# Antioxidant, Anti-inflammatory and Hypolipidemic Properties of Apple Flavonols

by

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# Dedication

I dedicate this document to my husband for his support, trust, motivation & being with me at every step of this journey.

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## ABSTRACT

Obesity is considered an underlying risk factor for metabolic disease including cardiovascular disease (CVD) and diabetes. The fractions containing flavonols from apple peel were evaluated for their antioxidant, anti-inflammatory, and hypolipidemic properties using *in vitro* and *in vivo* experimental model systems. The fractionated polyphenolics from apple peels showed a strong antioxidant property protecting against heat-induced oxidation of polyunsaturated fatty acids present in fish oil. Apple flavonols (AF), eicosapentaenoic acid (EPA) and the isoquercitrin-EPA ester (QE) significantly reduced serum triacylglycerols and elevated the high density lipoprotein (HDL)-cholesterol compared to the high fat control group. C-reactive protein and interleukin-6 were also reduced compared to the high fat control group and inflammation induced by lipopolysaccharides. Serum adiponectin and interferon-γ concentrations were significantly altered by QE treatment. Overall, AF and QE exhibited anti-inflammatory and hypolipidemic effects under *in vivo* conditions. These beneficial physiological properties and mode of action of AF and QE need to be further investigated.

# LIST OF ABBREVIATIONS USED

| | |
|---|---|
| AA | Arachidonic acid |
| AAPH | 2, 2′-azobis (2-amidinopropane) dihydrochloride |
| AF | Apple flavonols |
| ALA | Alpha-linolenic acid |
| ANOVA | Analysis of variance |
| BD | Becton Dickinson |
| BHT | Butylated hydroxyltoluene |
| BW | Body weight |
| $CaCl_2$ | Calcium chloride |
| CAE | Chlorogenic acid equivalent |
| CCC | Counter-current chromatography |
| CE | Cholesteryl ester |
| COX | Cyclooxygenase |
| CRP | C-reactive protein |
| CVD | Cardiovascular disease |
| DHA | Docosahexaenoic acid |
| DM | Dry matter |
| DNA | Deoxyribonucleic acid |
| DP | Dry peel |
| $DPPH^{\cdot}$ | 1, 1-diphenyl-2-picryl-hydrazyl radical |
| DW | Dry weight |
| EC | Epicatechin |
| ELISA | Enzyme-linked immunosorbant assay |
| eNOS | Endothelial nitric oxide synthase |
| EPA | Eicosapentaenoic acid |
| ESI | Electron spray ionization |
| FC | Free cholesterol |
| FC | Folin-ciocalteau |
| $FeCl_3$ | Ferric chloride |
| FP | Frozen peel |
| FRAP | Ferric reducing antioxidant power |
| FW | Frozen weight |
| HDL-C | High density lipoprotein cholesterol |
| HFC | High fat control |
| HFL | High fat control with lipopolysacchride treatment |
| HMG | 3-hydroxy-3-methyl-glutaryl |
| HPLC | High pressure liquid chromatography |
| HRP | Horse radish peroxidase |
| HSCCC | High speed counter-current chromatography |
| IC50 | 50% inhibitory concentration |
| ICAM-1 | Intercellular adhesion molecule-1 |
| IFN-γ | Interferon-gamma |

| | |
|---|---|
| IL | Interleukin |
| iNOS | Inducible nitric oxide synthase |
| JNK/AP-1 | c-Jun N-terminal kinase activator protein-1 |
| LC | Liquid chromatography |
| LDL-C | Low density lipoprotein cholesterol |
| LOX | Lipooxygenase |
| LPS | Lipopolysacchride |
| LSD | Least square deviation |
| MAPK | Mitogen activated protein kinase |
| MCP-1 | Monocyte chemotactic protein-1 |
| MRM | Multiple reaction-monitoring |
| mRNA | Messenger RNA |
| $Na_2CO_3$ | Sodium carbonate |
| NF-KB | Nuclear factor-kappa B |
| ng | Nanogram |
| NO | Nitric oxide |
| NS | Non-significant |
| NSAC | Nova scotia agricultural college |
| NSAIDS | Non-steroidal anti-inflammatory drugs |
| PEI | Prince Edward island |
| pg | Pictogram |
| PUFA | Polyunsaturated fatty acids |
| Q-3-G | Quercetin-3-*O*-glycosides |
| QE | Quercetin-3-O-glucoside-eicosapentaenoic acid ester |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| SD | Standard deviation |
| SFA | Saturated fatty acids |
| STAT-1 | Signal transducer and activator of transcription-1 |
| TBA | Thiobarbituric acid |
| TC | Total cholesterol |
| TCA | Trichloroacetic acid |
| TE | Trolox equivalent |
| TG | Triacylglycerols |
| Th-2 | T-helper cells-2 |
| TMB | Tetramethylbenzidine |
| TNF-α | Tumor necrosis factor-alpha |
| TP | Total phenolics |
| TPTZ | 2, 4, 6-tripyridyl-s-triazine |
| UPLC-MS/MS | Ultra-pressure liquid chromatography coupled with tandem mass spectrometry |
| USA | United States of America |
| UV | Ultra-violet |
| VCAM-1 | Vascular adhesion molecule-1 |
| VLDL | Very low density lipoprotein |
| Wt | Weight |

