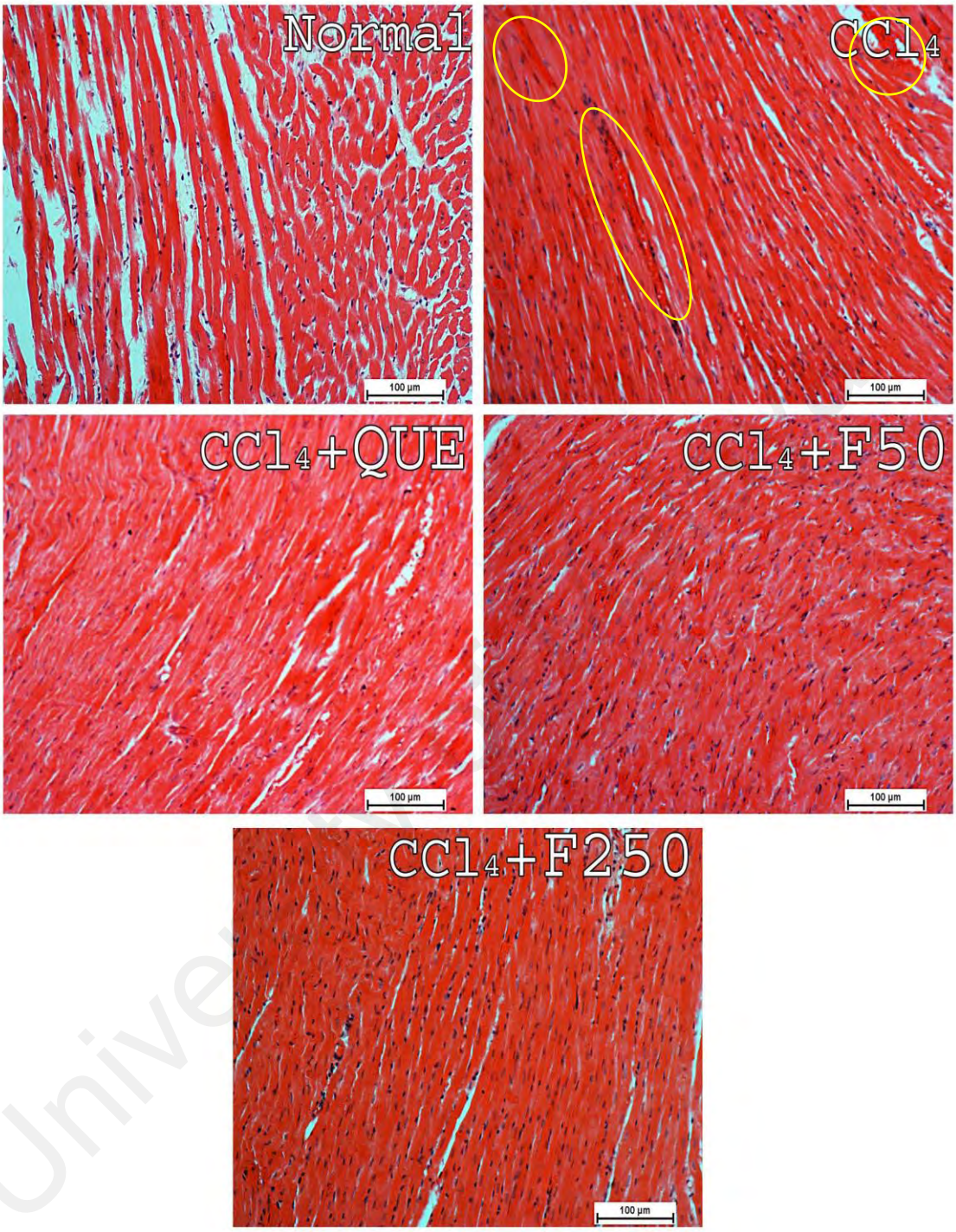


Figure 4.12: NADP concentration in atherogenic diet rats

4.2.4: Histopathology



Normal: tissue of normal rats
CCL_4: tissue of rats induced with carbon tetrachloride (CCL_4)
CCL_4 + QUE: tissue of rats pre-treated with quercetin followed by CCL_4 induction
CCL_4 + F50: tissue of rats pre-treated with 50 mg/ml of *F. deltoidea* followed by CCL_4 induction
CCL_4 + F250: tissue of rats pre-treated with 250 mg/ml of *F. deltoidea* followed by CCL_4 induction

The examination of cardiac tissue sections showed different results for the different groups, ranging from no damage in the normal rat heart architecture to mild damage as shown in the extract pre-treated groups and severe damage to heart tissue as shown in the CCL_4-induced rats. Normal rat's tissue has a normal myofibrillar structure, visible nuclei, and the absence of necrotic damage to the tissue. Rats induced with CCl_4 displayed extensive tissue damage (necrosis) in the areas circled with yellow. The same areas show a macrophages infiltrate due to the inflammation caused by CCl_4, and as supported by the TNF-α assay.

Positive control, quercetin, protected the heart tissue to some degree due to the absence of necrotic areas when compared to CCl_4 induced rats, and smoother fibrous tissue structure similar to normal rat's tissue. As for the extract pre-treated groups, the low and high dose treatment produced slightly different results. For the rats treated with low dose of *F. deltoidea* (50 mg/ml), the necrotic damage is halted to a greater extent than those treated with the higher dose of the extract (250 mg/ml). This is apparent from the absence of most necrotic damage in the low dose group's tissue as compared to the high dose group's tissue. However, these two group's tissue displays slight macrophage infiltrate due to inflammation in the cardiac tissue. These results are consistent with the TNF-α assay, which measures the amount of inflammation in the heart tissue, as the assay results suggest that only the CCl_4 group has high amount of TNF-α as a marker for inflammation. This assay acts as a visual confirmation and helps support the other *in-vivo* assays conducted and presented in the previous sections. The necrosis is quite visible to CCl_4-induced rats and is more or less prevented in the extract pre-treated groups. The histology of the extract pre-treated rats show lower signs of necrosis, which could be attributed to the extracts ability to prevent inflammation. This is in turn hypothized to be due to the phenolic content of the plant and its ability to act as an antioxidant by preventing lipid peroxidation and free radical build-up.

CHAPTER 5.0

CONCLUSION

It has already been established that *F.delotidea* plant was used as a traditional remedy for several conditions such as breast cancer and diabetes. The lack of scientific works concerning this plant makes it difficult to ascertain its ability to act as a bioactive compound for medicinal purposes. The few studies conducted gives promising but incomplete results on the role of this plant's extracts as an anti-inflammatory, antioxidant, and cholesterol lowering agent. This study aims to enhance the pool of research relating to the extracts of *F. deltoidea* and their uses as bioactive agents with an attempt to test its activity in animal models. Ethanolic extract (EE), water extract (WE) and water fraction (WF) were prepared according to well-established protocols, and the antioxidant, as well as the cholesterol lowering activity of the extracts were measured using 5 *in-vitro* assays. Although all extracts gave up-to par results, the EE produced the highest results, as compared to the other two extracts, in all the antioxidant assays. The HMG-CoA assay that was carried out to test the cholesterol lowering capacity of the extract gave highest activity in WE. Eventually, the EE was selected to be further used in animal experimentation as it possesses both, very high antioxidant capacity, and considerably high cholesterol lowering ability.

The animal studies were divided into two parts as described in the methodology, where one part of the experiment involved testing for cardioprotective potential of the extract through its antioxidant capacity, and the other part was carried out to test the cholesterol lowering ability of the extract. At the end of the experiment, the tissue and serum of all animals were tested using several *in-vivo* assays. Cardiac marker enzymes and lipid profile is the most standard assay that is carried out to determine any existing necrosis to the heart, as well as the level of cholesterol in the blood and tissue, whenever significant. The results of these assays confirm the presence of a cardioprotective

potential of the EE, although not as high when compared to other studies of other bioactive plants. Similarly, the cholesterol lowering ability of the plant is detected to some extent. The ability of EE to act as an anti-inflammatory agent is confirmed through the detection of tumor necrosis factor in the serum of the rats as shown in chapter 4. The NADP levels were also measured to assist the results of this study and to strengthen the theory that the EE possesses an ability to protect the heart muscles against induced necrosis due to its rich phenolics content. Finally, the histopathology of the heart tissue was carried out to visually detect the presence of any necrosis in tissue of unprotected rats. The results, as presented in chapter 4, show some degree of necrosis and fibrosis, and consequently a degree of protection against this necrosis in rats pretreated with EE. The results of the animal studies were statistically analyzed, and some of them were not significant due to the limitations of the study. The lack of animal studies concerning this plant makes it difficult to carry out the procedure, thus optimization is well needed to get more accurate results. However, the results obtained are sufficient to pursue a more in-depth research on this plants ability as an antioxidant, cardioprotective, and a cholesterol lowering agent.

CHAPTER 6.0

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CHAPTER 7.0

APPENDICES

APPENDIX A: Reagents and Equipments

3.1: Plant preparation and extraction

Reagents:

- 90% ethanol
- Distiller water

Equipment:

- Electrical blender
- Soxhlet apparatus (Favorit, Malaysia)
- Rotary evaporator (BÜCHI Rotavapor R-114, Switzerland)
- Glass containers
- Desiccator
- 1 L round bottom conical flask
- Centrifuge
- Freeze drier

3.2.1: DPPH

Reagents:

- DPPH
- Methanol
- Quercetin standard
- Distiller water

Equipment:

- Test tubes
- Spectrophotometer
- Pipettes

3.2.2: CUPRAC

Reagents:

- Neocuproine
- Ammonium acetate
- Ultra-pure water
- Quercetin standard

Equipment:

- Test tubes
- Pipettes
- Spectrophotometer

3.2.3: TPC

Reagents:

- Folin-Ciocalteu reagent
- Distilled water
- Sodium carbonate
- Gallic acid standard

Equipment:

- Test tubes
- Pipettes
- Incubator
- UV/Vis spectrophotometer

3.2.4: Lipid peroxidation

Reagents:

- Egg yolk
- Phosphate buffer saline
- Ferrous sulphate
- TCA
- TBA
- Distilled water
- Quercetin standard

Equipment:

- Test tubes
- Pipettes
- Incubator
- Centrifuge
- Spectrophotometer (Janero, 1990)

3.2.5: HMG-CoA

Reagents:

- Sigma Aldrich kit
- 0.1 M Sodium hydroxide
- 0.1 M HCl
- Assay buffer
- HMG-CoA
- NADPH
- HMG-CoA Reductase (HMGR)

Equipment:

- Eppendorf tubes
- Pipettes
- Incubator
- Spectrophotometer

3.3.2: Animal experiments setup

Reagents:

- Custom prepared pellets
- Distiller water
- CCL_4
- Quercetin (standard)
- Olive oil
- Saline
- 0.1 M tris-HCL buffer

Equipment:

- Syringes
- Plastic containers
- Weighing balance
- Cages
- Bedding
- Surgical knives and instruments
- Centrifuge
- Test tubes
- Pipettes

3.4.2: Inflammation (TNF-α)

Reagents:

- TNF-α human Enzyme Immunoassay kit (Cayman, USA)

Equipment:

- Test tubes
- Pipettes
- Spectrophotometer

3.4.3: Oxidative stress – NADP concentration

Reagents:

- NADP kit (AnaSpec Inc, USA)

Equipment:

- Test tubes
- Pipettes
- Spectrophotometer

3.4.4: Histopathology

Reagents:

- Formalin
- Paraffin
- Xylene
- Absolute, 95%, and 70% ethanol
- PBS
- Haematoxylin and Eosin dye (H&E)

- Eukitt

Equipment:

- Automated tissue processor (Leica TP1050, Leica Instruments GMbH, Nussloch, Germany).
- Blades
- Microscopic slide
- Incubator
- Test tubes
- Pipettes

APPENDIX B: Raw Data

Free radical scavenging activity (DPPH):

The absorbances measured at 515 nm are recorded in tables below for n=3.

For quercetin (positive control)

Concentration (µg/ml)	Absorbance			% Activity			Average % activity	Standard deviation
	1	2	3	1	2	3		
0.01	0.6	0.58	0.579	12.023	14.956	15.103	14.027[f]	1.737
0.02	0.437	0.462	0.458	35.924	32.258	32.845	33.675[e]	1.969
0.03	0.326	0.346	0.333	52.19941	49.267	51.173	50.880[d]	1.488
0.04	0.223	0.197	0.199	67.30205	71.114	70.821	69.746[c]	2.121
0.05	0.125	0.127	0.123	81.67155	81.378	81.965	81.672[b]	0.293
0.06	0.027	0.023	0.023	96.04106	96.628	96.628	96.432[a]	0.339

Values are mean, n=3 in each group. [a,b,c,d,e,f]Means in the same column not sharing a common superscript are significantly different (p<0.05).