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# CHAPTER 1.0 INTRODUCTION

During the last decade, the changing life style including reduced physical activity has increased the incidence of obesity, which contributes to the chronic diseases such as cardiovascular disease (CVD), type 2 diabetes, arthritis and asthma (Kuipers et al., 2011; Raymond et al., 2006). Obesity has also been considered a subclinical inflammation state resulting from chronic activation of the innate immune system (Bastard et al., 2006). Inflammation is a defensive immune response to a variety of stimuli including physical injury, ultra-violet radiation and microbial invasion (Gautam and Jacbak, 2009). The adipose tissue is recently recognized for its participation in various physiological and pathophysiological activities (Antuna-Puente et al., 2008). The pro-inflammatory state in obesity is characterised by elevated production of circulating inflammatory cytokines such as interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α) and C-reactive protein (CRP) (Smith et al., 2007). The concentration of inflammatory cytokines and degree of inflammation is directly associated with risk of insulin resistance and CVD (Hanley et al., 2004; Pradhan et al., 2001).

The high fat Western diet is considered vulnerable for CVD. The resulting hyperlipidemia is characterized by elevated concentrations of free fatty acids, triacylglycerols, low-density lipoprotein (LDL)-cholesterol and reduced high-density lipoprotein (HDL)-cholesterol in the bloodstream (Kolovou et al., 2005). The link between elevated levels of LDL-cholesterol and incidence of CVD has been well established (Gorden and Kannel, 1971; Holvoet et al., 2006). Therefore, the dietary choices need to be monitored as they mediate various metabolic steps like oxidative stress, endothelial dysfunction, inflammation and insulin sensitivity (Hu et al., 2002).

The most widely used strategy to down-regulate production of inflammatory cytokines is by intervention of anti-inflammatory compounds in multiple stages of immune cascade. Non-steroidal anti-inflammatory drug (NSAID) therapies are based on this principle. The NSAID have the ability to inhibit cyclo-oxygenase (COX) which is the key enzyme involved in the production of inflammatory prostaglandins (Rao and Knaus, 2008). A recent evidence showed that 86.6% of patients on oral NSAID therapies experienced increased risk of gastro-intestinal events (Lanas et al., 2010).

A preponderance of evidences from *in vitro* and epidemiological studies suggest that flavonoids could play an anti-inflammatory role (O'Leary et al., 2004; Ruiz et al., 2007). Quercetin (2-(3, 4-dihydroxyphenyl)-3, 5, 7-trihydroxy-4*H*-chromen-4-one) is a widely studied flavonol that is commonly present in fruits and vegetables. Quercetin glycosides constitute nearly 70% of all flavonols in the diet and the intake of quercetin in humans has been estimated to be around 25-40 mg per day (Liu et al., 2005; Hertog et al., 1993). Quercetin modulates endothelial dysfunction by exerting vasodilator effects through increasing nitric oxide synthase (NOS) activity (Perez-Vizcaino et al., 2006; Benito et al., 2002). Quercetin exhibits anti-inflammatory effects by down-regulating the production of inflammatory cytokines, IL-6, IL-4, IL-1α and TNF-α (Winterborne et al., 2009; Rivera et al., 2008; Stewart et al., 2008).

Dietary supplementation of omega-3 polyunsaturated fatty acids (ω3 PUFA) is another nutritional approach to alter the inflammatory pathway (Lombardo et al., 2006; Gil, 2002; Camuesco et al., 2005). Eicosapantenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) are two major ω3 PUFA which can be obtained from salmon and sea-weeds, among others. EPA and DHA have anti-inflammatory

effects (Djousse et al., 2001; Lopez-Garcia et al., 2004). They decrease the content of 2-series of eicosanoids from arachidonic acid (AA, C20:4 ω6) by acting as competitive substrate of COX (Micallef and Garg, 2009). AA is the precursor of the 2-series of eicosanoids whereas EPA is the precursor of the 3-series of eicosanoids. The 3-series eicosanoids produced by EPA are less inflammatory than the 2-series counterparts by AA. The ω3 PUFA have been reported to exhibit anti-inflammatory properties by inhibiting the production of leukotriene $B_4$, thromboxane $A_2$, prostaglandin $E_2$ (Gil, 2002; Camuesco et al., 2006; Lee et al., 2006). A number of human observational studies have found that the increased EPA and DHA intake results in lower plasma levels of TNF-α, CRP and IL-6 and lower CVD risk (Pischon et al., 2003; Lopez- Garcia et al., 2004).