For ethanolic extract:

Concentration (µg/ml)	Absorbance			% Activity			Average % activity	Standard deviation
	1	2	3	1	2	3		
0.25	0.536	0.538	0.532	19.760	19.461	20.359	19.860[e]	0.457
0.5	0.471	0.46	0.462	29.491	31.138	30.838	30.489[d]	0.877
1	0.309	0.274	0.29	53.743	58.982	56.587	56.437[c]	2.623
2	0.143	0.117	0.119	78.593	82.485	82.186	81.088[b]	2.166
3	0.073	0.068	0.057	89.072	89.820	91.467	90.120[a]	1.225
4	0.056	0.054	0.057	91.617	91.916	91.467	91.667[a]	0.229

Values are mean, n=3 in each group. [a,b,c,d,e]Means in the same column not sharing a common superscript are significantly different (p<0.05).

For water extract:

Concentration (µg/ml)	Absorbance			% Activity			Average % activity	Standard deviation
	1	2	3	1	2	3		
0.25	0.631	0.631	0.634	5.539	5.539	5.090	5.389[d]	0.259
0.5	0.606	0.604	0.625	9.281	9.581	6.437	8.433[d]	1.735
1	0.433	0.492	0.528	35.180	26.347	20.958	27.495[c]	7.180
2	0.501	0.515	0.511	25.000	22.904	23.503	23.802[c]	1.080
3	0.48	0.46	0.468	28.144	31.138	29.940	29.741[c]	1.507
4	0.437	0.375	0.44	34.581	43.862	34.132	37.525[b]	5.493
10	0.201	0.17	0.185	69.910	74.551	72.305	72.255[a]	2.321

Values are mean, n=3 in each group. [a,b,c,d]Means in the same column not sharing a common superscript are significantly different (p<0.05).

For water fraction:

Concentration (µg/ml)	Absorbance			% Activity			Average % activity	Standard deviation
	1	2	3	1	2	3		
0.25	0.626	0.627	0.633	6.287	6.138	5.240	5.888[e]	0.567
0.5	0.6	0.592	0.58	10.180	11.377	13.174	11.577[e]	1.507
1	0.538	0.561	0.522	19.461	16.018	21.856	19.112[d]	2.935
2	0.456	0.499	0.463	31.737	25.299	30.689	29.242[c]	3.454
3	0.429	0.341	0.423	35.778	48.952	36.677	40.469[b]	7.360
4	0.343	0.356	0.396	48.653	46.707	40.719	45.359[b]	4.135
10	0.125	0.132	0.155	81.287	80.240	76.796	79.441[a]	2.350

Values are mean, n=3 in each group. [a,b,c,d,e]Means in the same column not sharing a common superscript are significantly different (p<0.05).

The percentage activity is calculated using the following formula:

$$\%\ activity = \left(1 - \left(\frac{A_{sample}}{A_{blank}}\right)\right) X\ 100$$

Where the blank value = 0.668.

Cupric ion Reducing Antioxidant Capacity (CUPRAC):

The absorbances measured at 450 nm are recorded in tables below for n=3.

For quercetin (positive control):

Concentration (mg/ml)	Absorbance 1	Absorbance 2	Absorbance 3	Average absorbance	Standard deviation
0.01	0.269	0.267	0.278	0.271[e]	0.006
0.02	0.295	0.329	0.316	0.313[e]	0.017
0.03	0.509	0.557	0.588	0.551[d]	0.040
0.04	0.738	0.808	0.785	0.777[c]	0.037
0.05	0.921	1	0.979	0.967[a]	0.041
0.06	0.84	0.911	0.887	0.879[b]	0.036

Values are mean, n=3 in each group. [a,b,c,d,e]Means in the same column not sharing a common superscript are significantly different (p<0.05).

For ethanolic extract:

Concentration (mg/ml)	Absorbance 1	Absorbance 2	Absorbance 3	Average absorbance	Standard deviation
0.01	0.014	0.004	0.028	0.0153[d]	0.012
0.02	0.068	0.08	0.115	0.0877[c]	0.024
0.03	0.096	0.099	0.121	0.1053[c]	0.014
0.04	0.143	0.146	0.143	0.144[b]	0.002
0.05	0.218	0.243	0.249	0.237[a]	0.016
0.06	0.248	0.218	0.232	0.233[a]	0.0150

Values are mean, n=3 in each group. [a,b,c,d]Means in the same column not sharing a common superscript are significantly different (p<0.05).

For water extract:

Concentration (mg/ml)	Absorbance 1	Absorbance 2	Absorbance 3	Average absorbance	Standard deviation
0.01	0.014	0.004	0.028	0.015[d]	0.012
0.02	0.068	0.08	0.115	0.086[d]	0.024
0.03	0.096	0.099	0.121	0.105[c]	0.014
0.04	0.143	0.146	0.143	0.144[b]	0.002
0.05	0.218	0.243	0.249	0.237[a]	0.016
0.06	0.248	0.218	0.232	0.233[a]	0.015

Values are mean, n=3 in each group. [a,b,c,d]Means in the same column not sharing a common superscript are significantly different (p<0.05).

For water fraction:

Concentration (mg/ml)	Absorbance 1	Absorbance 2	Absorbance 3	Average absorbance	Standard deviation
0.01	0.02	0.023	0.015	0.019[e]	0.004
0.02	0.04	0.03	0.039	0.036[d]	0.006
0.03	0.1	0.095	0.099	0.098[c]	0.003
0.04	0.122	0.139	0.142	0.134[b]	0.011
0.05	0.164	0.195	0.18	0.180[a]	0.016
0.06	0.187	0.171	0.165	0.174[a]	0.011

Values are mean, n=3 in each group. [a,b,c,d,e]Means in the same column not sharing a common superscript are significantly different (p<0.05).

Total Phenolic content (TPC)

Sample	Absorbance			Concentration of gallic acid (mg/ml)		
	1	2	3	1	2	3
Quercetin (10 µg/ml)	0.203	0.182	0.205	0.005	0.004	0.005
Quercetin (100 µg/ml)	2.280	2.357	2.189	0.056	0.058	0.054
Ethanol extract (100 µg/ml)	0.425	0.417	0.410	0.010	0.010	0.010
Water extract (100 µg/ml)	0.420	0.389	0.368	0.010	0.010	0.009
Water fraction (100 µg/ml)	0.406	0.410	0.310	0.010	0.010	0.008

Concentration of gallic acid in above table is calculated using the standard curve of absorbance versus concentration of gallic acid. The equation of the line obtained from the standard curve is $y = 0.004x+0.0201$.

Y is replaced by the absorbance values to calculate the concentration of gallic acid present in each sample. Following this calculation, the concentration of gallic acid in mg/ml is converted to the total phenolic content of the sample in mg of gallic acid equivalents per gram of the extract/sample (mg GAE/g sample).

Sample	TPC (mg GAE/g sample)			Average TPC	Standard deviation
	1	2	3		
Quercetin (10 µg/ml)	496.069	445.700	502.211	481.327[c]	31.006
Quercetin (100 µg/ml)	559.975	578.894	537.617	558.829[a]	20.663
Ethanol extract (100 µg/ml)	104.202	102.236	100.516	102.317[c]	1.844
Water extract (100 µg/ml)	102.973	95.356	90.197	96.175[c]	6.427
Water fraction (100 µg/ml)	99.533	100.516	75.946	91.998[b]	13.910

Values are mean ± SEM, n=3 in each group. [a,b,c]Means in the same column not sharing a common superscript are significantly different (p<0.05).

Inhibition of lipid peroxidation:

For Quercetin:

Conc. (mg/ml)	Absorbance			%inhibition			Average % inhibition	Standard deviation
	1	2	3	1	2	3		
0.01	0.095	0.087	0.112	33.567	39.161	21.678	31.469[d]	8.928
0.05	0.089	0.097	0.082	37.762	32.168	42.657	37.529[cd]	5.249
0.1	0.073	0.082	0.074	48.951	42.657	48.252	46.620[bc]	3.450
0.2	0.082	0.071	0.077	42.657	50.350	46.154	46.387[bc]	3.851
0.3	0.062	0.068	0.066	56.643	52.448	53.846	54.312[ab]	2.136
0.4	0.071	0.047	0.053	50.350	67.133	62.937	60.140[a]	8.734

Values are mean, n=3 in each group. [a,b,c,d]Means in the same column not sharing a common superscript are significantly different (p<0.05).

For ethanolic extract:

Conc. (mg/ml)	Absorbance			%inhibition			Average % inhibition	Standard deviation
	1	2	3	1	2	3		
3	0.192	0.181	0.191	36.63	40.26	36.96	37.95[d]	0.020
5	0.147	0.154	0.157	51.49	49.17	50.17	50.28[c]	0.012
8	0.179	0.186	0.181	56.02	54.29	55.53	55.25[b]	0.009
10	0.117	0.116	0.123	61.39	61.72	59.41	60.84[a]	0.012
12	0.153	0.157	0.156	62.41	61.43	61.67	61.84[a]	0.005

Values are mean, n=3 in each group. [a,b,c,d]Means in the same column not sharing a common superscript are significantly different (p<0.05).

For water extract:

Conc. (mg/ml)	Absorbance			%inhibition			Average % inhibition	Standard deviation
	1	2	3	1	2	3		
2	0.2	0.209	0.206	30.31	27.18	28.22	28.57[e]	0.016
4	0.253	0.248	0.246	33.59	34.91	35.43	34.64[d]	0.009
5	0.079	0.081	0.078	49.03	47.74	49.68	48.82[c]	0.010
8	0.126	0.128	0.129	57.14	55.4	55.05	55.86[b]	0.011
10	0.089	0.089	0.09	68.99	68.99	68.64	68.87[a]	0.002

Values are mean, n=3 in each group. [a,b,c,d,e]Means in the same column not sharing a common superscript are significantly different (p<0.05).

For water fraction:

Conc. (mg/ml)	Absorbance			%inhibition			Average % inhibition	Standard deviation
	1	2	3	1	2	3		
2	0.18	0.18	0.183	37.28	37.28	36.24	36.93[e]	0.006
4	0.211	0.212	0.216	43.93	45.22	44.19	44.45[d]	0.007
5	0.076	0.078	0.082	50.97	49.67	47.09	49.24[c]	0.020
8	0.093	0.095	0.094	67.59	66.89	67.25	67.24[b]	0.004
10	0.112	0.118	0.118	70.6	69.03	69.03	69.55[a]	0.009

Values are mean, n=3 in each group. [a,b,c,d,e]Means in the same column not sharing a common superscript are significantly different (p<0.05).

Inhibition of HMG-CoA reductase:

For ethanol extract:

Concentration (mg/ml)	% inhibition			Average inhibition	Standard deviation
	1	2	3		
10	11.3	7.54	25.65	14.4[c]	9.56
20	23.62	16.38	11.88	17.39[c]	5.92
40	55.65	59.42	57.39	57.4[b]	1.89
80	71	87.39	-	79.7[a]	11.59

Values are mean, n=3 in each group. [a,b,c]Means in the same column not sharing a common superscript are significantly different (p<0.05).

For water extract:

Concentration (mg/ml)	% inhibition			Average inhibition	Standard deviation
	1	2	3		
10	20.93	37.83	31.53	30.1[c]	4.45
20	41.52	55.48	52.13	49.71[b]	2.37
40	79.82	81.89	-	80.85[a]	1.46
80	83.97	93.54	-	88.75[a]	6.77

Values are mean, n=3 in each group. [a,b,c]Means in the same column not sharing a common superscript are significantly different (p<0.05).

For water fraction:

Concentration (mg/ml)	% inhibition			Average inhibition	Standard deviation
	1	2	3		
10	24.72	14.63	8.7	11.67[c]	4.19
20	41.41	28.6	40.25	40.83[b]	0.82
40	44.64	58.36	44.52	44.58[b]	0.08
80	74.97	85.24	-	80[a]	7.26

Values are mean, n=3 in each group. [a,b,c]Means in the same column not sharing a common superscript are significantly different (p<0.05).

Normal diet rats:

Cardiac marker enzymes - serum

Group	No	ALP	AST	ALT	CK	LDH
Normal rats	1	316	97	66	340	248
	2	321	110	76	1079	301
	3	299	122	72	1839	431
	4	315	113	72	2160	435
	5	278	107	76	989	276
	6	212	76	53	485	238
Quercetin	1	265	177	98	1639	662
	2	250	3292	4175	1030	939
	3	226	8301	7486	917	16999
CCL_4	1	230	3573	2289	2078	2580
	2	258	167	97	1685	567
	3	278	379	199	735	521
	4	355	5776	4378	984	794
	5	257	119	88	925	243
	6	252	234	145	2737	670
Ethanolic extract (50mg/ml)	1	214	3533	2252	1286	831
	2	183	508	432	1214	362
	4	244	984	981	2543	391
	5	262	2898	2482	1575	544
	6	212	154	129	623	243
Ethanolic extract (250 mg/ml)	1	217	206	98	1437	364
	2	404	7460	5634	1519	1399
	3	214	204	98	1332	350
	4	406	8059	6150	1045	1332

Cardiac marker enzymes – tissue

Group	No	ALP	AST	ALT	CK	LDH
Normal rats	1	131	24926	761	2543	28046
	2	80	19730	593	2628	16727
	3	89	22030	617	2309	22107
	4	85	24179	666	2809	26839
Quercetin	1	171	32284	855	2704	36684
	2	127	23493	830	2019	25274
	3	139	30518	885	2242	31066
CCL_4	1	74	15183	544	1161	12143
	2	84	18288	612	832	27949
	3	75	16202	478	1561	22620
	4	84	19714	774	859	29194
Ethanolic extract (50mg/ml)	1	67	21933	675	1156	25635
	2	59	23240	699	1008	24996
	3	59	22678	817	890	23601
Ethanolic extract (250 mg/ml)	1	69	27695	899	1051	27262
	2	80	21115	669	1508	15500
	3	77	25086	711	1407	26616

Lipid profile – serum

Group	No	TG	TC	HDL
Normal rats	1	0.5	1.4	1.4
	2	0.4	1.3	1.11
	3	0.5	1.4	1.27
	4	0.6	1.6	1.51
	5	0.6	1.6	1.45
	6	0.4	1.3	0.9
Quercetin	1	0.8	1.7	1.55
	2	1.9	2.6	0.89
	3	1.5	3.0	0.75
CCL_4	1	0.4	1.3	1.04
	2	0.4	1.3	1.20
	3	0.5	2.3	2.08
	4	1.9	2.1	0.81
	5	0.4	1.5	1.42
	6	0.4	1.7	1.55
Ethanolic extract (50mg/ml)	1	1.5	2.1	0.91
	2	0.2	1.3	0.86
	4	1.3	1.9	0.85
	5	1.5	1.9	0.88
	6	0.3	1.3	0.98
Ethanolic extract (250 mg/ml)	1	0.3	1.3	1.03
	2	1.2	1.7	0.53
	3	0.3	1.3	0.98
	4	1.2	1.7	0.49

Lipid profile - tissue

Group	No	TG	TC	HDL
Normal rats	1	0.3	1.3	0.08
	2	0.2	1.3	0.08
	3	0.3	1.3	0.08
	4	0.3	1.3	0.08
Quercetin	1	0.5	1.3	0.08
	2	0.4	1.3	0.08
	3	0.5	1.3	0.08
CCL_4	1	0.4	1.3	0.08
	2	0.5	1.3	0.08
	3	0.3	1.3	0.08
	4	0.4	1.3	0.08
Ethanolic extract (50mg/ml)	1	0.3	1.3	0.08
	2	0.3	1.3	0.08
	3	0.3	1.3	0.08
Ethanolic extract (250 mg/ml)	1	0.3	1.3	0.08
	2	0.4	1.3	0.08
	3	0.5	1.3	0.08

Atherogenic diet rats:

Cardiac marker enzymes - serum

Group	No	ALP	AST	ALT	CK	LDH
Normal rats	1	316	97	66	340	248
	2	321	110	76	1079	301
	3	299	122	72	1839	431
	4	315	113	72	2160	435
	5	278	107	76	989	276
	6	212	76	53	485	238
Atherogenic diet	1	369	137	77	1629	436
	2	404	157	68	2669	487
	3	423	218	107	4438	1158
Atorvastatin (10 mg/ml)	1	284	126	70	2283	496
	2	299	135	67	717	509
	3	314	126	78	1188	370
	4	304	117	79	1432	247
	5	356	106	73	1230	300
	6	254	98	57	561	203
Ethanolic extract (50 mg/ml)	1	288	118	81	1229	443
	2	210	90	60	976	229
	3	248	113	71	1316	311
	4	288	117	78	2037	335
Ethanolic extract (250 mg/ml)	1	261	115	82	910	457
	2	218	95	57	780	233
	3	305	112	58	1350	378
	4	296	121	72	1425	401

Cardiac marker enzymes - tissue

Group	No	ALP	AST	ALT	CK	LDH
Normal rats	1	131	24926	761	2543	28046
	2	80	19730	593	2628	16727
	3	89	22030	617	2309	22107
	4	85	24179	666	2809	26839
Atherogenic diet	2	66	18687	542	1698	13357
	3	63	22692	710	3171	23425
	5	101	23022	676	2704	25471
	6	79	17146	433	1985	23915
Atorvastatin (10 mg/ml)	2	145	44889	719	1934	21737
	3	100	19608	992	2562	9528
	4	93	18001	715	2261	7914
	5	89	16471	695	1351	9620
Ethanolic extract (50 mg/ml)	1	119	20754	711	2148	12118
	2	136	15246	883	1518	13241
	3	146	25129	884	3003	20236
	4	166	29593	833	3156	15510
Ethanolic extract (250 mg/ml)	1	109	26739	713	2518	13589
	3	73	13124	559	1398	8514
	4	169	37827	1131	4041	22250
	6	111	24247	721	2210	11642

Lipid profile - serum

Group	No	TG	TC	HDL	LDL
Normal rats	1	0.5	1.4	1.4	0
	2	0.4	1.3	1.11	****
	3	0.5	1.4	1.27	0
	4	0.6	1.6	1.51	0
	5	0.6	1.6	1.45	0
	6	0.4	1.3	0.9	****
Atherogenic diet	1	0.7	3	2.45	0.23
	4	0.8	2.5	2.03	0.11
	5	0.4	1.8	1.59	0.03
Atorvastatin (10 mg/ml)	1	0.5	1.4	1.33	0
	2	0.5	1.4	1.35	0
	3	0.4	1.5	1.37	0
	4	0.9	2.2	1.94	0
	5	0.5	1.5	1.37	0
	6	0.6	2.2	2.01	0
Ethanolic extract (50 mg/ml)	1	0.5	1.3	1.22	0
	2	0.6	1.5	1.32	0
	4	0.4	1.9	1.55	0
	5	0.5	2.1	1.75	0.12
Ethanolic extract (250 mg/ml)	1	0.8	2.2	1.85	0
	2	0.7	2.2	1.93	0
	3	0.9	1.8	1.56	0
	6	0.5	2.5	2.12	0.15

Inflammation – TNF-α:

Serum values

Group	No	Absorbance	TNF-α concentration (pg/ml)	Average concentration (pg/ml)	Standard deviation
Normal rats	1	0.49	6		
	2	0.467	4.14		
	3	0.485	5.6		
	4	0.491	6.09		
	5	0.445	2.35		
	6	0.475	4.79	4.83[b]	1a.43
Quercetin	1	0.47	4.38		
	2	0.453	3		
	3	0.454	3.08	3.49[b]	0.77
CCL_4	1	0.598	14.79		
	2	0.595	14.54		
	3	0.57	12.51		
	4	0.489	5.93		
	5	0.566	12.19		
	6	0.563	11.94	12[a]	3.21
Ethanolic extract (50mg/ml)	1	0.47	4.38		
	2	0.566	12.19		
	3	0.483	5.44		
	4	0.492	6.17		
	5	0.483	5.44		
	6	0.479	5.11	6.46[b]	2.87
Ethanolic extract (250 mg/ml)	1	0.479	5.11		
	2	0.503	7.07	6.09[b]	1.39

Values are mean, n=3 in each group. [a,b]Means in the same column not sharing a common superscript are significantly different (p<0.05).

NADP concentration:

For serum of normal diet rats:

Group	No	Absorbance	NADP concentration (μM)	Average concentration (μM)	Standard deviation
Normal rats	1	0.304	1.47		
	2	0.283	1.28		
	3	0.238	0.88		
	4	0.278	1.24		
	5	0.296	1.4	1.25^{bc}	0.23
Quercetin	1	0.436	2.66		
	2	0.242	0.92		
	3	0.323	1.65	1.74^{ab}	0.88
CCL_4	1	0.351	1.90		
	2	0.347	1.86		
	3	0.364	2.02		
	4	0.397	2.31		
	5	0.322	1.64	1.94^{a}	0.25
Ethanolic extract (50mg/ml)	1	0.306	1.49		
	2	0.289	1.34		
	3	0.295	1.39		
	4	0.307	1.50		
	5	0.305	1.48		
	6	0.283	1.28	1.42^{abc}	0.09
Ethanolic extract (250 mg/ml)	1	0.28	1.26		
	2	0.246	0.95		
	3	0.211	0.64		
	4	0.328	1.69	1.13^{c}	0.45

Values are mean, n=3 in each group. ^{a,b,c}Means in the same column not sharing a common superscript are significantly different ($p<0.05$).

For serum of atherogenic diet rats:

Group	No	Absorbance	NADP concentration (μM)	Average concentration (μM)	Standard deviation
Normal rats	1	0.304	1.47		
	2	0.283	1.28		
	3	0.238	0.88		
	4	0.278	1.24		
	5	0.296	1.4	1.25^{b}	0.23
Atrovastatin	1	0.348	1.87		
	2	0.336	1.76		
	3	0.385	2.20		
	4	0.355	1.93		
	5	0.385	2.20	1.99^{a}	0.20
Atherogenic diet	1	0.386	2.21		
	2	0.429	2.60		
	3	0.357	1.95		
	4	0.317	1.59	2.09^{a}	0.43
Ethanolic extract (50mg/ml)	1	0.301	1.45		
	2	0.354	1.93		
	3	0.341	1.81		
	4	0.356	1.94		
	5	0.337	1.77	1.74^{ab}	0.20
Ethanolic extract (250 mg/ml)	1	0.355	1.93		
	2	0.366	2.03		
	3	0.287	1.32		
	4	0.416	2.48	1.94^{a}	0.48

Values are mean, n=3 in each group. [a,b]Means in the same column not sharing a common superscript are significantly different (p<0.05).

APPENDIX C: Statistical analysis

Free radical scavenging activity (DPPH):

```
------------
Sample = Q
------------

Summary Information
-----------------------------------------------------
FACTOR          NO. OF LEVELS   LEVELS
-----------------------------------------------------
Concentration   6               0.01, 0.02, ..., 0.06
-----------------------------------------------------
Number of Observations Read and Used: 18


ANOVA TABLE
Response Variable: DPPH.inhibition
-------------------------------------------------------------------
Source           DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------------
Concentration     5     14253.0554    2850.6111  1239.17  0.0000
Error            12        27.6051       2.3004
Total            17     14280.6605
-------------------------------------------------------------------

Summary Statistics
------------------------------
  CV(%)   DPPH.inhibition Mean
------------------------------
   2.63                 57.74
------------------------------

Standard Errors
------------------------
Effects          StdErr
------------------------
Concentration      1.24
------------------------
```

Pairwise Mean Comparison of Concentration

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	2.3004

	LSD	HSD	scheffe
Critical Value	2.1788	4.7502	3.1059
Test Statistic	2.6982	4.1597	4.8802

Summary:

Concentration	means	N	LSD	HSD	scheffe
0.01	14.0267	3	f	f	f
0.02	33.6733	3	e	e	e
0.03	50.8800	3	d	d	d
0.04	69.7433	3	c	c	c
0.05	81.6700	3	b	b	b
0.06	96.4333	3	a	a	a

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	2.3004

Number of Means	2	3	4	5	6
Tabular Value	3.0813	3.2252	3.3125	3.3702	3.4102
Test Statistics	2.6982	2.8243	2.9006	2.9512	2.9862

Summary of the Result:

Concentration	means	N	group
0.01	14.03	3	f
0.02	33.67	3	e
0.03	50.88	3	d
0.04	69.74	3	c
0.05	81.67	3	b
0.06	96.43	3	a

Means with the same letter are not significantly different.

```
------------
Sample = E
------------

Summary Information
---------------------------------------------------
FACTOR          NO. OF LEVELS   LEVELS
---------------------------------------------------
Concentration   6               0.25, 0.5, ..., 4
---------------------------------------------------
Number of Observations Read and Used: 18


ANOVA TABLE
Response Variable: DPPH.inhibition
-------------------------------------------------------------------
Source           DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------------
Concentration     5     14501.7972    2900.3594  1234.03  0.0000
Error            12        28.2038       2.3503
Total            17     14530.0010
-------------------------------------------------------------------

Summary Statistics
------------------------------
  CV(%)   DPPH.inhibition Mean
------------------------------
   2.49                 61.61
------------------------------

Standard Errors
------------------------
Effects           StdErr
------------------------
Concentration       1.25
```

Pairwise Mean Comparison of Concentration

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	2.3503

	LSD	HSD	scheffe
Critical Value	2.1788	4.7502	3.1059
Test Statistic	2.7273	4.2045	4.9328

Summary:

Concentration	means	N	LSD	HSD	scheffe
0.25	19.8600	3	e	e	e
0.5	30.4890	3	d	d	d
1	56.4373	3	c	c	c
2	81.0880	3	b	b	b
3	90.1197	3	a	a	a
4	91.6667	3	a	a	a

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	2.3503

Number of Means	2	3	4	5	6
Tabular Value	3.0813	3.2252	3.3125	3.3702	3.4102
Test Statistics	2.7273	2.8547	2.9319	2.9830	3.0184

Summary of the Result:

Concentration	means	N	group
0.25	19.86	3	e
0.5	30.49	3	d
1	56.44	3	c
2	81.09	3	b
3	90.12	3	a
4	91.67	3	a

Means with the same letter are not significantly different.

```
-------------
Sample = WE
-------------

Summary Information
-----------------------------------------------------
FACTOR          NO. OF LEVELS   LEVELS
-----------------------------------------------------
Concentration   7               0.25, 0.5, ..., 10
-----------------------------------------------------
Number of Observations Read and Used: 21


ANOVA TABLE
Response Variable: DPPH.inhibition
-------------------------------------------------------------------
Source           DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------------
Concentration     6      8860.8281    1476.8047   110.42  0.0000
Error            14       187.2478      13.3748
Total            20      9048.0759
-------------------------------------------------------------------

Summary Statistics
------------------------------
  CV(%)   DPPH.inhibition Mean
------------------------------
  12.51                 29.23
------------------------------

Standard Errors
-------------------------
Effects           StdErr
-------------------------
Concentration       2.99
-------------------------
```

Pairwise Mean Comparison of Concentration

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	13.3748

	LSD	HSD	scheffe
Critical Value	2.1448	4.8290	2.8477
Test Statistic	6.4045	10.1962	12.3431

Summary:

Concentration	means	N	LSD	HSD	scheffe
0.25	5.3893	3	d	d	d
0.5	8.4330	3	d	d	d
1	27.4950	3	c	bc	c
2	23.8023	3	c	c	c
3	29.7407	3	c	bc	bc
4	37.5250	3	b	b	b
10	72.2553	3	a	a	a

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	13.3748

Number of Means	2	3	4	5	6	7
Tabular Value	3.0332	3.1783	3.2679	3.3284	3.3714	3.4029
Test Statistics	6.4045	6.7109	6.9000	7.0278	7.1186	7.1852

Summary of the Result:

Concentration	means	N	group
0.25	5.39	3	d
0.5	8.43	3	d
1	27.50	3	c
2	23.80	3	c
3	29.74	3	c
4	37.52	3	b
10	72.26	3	a

Means with the same letter are not significantly different.

```
-------------
Sample = WF
-------------

Summary Information
-----------------------------------------------------
FACTOR          NO. OF LEVELS   LEVELS
-----------------------------------------------------
Concentration   7               0.25, 0.5, ..., 10
-----------------------------------------------------
Number of Observations Read and Used: 21


ANOVA TABLE
Response Variable: DPPH.inhibition
-------------------------------------------------------------------
Source           DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------------
Concentration     6     11299.2330    1883.2055   131.92  0.0000
Error            14       199.8620      14.2759
Total            20     11499.0950
-------------------------------------------------------------------

Summary Statistics
------------------------------
  CV(%)   DPPH.inhibition Mean
------------------------------
  11.45                 33.01
------------------------------

Standard Errors
------------------------
Effects           StdErr
------------------------
Concentration       3.09
------------------------
```

Pairwise Mean Comparison of Concentration

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	14.2759

	LSD	HSD	scheffe
Critical Value	2.1448	4.8290	2.8477
Test Statistic	6.6167	10.5340	12.7521

Summary:

Concentration	means	N	LSD	HSD	scheffe
0.25	5.8883	3	e	e	e
0.5	11.5747	3	e	de	de
1	19.1117	3	d	cd	d
2	29.2417	3	c	c	c
3	40.4690	3	b	b	b
4	45.3597	3	b	b	b
10	79.4410	3	a	a	a

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	14.2759

Number of Means	2	3	4	5	6	7
Tabular Value	3.0332	3.1783	3.2679	3.3284	3.3714	3.4029
Test Statistics	6.6167	6.9332	7.1286	7.2606	7.3545	7.4232

Summary of the Result:

Concentration	means	N	group
0.25	5.89	3	e
0.5	11.57	3	e
1	19.11	3	d
2	29.24	3	c
3	40.47	3	b
4	45.36	3	b
10	79.44	3	a

Means with the same letter are not significantly different.

Cupric ion Reducing Antioxidant Capacity (CUPRAC):

```
------------
Sample = Q
------------

Summary Information
-----------------------------------------------------
FACTOR         NO. OF LEVELS  LEVELS
-----------------------------------------------------
Concentration  6              0.01, 0.02, ..., 0.06
-----------------------------------------------------
Number of Observations Read and Used: 18

ANOVA TABLE
Response Variable: CUPRAC
-------------------------------------------------------------------
Source           DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------------
Concentration     5         1.2965       0.2593   252.37  0.0000
Error            12         0.0123       0.0010
Total            17         1.3088
-------------------------------------------------------------------

Summary Statistics
-------------------
 CV(%)  CUPRAC Mean
-------------------
  5.12       0.6265
-------------------

Standard Errors
----------------------
Effects         StdErr
----------------------
Concentration   0.0262
----------------------
```

Pairwise Mean Comparison of Concentration

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.0010

	LSD	HSD
Critical Value	2.1788	4.7502
Test Statistic	0.0570	0.0879

Summary:

Concentration	means	N	LSD	HSD
0.01	0.2713	3	e	d
0.02	0.3133	3	e	d
0.03	0.5513	3	d	c
0.04	0.7770	3	c	b
0.05	0.9667	3	a	a
0.06	0.8793	3	b	a

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.0010

Number of Means	2	3	4	5	6
Tabular Value	3.0813	3.2252	3.3125	3.3702	3.4102
Test Statistics	0.0570	0.0597	0.0613	0.0624	0.0631

Summary of the Result:

Concentration	means	N	group
0.01	0.2713	3	e
0.02	0.3133	3	e
0.03	0.5513	3	d
0.04	0.7770	3	c
0.05	0.9667	3	a
0.06	0.8793	3	b

Means with the same letter are not significantly different.

```
------------
Sample = E
------------

Summary Information
-----------------------------------------------------
FACTOR         NO. OF LEVELS  LEVELS
-----------------------------------------------------
Concentration  6              0.01, 0.02, ..., 0.06
-----------------------------------------------------
Number of Observations Read and Used: 18

ANOVA TABLE
Response Variable: CUPRAC
-------------------------------------------------------------------
Source           DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------------
Concentration     5         0.1121       0.0224    94.31  0.0000
Error            12         0.0029       0.0002
Total            17         0.1150
-------------------------------------------------------------------

Summary Statistics
-------------------
 CV(%)  CUPRAC Mean
-------------------
 11.26       0.1369
-------------------

Standard Errors
----------------------
Effects         StdErr
----------------------
Concentration   0.0126
----------------------
```

Pairwise Mean Comparison of Concentration

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.0002

	LSD	HSD
Critical Value	2.1788	4.7502
Test Statistic	0.0274	0.0423

Summary:

Concentration	means	N	LSD	HSD
0.01	0.0153	3	d	d
0.02	0.0877	3	c	c
0.03	0.1053	3	c	bc
0.04	0.1440	3	b	b
0.05	0.2367	3	a	a
0.06	0.2327	3	a	a

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.0002

Number of Means	2	3	4	5	6
Tabular Value	3.0813	3.2252	3.3125	3.3702	3.4102
Test Statistics	0.0274	0.0287	0.0295	0.0300	0.0304

Summary of the Result:

Concentration	means	N	group
0.01	0.0153	3	d
0.02	0.0877	3	c
0.03	0.1053	3	c
0.04	0.1440	3	b
0.05	0.2367	3	a
0.06	0.2327	3	a

Means with the same letter are not significantly different.

```
-------------
Sample = WE
-------------

Summary Information
-----------------------------------------------------
FACTOR         NO. OF LEVELS  LEVELS
-----------------------------------------------------
Concentration  6              0.01, 0.02, ..., 0.06
-----------------------------------------------------
Number of Observations Read and Used: 18

ANOVA TABLE
Response Variable: CUPRAC
-------------------------------------------------------------------
Source           DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------------
Concentration     5         0.0624       0.0125    53.68  0.0000
Error            12         0.0028       0.0002
Total            17         0.0652
-------------------------------------------------------------------

Summary Statistics
-------------------
 CV(%)  CUPRAC Mean
-------------------
 14.24       0.1071
-------------------

Standard Errors
----------------------
Effects         StdErr
----------------------
Concentration   0.0125
----------------------
```

Pairwise Mean Comparison of Concentration

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.0002

	LSD	HSD
Critical Value	2.1788	4.7502
Test Statistic	0.0271	0.0418

Summary:

Concentration	means	N	LSD	HSD
0.01	0.0253	3	d	c
0.02	0.0477	3	d	c
0.03	0.0913	3	c	b
0.04	0.1223	3	b	b
0.05	0.1837	3	a	a
0.06	0.1723	3	a	a

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.0002

Number of Means	2	3	4	5	6
Tabular Value	3.0813	3.2252	3.3125	3.3702	3.4102
Test Statistics	0.0271	0.0284	0.0292	0.0297	0.0300

Summary of the Result:

Concentration	means	N	group
0.01	0.0253	3	d
0.02	0.0477	3	d
0.03	0.0913	3	c
0.04	0.1223	3	b
0.05	0.1837	3	a
0.06	0.1723	3	a

Means with the same letter are not significantly different.

```
-------------
Sample = WF
-------------

Summary Information
-----------------------------------------------------
FACTOR         NO. OF LEVELS  LEVELS
-----------------------------------------------------
Concentration  6              0.01, 0.02, ..., 0.06
-----------------------------------------------------
Number of Observations Read and Used: 18

ANOVA TABLE
Response Variable: CUPRAC
-------------------------------------------------------------------
Source           DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------------
Concentration     5         0.0652       0.0130    95.45  0.0000
Error            12         0.0016       0.0001
Total            17         0.0669
-------------------------------------------------------------------

Summary Statistics
--------------------
 CV(%)  CUPRAC Mean
--------------------
 10.70       0.1092
--------------------

Standard Errors
-----------------------
Effects         StdErr
-----------------------
Concentration   0.0095
-----------------------
```

Pairwise Mean Comparison of Concentration

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.0001

	LSD	HSD
Critical Value	2.1788	4.7502
Test Statistic	0.0208	0.0321

Summary:

Concentration	means	N	LSD	HSD
0.01	0.0243	3	e	d
0.02	0.0483	3	d	d
0.03	0.0900	3	c	c
0.04	0.1350	3	b	b
0.05	0.1860	3	a	a
0.06	0.1717	3	a	a

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.0001

Number of Means	2	3	4	5	6
Tabular Value	3.0813	3.2252	3.3125	3.3702	3.4102
Test Statistics	0.0208	0.0218	0.0224	0.0227	0.0230

Summary of the Result:

Concentration	means	N	group
0.01	0.0243	3	e
0.02	0.0483	3	d
0.03	0.0900	3	c
0.04	0.1350	3	b
0.05	0.1860	3	a
0.06	0.1717	3	a

Means with the same letter are not significantly different.

Total Phenolic content (TPC):

```
========================================
ANALYSIS FOR RESPONSE VARIABLE: TPC
========================================

Summary Information
-------------------------------------------------------
FACTOR  NO. OF LEVELS  LEVELS
-------------------------------------------------------
Sample  5              100E, 100Q, 100WE, 100WF, 10Q
-------------------------------------------------------
Number of Observations Read and Used: 15

ANOVA TABLE
Response Variable: TPC
-------------------------------------------------------------
Source    DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------
Sample     4    654066.0116  163516.5029   502.73  0.0000
Error     10      3252.5405     325.2541
Total     14    657318.5521
-------------------------------------------------------------

Summary Statistics
----------------
 CV(%)  TPC Mean
----------------
  6.78    266.13
----------------

Standard Errors
----------------
Effects   StdErr
----------------
Sample    14.73
----------------
```

Pairwise Mean Comparison of Sample

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	325.2541

	LSD	HSD
Critical Value	2.2281	4.6543
Test Statistic	32.8101	48.4623

Summary:

Sample	means	N	LSD	HSD
100E	102.3200	3	c	c
100Q	558.8300	3	a	a
100WE	92.0000	3	c	c
100WF	96.1767	3	c	c
10Q	481.3267	3	b	b

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	325.2541

Number of Means	2	3	4	5
Tabular Value	3.1511	3.2928	3.3763	3.4297
Test Statistics	32.8101	34.2863	35.1552	35.7110

Summary of the Result:

Sample	means	N	group
100E	102.32	3	c
100Q	558.83	3	a
100WE	92.00	3	c
100WF	96.18	3	c
10Q	481.33	3	b

Means with the same letter are not significantly different.

Inhibition of lipid peroxidation:

```
------------
Sample = Q
------------

Summary Information
-------------------------------------------------------
FACTOR          NO. OF LEVELS   LEVELS
-------------------------------------------------------
Concentration   6               0.01, 0.05, ..., 0.4
-------------------------------------------------------
Number of Observations Read and Used: 18


ANOVA TABLE
Response Variable: L.peroxidation.inhibition
-------------------------------------------------------------------
Source           DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------------
Concentration     5      1656.9053     331.3811     9.26  0.0008
Error            12       429.5999      35.8000
Total            17      2086.5052
-------------------------------------------------------------------

Summary Statistics
----------------------------------------
  CV(%)   L.peroxidation.inhibition Mean
----------------------------------------
  12.99                           46.08
----------------------------------------

Standard Errors
------------------------
Effects          StdErr
------------------------
Concentration      4.89
------------------------
```

Pairwise Mean Comparison of Concentration

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	35.8000

	LSD	HSD	scheffe
Critical Value	2.1788	4.7502	3.1059
Test Statistic	10.6443	16.4095	19.2519

Summary:

Concentration	means	N	LSD	HSD	scheffe
0.01	31.4700	3	d	b	b
0.05	37.5300	3	cd	b	b
0.1	46.6200	3	bc	ab	ab
0.2	46.3867	3	bc	ab	ab
0.3	54.3067	3	ab	a	a
0.4	60.1400	3	a	a	a

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	35.8000

Number of Means	2	3	4	5	6
Tabular Value	3.0813	3.2252	3.3125	3.3702	3.4102
Test Statistics	10.6443	11.1415	11.4428	11.6421	11.7804

Summary of the Result:

Concentration	means	N	group
0.01	31.47	3	d
0.05	37.53	3	cd
0.1	46.62	3	bc
0.2	46.39	3	bc
0.3	54.31	3	ab
0.4	60.14	3	a

Means with the same letter are not significantly different.

```
------------
Sample = E
------------

Summary Information
-------------------------------------------------
FACTOR          NO. OF LEVELS   LEVELS
-------------------------------------------------
Concentration   5               3, 5, 8, 10, 12
-------------------------------------------------
Number of Observations Read and Used: 15


ANOVA TABLE
Response Variable: L.peroxidation.inhibition
-------------------------------------------------------------------
Source           DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------------
Concentration     4      1135.1690     283.7922   177.36  0.0000
Error            10        16.0007       1.6001
Total            14      1151.1697
-------------------------------------------------------------------

Summary Statistics
----------------------------------------
  CV(%)   L.peroxidation.inhibition Mean
----------------------------------------
   2.38                           53.24
----------------------------------------

Standard Errors
------------------------
Effects           StdErr
------------------------
Concentration       1.03
------------------------
```

Pairwise Mean Comparison of Concentration

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	1.6001

	LSD	HSD	scheffe
Critical Value	2.2281	4.6543	3.4780
Test Statistic	2.3013	3.3991	3.8523

Summary:

Concentration	means	N	LSD	HSD	scheffe
3	37.9500	3	d	d	d
5	50.2767	3	c	c	c
8	55.2800	3	b	b	b
10	60.8400	3	a	a	a
12	61.8367	3	a	a	a

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	1.6001

Number of Means	2	3	4	5
Tabular Value	3.1511	3.2928	3.3763	3.4297
Test Statistics	2.3013	2.4048	2.4657	2.5047

Summary of the Result:

Concentration	means	N	group
3	37.95	3	d
5	50.28	3	c
8	55.28	3	b
10	60.84	3	a
12	61.84	3	a

Means with the same letter are not significantly different.

```
-------------
Sample = WE
-------------

Summary Information
-----------------------------------------------
FACTOR          NO. OF LEVELS   LEVELS
-----------------------------------------------
Concentration   5               0, 2, 4, 5, 8
-----------------------------------------------
Number of Observations Read and Used: 15


ANOVA TABLE
Response Variable: L.peroxidation.inhibition
-------------------------------------------------------------------
Source           DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------------
Concentration     4      3156.1767     789.0442   694.86  0.0000
Error            10        11.3555       1.1355
Total            14      3167.5322
-------------------------------------------------------------------

Summary Statistics
----------------------------------------
  CV(%)   L.peroxidation.inhibition Mean
----------------------------------------
   2.25                           47.36
----------------------------------------

Standard Errors
------------------------
Effects          StdErr
------------------------
Concentration    0.8701
------------------------
```

Pairwise Mean Comparison of Concentration

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	1.1355

	LSD	HSD	scheffe
Critical Value	2.2281	4.6543	3.4780
Test Statistic	1.9386	2.8635	3.2453

Summary:

Concentration	means	N	LSD	HSD	scheffe
0	68.8733	3	a	a	a
2	28.5700	3	e	e	e
4	34.6433	3	d	d	d
5	48.8267	3	c	c	c
8	55.8633	3	b	b	b

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	1.1355

Number of Means	2	3	4	5
Tabular Value	3.1511	3.2928	3.3763	3.4297
Test Statistics	1.9386	2.0259	2.0772	2.1101

Summary of the Result:

Concentration	means	N	group
0	68.87	3	a
2	28.57	3	e
4	34.64	3	d
5	48.83	3	c
8	55.86	3	b

Means with the same letter are not significantly different.

```
-------------
Sample = WF
-------------

Summary Information
-------------------------------------------------
FACTOR          NO. OF LEVELS   LEVELS
-------------------------------------------------
Concentration   5               2, 4, 5, 8, 10
-------------------------------------------------
Number of Observations Read and Used: 15


ANOVA TABLE
Response Variable: L.peroxidation.inhibition
-----------------------------------------------------------------------
Source           DF  Sum of Square  Mean Square  F Value Pr(> F)
-----------------------------------------------------------------------
Concentration     4      2463.3718     615.8430   543.05  0.0000
Error            10        11.3405       1.1341
Total            14      2474.7124
-----------------------------------------------------------------------

Summary Statistics
----------------------------------------
  CV(%)   L.peroxidation.inhibition Mean
----------------------------------------
   1.99                           53.48
----------------------------------------

Standard Errors
------------------------
Effects           StdErr
------------------------
Concentration     0.8695
------------------------
```

Pairwise Mean Comparison of Concentration

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	1.1341

	LSD	HSD	scheffe
Critical Value	2.2281	4.6543	3.4780
Test Statistic	1.9374	2.8616	3.2432

Summary:

Concentration	means	N	LSD	HSD	scheffe
2	36.9333	3	e	d	d
4	44.4467	3	d	c	c
5	49.2433	3	c	b	b
8	67.2433	3	b	a	a
10	69.5533	3	a	a	a

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	1.1341

Number of Means	2	3	4	5
Tabular Value	3.1511	3.2928	3.3763	3.4297
Test Statistics	1.9374	2.0245	2.0758	2.1087

Summary of the Result:

Concentration	means	N	group
2	36.93	3	e
4	44.45	3	d
5	49.24	3	c
8	67.24	3	b
10	69.55	3	a

Means with the same letter are not significantly different.

Inhibition of HMG-CoA reductase:

```
--------------
Sample = LOV
--------------

Summary Information
-----------------------------------------------
FACTOR          NO. OF LEVELS   LEVELS
-----------------------------------------------
Concentration   3               50, 100, 200
-----------------------------------------------
Number of Observations Read and Used: 8

ANOVA TABLE
Response Variable: X.inhibition
-------------------------------------------------------------------
Source           DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------------
Concentration     2         1.8497       0.9249    29.33  0.0017
Error             5         0.1577       0.0315
Total             7         2.0074
-------------------------------------------------------------------

Summary Statistics
----------------------------
   CV(%)   X.inhibition Mean
----------------------------
  0.1936              91.75
----------------------------


Pairwise Mean Comparison of Concentration

Alpha                        0.05
Error Degrees of Freedom        5
Error Mean Square          0.0315

----------------------------------------
                     LSD    HSD scheffe
----------------------------------------
Critical Value    2.5706  4.6017  5.7861
Test Statistic    0.4026  0.5096  0.5327
----------------------------------------

Summary:
-------------------------------------------------------
Concentration      means       N LSD   HSD   scheffe
-------------------------------------------------------
50              91.2833  2.5714   c    b     b
100             91.6900  2.5714  b     b    ab
200             92.5200  2.5714 a     a     a
-------------------------------------------------------
Means with the same letter are not significantly different
```

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	5
Error Mean Square	0.0315
Harmonic Mean of Cell Sizes	2.5714

Number of Means	2	3
Tabular Value	3.6354	3.7485
Test Statistics	0.4026	0.4151

Summary of the Result:

Concentration	means	N	group
50	91.28	2.57	c
100	91.69	2.57	b
200	92.52	2.57	a

Means with the same letter are not significantly different.

Sample = E

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Concentration	4	10, 20, 60, 80

Number of Observations Read and Used: 11

ANOVA TABLE
Response Variable: X.inhibition

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Concentration	3	7422.3634	2474.1211	43.93	0.0001
Error	7	394.2790	56.3256		
Total	10	7816.6424			

Summary Statistics

CV(%)	X.inhibition Mean
19.32	38.84

Pairwise Mean Comparison of Concentration

Alpha	0.05
Error Degrees of Freedom	7
Error Mean Square	56.3256

	LSD	HSD	scheffe
Critical Value	2.3646	4.6813	4.3468
Test Statistic	15.3690	21.5146	23.4709

Summary:

Concentration	means	N	LSD	HSD	scheffe
10	14.8300	2.6667	c	c	b
20	17.2933	2.6667	c	c	b
60	57.4867	2.6667	b	b	a
80	79.1950	2.6667	a	a	a

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	7
Error Mean Square	56.3256
Harmonic Mean of Cell Sizes	2.6667

Number of Means	2	3	4
Tabular Value	3.3441	3.4772	3.5483
Test Statistics	15.3690	15.9806	16.3073

Summary of the Result:

Concentration	means	N	group
10	14.83	2.67	c
20	17.29	2.67	c
60	57.49	2.67	b
80	79.19	2.67	a

Means with the same letter are not significantly different.

```
-------------
Sample = WE
-------------

Summary Information
-------------------------------------------------
FACTOR          NO. OF LEVELS   LEVELS
-------------------------------------------------
Concentration   4               10, 20, 60, 80
-------------------------------------------------
Number of Observations Read and Used: 12


ANOVA TABLE
Response Variable: X.inhibition
-----------------------------------------------------------------------
Source           DF  Sum of Square  Mean Square  F Value Pr(> F)
-----------------------------------------------------------------------
Concentration     3      3439.2219    1146.4073     3.34  0.0767
Error             8      2746.2778     343.2847
Total            11      6185.4997
-----------------------------------------------------------------------

Summary Statistics
----------------------------
  CV(%)   X.inhibition Mean
----------------------------
  33.51               55.28
----------------------------

Standard Errors
------------------------
Effects           StdErr
------------------------
Concentration      15.13
------------------------
```

Pairwise Mean Comparison of Concentration

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	50.0078

	LSD	HSD	scheffe
Critical Value	2.4469	4.8956	4.7571
Test Statistic	15.7960	22.3470	24.3870

Summary:

Concentration	means	N	LSD	HSD	scheffe
10	30.0967	2.4000	c	b	b
20	49.7100	2.4000	b	b	b
60	80.8550	2.4000	a	a	a
80	88.7550	2.4000	a	a	a

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	50.0078
Harmonic Mean of Cell Sizes	2.4000

Number of Means	2	3	4
Tabular Value	3.4605	3.5865	3.6489
Test Statistics	15.7960	16.3713	16.6563

Summary of the Result:

Concentration	means	N	group
10	30.10	2.40	c
20	49.71	2.40	b
60	80.85	2.40	a
80	88.75	2.40	a

Means with the same letter are not significantly different.

```
-------------
Sample = WF
-------------

Summary Information
-----------------------------------------------
FACTOR          NO. OF LEVELS   LEVELS
-----------------------------------------------
Concentration   4               10, 20, 60, 80
-----------------------------------------------
Number of Observations Read and Used: 11

ANOVA TABLE
Response Variable: X.inhibition
-----------------------------------------------------------------
Source           DF  Sum of Square  Mean Square  F Value Pr(> F)
-----------------------------------------------------------------
Concentration     3      5164.8518    1721.6173    29.33  0.0002
Error             7       410.9284      58.7041
Total            10      5575.7802
-----------------------------------------------------------------

Summary Statistics
---------------------------
  CV(%)   X.inhibition Mean
---------------------------
  18.08              42.37
---------------------------


Pairwise Mean Comparison of Concentration

Alpha                          0.05
Error Degrees of Freedom          7
Error Mean Square           58.7041

-------------------------------------------
                     LSD      HSD  scheffe
-------------------------------------------
Critical Value     2.3646   4.6813   4.3468
Test Statistic    15.6901  21.9641  23.9614
-------------------------------------------

Summary:
-------------------------------------------------------
Concentration      means       N LSD   HSD   scheffe
-------------------------------------------------------
10               16.0167  2.6667   c     c     c
20               36.7533  2.6667  b     bc    bc
60               49.1733  2.6667  b     b     b
80               80.1050  2.6667 a     a     a
-------------------------------------------------------
Means with the same letter are not significantly different
```

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	7
Error Mean Square	58.7041
Harmonic Mean of Cell Sizes	2.6667

Number of Means	2	3	4
Tabular Value	3.3441	3.4772	3.5483
Test Statistics	15.6901	16.3145	16.6481

Summary of the Result:

Concentration	means	N	group
10	16.02	2.67	c
20	36.75	2.67	b
60	49.17	2.67	b
80	80.10	2.67	a

Means with the same letter are not significantly different.

Normal diet rats:

Cardiac marker enzymes – serum

```
ANALYSIS FOR RESPONSE VARIABLE: ALP
Summary Information
-------------------------------------------
FACTOR   NO. OF LEVELS   LEVELS
-------------------------------------------
sample   5               CCL4, F, Ff, N, Q
-------------------------------------------
Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: ALP
-----------------------------------------------------------
Source    DF  Sum of Square  Mean Square  F Value Pr(> F)
-----------------------------------------------------------
sample     4     21563.0833    5390.7708     1.75  0.1808
Error     19     58534.9167    3080.7851
Total     23     80098.0000
-----------------------------------------------------------

Summary Statistics
------------------
  CV(%)   ALP Mean
------------------
  20.60     269.50
------------------

Table of Means
--------------------
sample     ALP Means
--------------------
CCL4           271.67
F              223.00
Ff             310.25
N              290.17
Q              247.00
--------------------
```

ANALYSIS FOR RESPONSE VARIABLE: AST

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
sample	5	CCL4, F, Ff, N, Q

Number of Observations Read and Used: 24

ANOVA TABLE

Response Variable: AST

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
sample	4	49555117.5083	12388779.3771	1.83	0.1652
Error	19	128797027.4500	6778790.9184		
Total	23	178352144.9583			

Summary Statistics

CV(%)	AST Mean
133.95	1943.71

Table of Means

sample	AST Means
CCL4	1708.00
F	1615.40
Ff	3982.25
N	104.17
Q	3923.33

ANALYSIS FOR RESPONSE VARIABLE: ALT
Summary Information

FACTOR	NO. OF LEVELS	LEVELS
sample	5	CCL4, F, Ff, N, Q

Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: ALT

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
sample	4	39520040.8667	9880010.2167	2.30	0.0958
Error	19	81466609.6333	4287716.2965		
Total	23	120986650.5000			

Summary Statistics

CV(%)	ALT Mean
132.08	1567.75

Table of Means

sample	ALT Means
CCL4	1199.33
F	1255.20
Ff	2995.00
N	69.17
Q	3919.67

ANALYSIS FOR RESPONSE VARIABLE: CK

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
sample	5	CCL4, F, Ff, N, Q

Number of Observations Read and Used: 24

ANOVA TABLE

Response Variable: CK

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
sample	4	544236.4500	136059.1125	0.32	0.8618
Error	19	8108005.5500	426737.1342		
Total	23	8652242.0000			

Summary Statistics

CV(%)	CK Mean
48.70	1341.50

Table of Means

sample	CK Means
CCL4	1524.00
F	1448.20
Ff	1333.25
N	1148.67
Q	1195.33

ANALYSIS FOR RESPONSE VARIABLE: LDH

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
sample	5	CCL4, F, Ff, N, Q

Number of Observations Read and Used: 24

ANOVA TABLE

Response Variable: LDH

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
sample	4	82923345.4500	20730836.3625	2.19	0.1089
Error	19	179802871.8833	9463309.0465		
Total	23	262726217.3333			

Summary Statistics

CV(%)	LDH Mean
232.76	1321.67

Table of Means

sample	LDH Means
CCL4	895.83
F	474.20
Ff	861.25
N	321.50
Q	6200.00

Cardiac marker enzymes – tissue

```
========================================
ANALYSIS FOR RESPONSE VARIABLE: ALP
========================================

Summary Information
-------------------------------------------
FACTOR   NO. OF LEVELS   LEVELS
-------------------------------------------
sample   5               CCL4, F, Ff, N, Q
-------------------------------------------
Number of Observations Read and Used: 17


ANOVA TABLE
Response Variable: ALP
-------------------------------------------------------------
Source    DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------
sample     4     12944.9706    3236.2426    13.47  0.0002
Error     12      2883.5000     240.2917
Total     16     15828.4706
-------------------------------------------------------------

Summary Statistics
------------------
  CV(%)   ALP Mean
------------------
  17.00      91.18
------------------
```

Pairwise Mean Comparison of sample

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	240.2917

	LSD	HSD	scheffe
Critical Value	2.1788	4.5077	3.2592
Test Statistic	26.1616	38.2724	43.3539

Summary:

sample	means	N	LSD	HSD	scheffe
CCL4	79.2500	3.3333	bc	b	b
F	61.6667	3.3333	c	b	b
Ff	75.3333	3.3333	bc	b	b
N	96.2500	3.3333	b	b	b
Q	145.6667	3.3333	a	a	a

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	240.2917
Harmonic Mean of Cell Sizes	3.3333

Number of Means	2	3	4	5
Tabular Value	3.0813	3.2252	3.3125	3.3702
Test Statistics	26.1616	27.3837	28.1242	28.6142

Summary of the Result:

sample	means	N	group
CCL4	79.25	3.33	bc
F	61.67	3.33	c
Ff	75.33	3.33	bc
N	96.25	3.33	b
Q	145.67	3.33	a

Means with the same letter are not significantly different.

```
========================================
ANALYSIS FOR RESPONSE VARIABLE: AST
========================================

Summary Information
-------------------------------------------
FACTOR   NO. OF LEVELS   LEVELS
-------------------------------------------
sample   5               CCL4, F, Ff, N, Q
-------------------------------------------
Number of Observations Read and Used: 17


ANOVA TABLE
Response Variable: AST
-------------------------------------------------------------------
Source    DF   Sum of Square    Mean Square  F Value Pr(> F)
-------------------------------------------------------------------
sample     4  235864280.9706  58966070.2426     7.45  0.0030
Error     12   94960785.5000   7913398.7917
Total     16  330825066.4706
-------------------------------------------------------------------

Summary Statistics
------------------
  CV(%)   AST Mean
------------------
  12.32   22840.82
------------------
```

Pairwise Mean Comparison of sample

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	7913398.7917

	LSD	HSD	scheffe
Critical Value	2.1788	4.5077	3.2592
Test Statistic	4747.6323	6945.4136	7867.5667

Summary:

sample	means	N	LSD	HSD	scheffe
CCL4	17346.7500	3.3333	c	b	b
F	22617.0000	3.3333	b	ab	ab
Ff	24632.0000	3.3333	ab	a	a
N	22716.2500	3.3333	b	ab	ab
Q	28765.0000	3.3333	a	a	a

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	7913398.7917
Harmonic Mean of Cell Sizes	3.3333

Number of Means	2	3	4	5
Tabular Value	3.0813	3.2252	3.3125	3.3702
Test Statistics	4747.6323	4969.4082	5103.7793	5192.7113

Summary of the Result:

sample	means	N	group
CCL4	17346.75	3.33	c
F	22617.00	3.33	b
Ff	24632.00	3.33	ab
N	22716.25	3.33	b
Q	28765.00	3.33	a

Means with the same letter are not significantly different.

```
========================================
ANALYSIS FOR RESPONSE VARIABLE: ALT
========================================

Summary Information
-------------------------------------------
FACTOR   NO. OF LEVELS   LEVELS
-------------------------------------------
sample   5               CCL4, F, Ff, N, Q
-------------------------------------------
Number of Observations Read and Used: 17


ANOVA TABLE
Response Variable: ALT
-------------------------------------------------------------
Source    DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------
sample     4    130119.0147   32529.7537     3.61  0.0373
Error     12    108070.7500    9005.8958
Total     16    238189.7647
-------------------------------------------------------------

Summary Statistics
------------------
  CV(%)   ALT Mean
------------------
  13.35     710.88
------------------
```

Pairwise Mean Comparison of sample

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	9005.8958

	LSD	HSD	scheffe
Critical Value	2.1788	4.5077	3.2592
Test Statistic	160.1618	234.3042	265.4131

Summary:

sample	means	N	LSD	HSD	scheffe
CCL4	602.0000	3.3333	b	b	b
F	730.3333	3.3333	ab	ab	ab
Ff	759.6667	3.3333	ab	ab	ab
N	659.2500	3.3333	b	ab	ab
Q	856.6667	3.3333	a	a	a

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	9005.8958
Harmonic Mean of Cell Sizes	3.3333

Number of Means	2	3	4	5
Tabular Value	3.0813	3.2252	3.3125	3.3702
Test Statistics	160.1618	167.6435	172.1765	175.1766

Summary of the Result:

sample	means	N	group
CCL4	602.00	3.33	b
F	730.33	3.33	ab
Ff	759.67	3.33	ab
N	659.25	3.33	b
Q	856.67	3.33	a

Means with the same letter are not significantly different.

```
=======================================
ANALYSIS FOR RESPONSE VARIABLE: CK
=======================================

Summary Information
-------------------------------------------
FACTOR   NO. OF LEVELS   LEVELS
-------------------------------------------
sample   5               CCL4, F, Ff, N, Q
-------------------------------------------
Number of Observations Read and Used: 17


ANOVA TABLE
Response Variable: CK
-------------------------------------------------------------
Source    DF  Sum of Square   Mean Square  F Value Pr(> F)
-------------------------------------------------------------
sample     4   7448486.0686  1862121.5172    25.67  0.0000
Error     12    870342.1667    72528.5139
Total     16   8318828.2353
-------------------------------------------------------------

Summary Statistics
-----------------
  CV(%)   CK Mean
-----------------
  15.96   1687.47
-----------------
```

Pairwise Mean Comparison of sample

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	72528.5139

	LSD	HSD	scheffe
Critical Value	2.1788	4.5077	3.2592
Test Statistic	454.5168	664.9224	753.2052

Summary:

sample	means	N	LSD	HSD	scheffe
CCL4	1103.2500	3.3333	b	b	b
F	1018.0000	3.3333	b	b	b
Ff	1322.0000	3.3333	b	b	b
N	2572.2500	3.3333	a	a	a
Q	2321.6667	3.3333	a	a	a

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	72528.5139
Harmonic Mean of Cell Sizes	3.3333

Number of Means	2	3	4	5
Tabular Value	3.0813	3.2252	3.3125	3.3702
Test Statistics	454.5168	475.7486	488.6127	497.1266

Summary of the Result:

sample	means	N	group
CCL4	1103.25	3.33	b
F	1018.00	3.33	b
Ff	1322.00	3.33	b
N	2572.25	3.33	a
Q	2321.67	3.33	a

Means with the same letter are not significantly different.

ANALYSIS FOR RESPONSE VARIABLE: LDH

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
sample	5	CCL4, F, Ff, N, Q

Number of Observations Read and Used: 17

ANOVA TABLE

Response Variable: LDH

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
sample	4	144819431.3088	36204857.8272	1.05	0.4238
Error	12	415189553.7500	34599129.4792		
Total	16	560008985.0588			

Summary Statistics

CV(%)	LDH Mean
23.68	24838.76

Table of Means

sample	LDH Means
CCL4	22976.50
F	24744.00
Ff	23126.00
N	23429.75
Q	31008.00

Lipid profile – serum

```
ANALYSIS FOR RESPONSE VARIABLE: TG
Summary Information
-------------------------------------------
FACTOR   NO. OF LEVELS   LEVELS
-------------------------------------------
Sample   5               CCL4, F, Ff, N, Q
-------------------------------------------
Number of Observations Read and Used: 24

ANOVA TABLE
Response Variable: TG
-------------------------------------------------------------
Source    DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------
Sample     4         1.8630       0.4657     1.76  0.1777
Error     19         5.0153       0.2640
Total     23         6.8783
-------------------------------------------------------------

Summary Statistics
-----------------
  CV(%)   TG Mean
-----------------
  64.90    0.7917
-----------------

Table of Means
-------------------
Sample     TG Means
-------------------
CCL4           0.67
F              0.96
Ff             0.75
N              0.50
Q              1.40
-------------------
```

```
======================================
ANALYSIS FOR RESPONSE VARIABLE: TC
======================================

Summary Information
-------------------------------------------
FACTOR   NO. OF LEVELS   LEVELS
-------------------------------------------
Sample   5               CCL4, F, Ff, N, Q
-------------------------------------------
Number of Observations Read and Used: 24


ANOVA TABLE
Response Variable: TC
-----------------------------------------------------------
Source    DF  Sum of Square  Mean Square  F Value Pr(> F)
-----------------------------------------------------------
Sample     4         2.1983       0.5496     4.05  0.0154
Error     19         2.5800       0.1358
Total     23         4.7783
-----------------------------------------------------------

Summary Statistics
-----------------
  CV(%)   TC Mean
-----------------
  21.78      1.69
-----------------




Pairwise Mean Comparison of Sample

Alpha                      0.05
Error Degrees of Freedom     19
Error Mean Square        0.1358

----------------------------------------
                  LSD     HSD scheffe
----------------------------------------
Critical Value    2.0930  4.2528  2.8951
Test Statistic    0.5155  0.7406  0.8381
----------------------------------------

Summary:
-----------------------------------------------
Sample     means       N LSD   HSD   scheffe
-----------------------------------------------
CCL4      1.7000  4.4776  b    ab     b
F         1.7000  4.4776  b    ab     b
Ff        1.5000  4.4776  b     b     b
N         1.4333  4.4776  b     b     b
Q         2.4333  4.4776 a     a     a
-----------------------------------------------
Means with the same letter are not significantly different
```

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	19
Error Mean Square	0.1358
Harmonic Mean of Cell Sizes	4.4776

Number of Means	2	3	4	5
Tabular Value	2.9600	3.1064	3.1991	3.2636
Test Statistics	0.5155	0.5410	0.5571	0.5683

Summary of the Result:

Sample	means	N	group
CCL4	1.70	4.48	b
F	1.70	4.48	b
Ff	1.50	4.48	b
N	1.43	4.48	b
Q	2.43	4.48	a

Means with the same letter are not significantly different.

ANALYSIS FOR RESPONSE VARIABLE: HDL

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Sample	5	CCL4, F, Ff, N, Q

Number of Observations Read and Used: 24

ANOVA TABLE

Response Variable: HDL

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Sample	4	1.2365	0.3091	3.12	0.0392
Error	19	1.8804	0.0990		
Total	23	3.1169			

Summary Statistics

CV(%)	HDL Mean
28.56	1.10

```
Pairwise Mean Comparison of Sample

Least Significant Difference (LSD) Test

Alpha                            0.05
Error Degrees of Freedom           19
Error Mean Square              0.0990
Critical Value                 2.0930
Test Statistics                0.4401
Harmonic Mean of Cell Sizes    4.4776

Summary of the Result:
------------------------------
Sample     means      N group
------------------------------
CCL4        1.35   4.48 a
F           0.90   4.48  bc
Ff          0.76   4.48   c
N           1.27   4.48 ab
Q           1.06   4.48 abc
------------------------------
Means with the same letter are not significantly different.
```

Lipid profile – tissue

```
========================================
ANALYSIS FOR RESPONSE VARIABLE: TG
========================================

Summary Information
-------------------------------------------
FACTOR   NO. OF LEVELS   LEVELS
-------------------------------------------
Sample   5               CCL4, F, Ff, N, Q
-------------------------------------------
Number of Observations Read and Used: 17


ANOVA TABLE
Response Variable: TG
-----------------------------------------------------------
Source    DF  Sum of Square  Mean Square  F Value Pr(> F)
-----------------------------------------------------------
Sample     4         0.0847       0.0212     4.69  0.0164
Error     12         0.0542       0.0045
Total     16         0.1388
-----------------------------------------------------------

Summary Statistics
-----------------
  CV(%)   TG Mean
-----------------
  18.42    0.3647
-----------------
```

Pairwise Mean Comparison of Sample

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.0045

	LSD	HSD	scheffe
Critical Value	2.1788	4.5077	3.2592
Test Statistic	0.1134	0.1659	0.1879

Summary:

Sample	means	N	LSD	HSD	scheffe
CCL4	0.4000	3.3333	ab	ab	ab
F	0.3000	3.3333	bc	b	ab
Ff	0.4000	3.3333	ab	ab	ab
N	0.2750	3.3333	c	b	b
Q	0.4667	3.3333	a	a	a

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.0045
Harmonic Mean of Cell Sizes	3.3333

Number of Means	2	3	4	5
Tabular Value	3.0813	3.2252	3.3125	3.3702
Test Statistics	0.1134	0.1187	0.1219	0.1240

Summary of the Result:

Sample	means	N	group
CCL4	0.4000	3.33	ab
F	0.3000	3.33	bc
Ff	0.4000	3.33	ab
N	0.2750	3.33	c
Q	0.4667	3.33	a

Means with the same letter are not significantly different.

Atherogenic diet rats:

Cardiac marker enzymes – serum

```
========================================
ANALYSIS FOR RESPONSE VARIABLE: ALP
========================================

Summary Information
-----------------------------------------
FACTOR   NO. OF LEVELS   LEVELS
-----------------------------------------
Sample   5               A, AT, F, Ff, N
-----------------------------------------
Number of Observations Read and Used: 20


ANOVA TABLE
Response Variable: ALP
-------------------------------------------------------------
Source    DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------
Sample     4     48162.7000   12040.6750     6.43  0.0032
Error     15     28079.5000    1871.9667
Total     19     76242.2000
-------------------------------------------------------------

Summary Statistics
------------------
  CV(%)   ALP Mean
------------------
  13.77     314.30
------------------

Standard Errors
------------------
Effects     StdErr
------------------
Sample       30.59
------------------
```

Pairwise Mean Comparison of Sample

Alpha	0.05
Error Degrees of Freedom	15
Error Mean Square	1871.9667

	LSD	HSD	scheffe
Critical Value	2.1314	4.3670	3.0556
Test Statistic	65.2092	94.4715	106.9572

Summary:

Sample	means	N	LSD	HSD	scheffe
A	318.2500	4	b	ab	ab
AT	405.0000	4	a	a	a
F	263.2500	4	b	b	b
Ff	281.5000	4	b	b	b
N	303.5000	4	b	b	b

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	15
Error Mean Square	1871.9667

Number of Means	2	3	4	5
Tabular Value	3.0143	3.1598	3.2502	3.3118
Test Statistics	65.2092	68.3569	70.3130	71.6456

Summary of the Result:

Sample	means	N	group
A	318.25	4	b
AT	405.00	4	a
F	263.25	4	b
Ff	281.50	4	b
N	303.50	4	b

Means with the same letter are not significantly different.

ANALYSIS FOR RESPONSE VARIABLE: AST

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Sample	5	A, AT, F, Ff, N

Number of Observations Read and Used: 20

ANOVA TABLE

Response Variable: AST

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Sample	4	2646.3000	661.5750	1.01	0.4336
Error	15	9832.2500	655.4833		
Total	19	12478.5500			

Summary Statistics

CV(%)	AST Mean
21.49	119.15

Table of Means

Sample	AST Means
A	121.00
AT	139.75
F	107.75
Ff	118.25
N	109.00

ANALYSIS FOR RESPONSE VARIABLE: ALT

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Sample	5	A, AT, F, Ff, N

Number of Observations Read and Used: 20

ANOVA TABLE

Response Variable: ALT

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Sample	4	39.7000	9.9250	0.06	0.9928
Error	15	2510.5000	167.3667		
Total	19	2550.2000			

Summary Statistics

CV(%)	ALT Mean
17.32	74.70

Table of Means

Sample	ALT Means
A	74.25
AT	76.75
F	75.50
Ff	74.50
N	72.50

ANALYSIS FOR RESPONSE VARIABLE: CK

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Sample	5	A, AT, F, Ff, N

Number of Observations Read and Used: 20

ANOVA TABLE

Response Variable: CK

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Sample	4	5005866.3000	1251466.5750	2.20	0.1183
Error	15	8531783.5000	568785.5667		
Total	19	13537649.8000			

Summary Statistics

CV(%)	CK Mean
55.99	1347.10

Table of Means

Sample	CK Means
A	1141.75
AT	2325.50
F	942.50
Ff	1264.00
N	1061.75

ANALYSIS FOR RESPONSE VARIABLE: LDH

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Sample	5	A, AT, F, Ff, N

Number of Observations Read and Used: 20

ANOVA TABLE

Response Variable: LDH

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Sample	4	144705.2000	36176.3000	0.88	0.4999
Error	15	617725.0000	41181.6667		
Total	19	762430.2000			

Summary Statistics

CV(%)	LDH Mean
53.36	380.30

Table of Means

Sample	LDH Means
A	356.50
AT	541.50
F	308.00
Ff	381.50
N	314.00

Cardiac marker enzymes – tissue

```
========================================
ANALYSIS FOR RESPONSE VARIABLE: ALP
========================================

Summary Information
------------------------------------------
FACTOR   NO. OF LEVELS   LEVELS
------------------------------------------
Sample   5               A, AT, F, Ff, N
------------------------------------------
Number of Observations Read and Used: 20


ANOVA TABLE
Response Variable: ALP
-------------------------------------------------------------
Source    DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------
Sample     4      9117.0000    2279.2500     3.27  0.0407
Error     15     10448.0000     696.5333
Total     19     19565.0000
-------------------------------------------------------------

Summary Statistics
------------------
  CV(%)   ALP Mean
------------------
  24.55     107.50
------------------

Standard Errors
------------------
Effects    StdErr
------------------
Sample       18.66
------------------
```

Pairwise Mean Comparison of Sample

Alpha	0.05
Error Degrees of Freedom	15
Error Mean Square	696.5333

	LSD	HSD	scheffe
Critical Value	2.1314	4.3670	3.0556
Test Statistic	39.7769	57.6266	65.2427

Summary:

Sample	means	N	LSD	HSD	scheffe
A	106.7500	4	ab	ab	ab
AT	77.2500	4	b	b	b
F	141.7500	4	a	a	a
Ff	115.5000	4	ab	ab	ab
N	96.2500	4	b	ab	ab

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	15
Error Mean Square	696.5333

Number of Means	2	3	4	5
Tabular Value	3.0143	3.1598	3.2502	3.3118
Test Statistics	39.7769	41.6969	42.8901	43.7030

Summary of the Result:

Sample	means	N	group
A	106.75	4	ab
AT	77.25	4	b
F	141.75	4	a
Ff	115.50	4	ab
N	96.25	4	b

Means with the same letter are not significantly different.

ANALYSIS FOR RESPONSE VARIABLE: AST

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Sample	5	A, AT, F, Ff, N

Number of Observations Read and Used: 20

ANOVA TABLE

Response Variable: AST

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Sample	4	64058332.0000	16014583.0000	0.24	0.9124
Error	15	1009157122.0000	67277141.4667		
Total	19	1073215454.0000			

Summary Statistics

CV(%)	AST Mean
35.35	23202

Table of Means

Sample	AST Means
A	24742.25
AT	20386.75
F	22680.50
Ff	25484.25
N	22716.25

ANALYSIS FOR RESPONSE VARIABLE: ALT

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Sample	5	A, AT, F, Ff, N

Number of Observations Read and Used: 20

ANOVA TABLE

Response Variable: ALT

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Sample	4	156761.2000	39190.3000	1.81	0.1800
Error	15	325319.0000	21687.9333		
Total	19	482080.2000			

Summary Statistics

CV(%)	ALT Mean
20.24	727.70

Table of Means

Sample	ALT Means
A	780.25
AT	590.25
F	827.75
Ff	781.00
N	659.25

ANALYSIS FOR RESPONSE VARIABLE: CK

Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Sample	5	A, AT, F, Ff, N

Number of Observations Read and Used: 20

ANOVA TABLE

Response Variable: CK

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Sample	4	768525.3000	192131.3250	0.37	0.8240
Error	15	7717819.2500	514521.2833		
Total	19	8486344.5500			

Summary Statistics

CV(%)	CK Mean
29.92	2397.35

Table of Means

Sample	CK Means
A	2027.00
AT	2389.50
F	2456.25
Ff	2541.75
N	2572.25

ANALYSIS FOR RESPONSE VARIABLE: LDH
Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Sample	5	A, AT, F, Ff, N

Number of Observations Read and Used: 20

ANOVA TABLE
Response Variable: LDH

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Sample	4	386296769.2000	96574192.3000	3.31	0.0391
Error	15	436995827.0000	29133055.1333		
Total	19	823292596.2000			

Summary Statistics

CV(%)	LDH Mean
31.22	17289.30

Standard Errors

Effects	StdErr
Sample	3816.61

Pairwise Mean Comparison of Sample

Least Significant Difference (LSD) Test

Alpha	0.05
Error Degrees of Freedom	15
Error Mean Square	29133055.1333
Critical Value	2.1314
Test Statistics	8134.9160

Summary of the Result:

Sample	means	N	group
A	12199.75	4	c
AT	21542.00	4	ab
F	15276.25	4	bc
Ff	13998.75	4	bc
N	23429.75	4	a

Means with the same letter are not significantly different.

Lipid profile – serum

```
ANALYSIS FOR RESPONSE VARIABLE: TG
Summary Information
-----------------------------------------
FACTOR   NO. OF LEVELS   LEVELS
-----------------------------------------
Sample   5               A, AT, F, Ff, N
-----------------------------------------
Number of Observations Read and Used: 23

ANOVA TABLE
Response Variable: TG
-------------------------------------------------------------
Source    DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------
Sample     4         0.1568       0.0392     1.82  0.1687
Error     18         0.3875       0.0215
Total     22         0.5443
-------------------------------------------------------------

Summary Statistics
-----------------
  CV(%)   TG Mean
-----------------
  25.57    0.5739
-----------------

Table of Means
-------------------
Sample     TG Means
-------------------
A             0.5667
AT            0.6333
F             0.5000
Ff            0.7250
N             0.5000
-------------------
```

```
=======================================
ANALYSIS FOR RESPONSE VARIABLE: TC
=======================================

Summary Information
-----------------------------------------
FACTOR   NO. OF LEVELS   LEVELS
-----------------------------------------
Sample   5               A, AT, F, Ff, N
-----------------------------------------
Number of Observations Read and Used: 23


ANOVA TABLE
Response Variable: TC
-----------------------------------------------------------
Source    DF  Sum of Square  Mean Square  F Value Pr(> F)
-----------------------------------------------------------
Sample     4         2.6708       0.6677     5.40  0.0049
Error     18         2.2275       0.1238
Total     22         4.8983
-----------------------------------------------------------

Summary Statistics
-----------------
  CV(%)   TC Mean
-----------------
  19.45      1.81
-----------------
```

Pairwise Mean Comparison of Sample

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	0.1238

	LSD	HSD	scheffe
Critical Value	2.1009	4.2763	2.9277
Test Statistic	0.5049	0.7267	0.8224

Summary:

Sample	means	N	LSD	HSD	scheffe
A	1.7000	4.2857	bc	bc	bc
AT	2.4333	4.2857	a	a	a
F	1.7000	4.2857	bc	bc	bc
Ff	2.1750	4.2857	ab	ab	ab
N	1.4333	4.2857	c	c	c

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	0.1238
Harmonic Mean of Cell Sizes	4.2857

Number of Means	2	3	4	5
Tabular Value	2.9712	3.1174	3.2097	3.2736
Test Statistics	0.5049	0.5297	0.5454	0.5563

Summary of the Result:

Sample	means	N	group
A	1.70	4.29	bc
AT	2.43	4.29	a
F	1.70	4.29	bc
Ff	2.18	4.29	ab
N	1.43	4.29	c

Means with the same letter are not significantly different.

```
========================================
ANALYSIS FOR RESPONSE VARIABLE: HDL
========================================

Summary Information
------------------------------------------
FACTOR   NO. OF LEVELS   LEVELS
------------------------------------------
Sample   5               A, AT, F, Ff, N
------------------------------------------
Number of Observations Read and Used: 23


ANOVA TABLE
Response Variable: HDL
-------------------------------------------------------------
Source    DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------
Sample     4         1.5383       0.3846     4.65  0.0093
Error     18         1.4872       0.0826
Total     22         3.0255
-------------------------------------------------------------

Summary Statistics
------------------
  CV(%)   HDL Mean
------------------
  18.17       1.58
------------------
```

Pairwise Mean Comparison of Sample

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	0.0826

	LSD	HSD	scheffe
Critical Value	2.1009	4.2763	2.9277
Test Statistic	0.4125	0.5937	0.6720

Summary:

Sample	means	N	LSD	HSD	scheffe
A	1.5617	4.2857	bc	ab	ab
AT	2.0233	4.2857	a	a	a
F	1.4600	4.2857	bc	ab	ab
Ff	1.8650	4.2857	ab	ab	a
N	1.2733	4.2857	c	b	b

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	0.0826
Harmonic Mean of Cell Sizes	4.2857

Number of Means	2	3	4	5
Tabular Value	2.9712	3.1174	3.2097	3.2736
Test Statistics	0.4125	0.4328	0.4456	0.4545

Summary of the Result:

Sample	means	N	group
A	1.56	4.29	bc
AT	2.02	4.29	a
F	1.46	4.29	bc
Ff	1.86	4.29	ab
N	1.27	4.29	c

Means with the same letter are not significantly different.

ANALYSIS FOR RESPONSE VARIABLE: LDL
Summary Information

FACTOR	NO. OF LEVELS	LEVELS
Sample	5	A, AT, F, Ff, N

Number of Observations Read and Used: 23

ANOVA TABLE
Response Variable: LDL

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Sample	4	0.0370	0.0093	3.48	0.0285
Error	18	0.0479	0.0027		
Total	22	0.0850			

Summary Statistics

CV(%)	LDL Mean
185.47	0.0278

Pairwise Mean Comparison of Sample

Least Significant Difference (LSD) Test

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	0.0027
Critical Value	2.1009
Test Statistics	0.0741
Harmonic Mean of Cell Sizes	4.2857

Summary of the Result:

Sample	means	N	group
A	0.0000	4.29	b
AT	0.1233	4.29	a
F	0.0300	4.29	b
Ff	0.0375	4.29	b
N	0.0000	4.29	b

Means with the same letter are not significantly different.

Inflammation – TNF-α:

Serum values

```
================================================
ANALYSIS FOR RESPONSE VARIABLE: TNF.conc.
================================================

Summary Information
----------------------------------------
FACTOR   NO. OF LEVELS   LEVELS
----------------------------------------
Sample   4               CCL4, F, N, Q
----------------------------------------
Number of Observations Read and Used: 23


ANOVA TABLE
Response Variable: TNF.conc.
-------------------------------------------------------------
Source    DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------
Sample     3         0.0329       0.0110    12.99  0.0001
Error     19         0.0160       0.0008
Total     22         0.0489
-------------------------------------------------------------

Summary Statistics
------------------------
  CV(%)   TNF.conc. Mean
------------------------
   5.78           0.5029
------------------------
```

Pairwise Mean Comparison of Sample

Alpha	0.05
Error Degrees of Freedom	19
Error Mean Square	0.0008

	LSD	HSD	scheffe
Critical Value	2.0930	3.9766	3.1274
Test Statistic	0.0383	0.0514	0.0560

Summary:

Sample	means	N	LSD	HSD	scheffe
CCL4	0.5635	5.0526	a	a	a
F	0.4944	5.0526	b	b	b
N	0.4755	5.0526	b	b	b
Q	0.4590	5.0526	b	b	b

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	19
Error Mean Square	0.0008
Harmonic Mean of Cell Sizes	5.0526

Number of Means	2	3	4
Tabular Value	2.9600	3.1064	3.1991
Test Statistics	0.0383	0.0402	0.0414

Summary of the Result:

Sample	means	N	group
CCL4	0.5635	5.05	a
F	0.4944	5.05	b
N	0.4755	5.05	b
Q	0.4590	5.05	b

Means with the same letter are not significantly different.

NADP concentration:

For serum of normal diet rats:

```
================================================
ANALYSIS FOR RESPONSE VARIABLE: NADP.conc.
================================================

Summary Information
------------------------------------------------
FACTOR   NO. OF LEVELS   LEVELS
------------------------------------------------
Sample   5               CCL4, F, Ff, N, Q
------------------------------------------------
Number of Observations Read and Used: 23


ANOVA TABLE
Response Variable: NADP.conc.
-------------------------------------------------------------
Source    DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------
Sample     4         2.0500       0.5125     3.52  0.0273
Error     18         2.6211       0.1456
Total     22         4.6712
-------------------------------------------------------------

Summary Statistics
-------------------------
  CV(%)   NADP.conc. Mean
-------------------------
  25.63             1.49
-------------------------
```

Pairwise Mean Comparison of Sample

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	0.1456

	LSD	HSD	scheffe
Critical Value	2.1009	4.2763	2.9277
Test Statistic	0.5437	0.7826	0.8857

Summary:

Sample	means	N	LSD	HSD	scheffe
CCL4	1.9460	4.3478	a	a	a
F	1.4133	4.3478	abc	ab	ab
Ff	1.1350	4.3478	c	b	b
N	1.2540	4.3478	bc	ab	ab
Q	1.7433	4.3478	ab	ab	ab

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	0.1456
Harmonic Mean of Cell Sizes	4.3478

Number of Means	2	3	4	5
Tabular Value	2.9712	3.1174	3.2097	3.2736
Test Statistics	0.5437	0.5705	0.5874	0.5991

Summary of the Result:

Sample	means	N	group
CCL4	1.95	4.35	a
F	1.41	4.35	abc
Ff	1.14	4.35	c
N	1.25	4.35	bc
Q	1.74	4.35	ab

Means with the same letter are not significantly different.

For serum of atherogenic diet rats:

```
================================================
ANALYSIS FOR RESPONSE VARIABLE: NADP.conc.
================================================

Summary Information
-----------------------------------------
FACTOR   NO. OF LEVELS   LEVELS
-----------------------------------------
Sample   5               A, AT, F, Ff, N
-----------------------------------------
Number of Observations Read and Used: 23


ANOVA TABLE
Response Variable: NADP.conc.
-------------------------------------------------------------
Source    DF  Sum of Square  Mean Square  F Value Pr(> F)
-------------------------------------------------------------
Sample     4         2.0851       0.5213     5.33  0.0052
Error     18         1.7608       0.0978
Total     22         3.8458
-------------------------------------------------------------

Summary Statistics
-------------------------
  CV(%)   NADP.conc. Mean
-------------------------
  17.45             1.79
-------------------------
```

Pairwise Mean Comparison of Sample

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	0.0978

	LSD	HSD	scheffe
Critical Value	2.1009	4.2763	2.9277
Test Statistic	0.4359	0.6273	0.7100

Summary:

Sample	means	N	LSD	HSD	scheffe
A	1.9920	4.5455	a	a	a
AT	2.0875	4.5455	a	a	a
F	1.7780	4.5455	a	ab	ab
Ff	1.9400	4.5455	a	a	a
N	1.2540	4.5455	b	b	b

Means with the same letter are not significantly different

Duncan's Multiple Range Test (DMRT)

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	0.0978
Harmonic Mean of Cell Sizes	4.5455

Number of Means	2	3	4	5
Tabular Value	2.9712	3.1174	3.2097	3.2736
Test Statistics	0.4359	0.4573	0.4709	0.4802

Summary of the Result:

Sample	means	N	group
A	1.99	4.55	a
AT	2.09	4.55	a
F	1.78	4.55	a
Ff	1.94	4.55	a
N	1.25	4.55	b

Means with the same letter are not significantly different.