Apples are one of the most widely included fruit in North American and European diet and a significant source of flavonols (Boyer and Liu, 2004). The apple peel contains about 80% of the polyphenolics and 3-6-fold of total flavonols compared to apple flesh (Leccese et al., 2009; Rupasinghe et al., 2010). Apple peel is considered a waste product from apple processing of juice, sauce and pies. About 2-3 million kilograms of apple peels are produced every year in Nova Scotia, Canada (Rupasinghe et al., 2010). The flavonol compounds, quercetin-3-glycosides could be isolated from the apple peel for nutraceutical applications. However, the incorporation of flavonols in lipid-based food and nutraceuticals is limited due to their slightly hydrophilic nature. The application of these compounds in the food and nutraceutical market could be enhanced through increased miscibility in apolar media (Cazarolli et al., 2008, Figueroa and Villeneuve, 2005). Furthermore, improved degree of lipophilicity of quercetin glycosides can increase their ability to cross phospholipid membrane to reach the site of free radical reaction and

interact with target proteins and enzymes (Massaeli et al., 1999; Heim et al., 2002). A flavonol rich fraction was prepared from apple peel using reverse-phase chromatography in the Tree-fruit Bio-products research laboratory at the Nova Scotia Agricultural College. To investigate synergistic benefits, the quercetin-3-*O*-glucoside (isoquercitrin) was acylated with EPA. The aim was to produce structurally modified isoquercitrin analog via esterification of the hydroxyl groups with fatty acids. The resultant esterified compound was examined for its anti-inflammatory properties, compared to the parent compounds, quercetin-3-*O*-glucoside and EPA. The study was designed in which rats were fed with a high fat diet with or without supplementation of apple flavonols, EPA, isoquercitrin-EPA esters for a four-week period. Inflammation was induced by LPS five hours prior to the sacrifice, and serum and hepatic ipid profiles along with serum inflammatory biomarkers (CRP, TNF-α, Interferon-γ, adiponectin, IL-1α, IL-2, IL-4, IL-6, IL-10).

# CHAPTER 2.0 OBJECTIVES

A clear link between chronic inflammation and metabolic diseases such as obesity, diabetes, CVD has been established. The pro-inflammatory cytokines released by adipose tissue is termed as silent inflammation (Sears, 2009). Currently, a diet rich in polyphenolics is well accepted for controlling CVD and inflammation (Pandey and Rizvi, 2009). Apples, particularly apple peels, are considered a substantial source of polyphenolics. Quercetin, known for its antioxidant and anti-inflammatory properties, is a major flavonol found in apple peel. As the available NSAID in the market are reported for serious side effects, there is a need for identifying efficacious and natural anti-inflammatory compounds with minimum side effects.

The overall objective of this research was to assess antioxidant, anti-inflammatory and hypolipidemic properties of apple flavonols, isoquercitrin-EPA ester, EPA individually and in combination with apple flavonols using an experimental rat model system. To execute the first specific objective, apple flavonols were fractionated using reverse-phase chromatography and the antioxidant capacity was determined using ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$) scavenging activity and inhibition of fish oil oxidation using thiobarbituric acid reactive substances (TBARS) assays (Rupasinghe et al., 2010). To fulfill the second specific objective, anti-inflammatory and hypolipidemic activities of flavonol rich fractions, PUFA and isoquercitrin-EPA ester (quercetin-3-*O*-glucoside was esterified using EPA acyl donor) were investigated using a diet-induced hyperlipidemia and LPS-induced inflammation in a rat model.

# CHAPTER 3.0 LITERATURE REVIEW

## 3.1 INFLAMMATION DISORDERS

Obesity is considered a pro-inflammatory condition with increased production of circulating cytokines such as CRP, IL-6, IL-8 and monocyte chemotactic protein-1 (MCP-1) (Smith et al., 2007), leptin and resistin (Bastard et al., 2006). High body weight with accumulation of visceral fat is a predictive marker of insulin resistance, diabetes, coagulation and atherosclerosis (Kopelman, 2000; Lau, 2000). However, weight loss by adopting healthy life style leads to decreased expression of inflammatory cytokines (Clement et al., 2004).

The adipose cells have the ability to store excess of lipids by increasing their volume. The expansion of adipose cells increases the production of IL-8 and MCP-1, further enhancing the infiltration of monocytes and macrophages (Weisberg et al., 2003; Smith et al., 2007) and reduces the secretion of anti-inflammatory adipokine, adiponectin (Rotter et al., 2003). Presence of increased number of macrophages is a biomarker for low-grade inflammation. Preadipocytes, not the mature adipocytes have been suggested as a source of production of adipokines (Charrisere et al., 2003; Bastard et al., 2006) (Fig. 2.1).

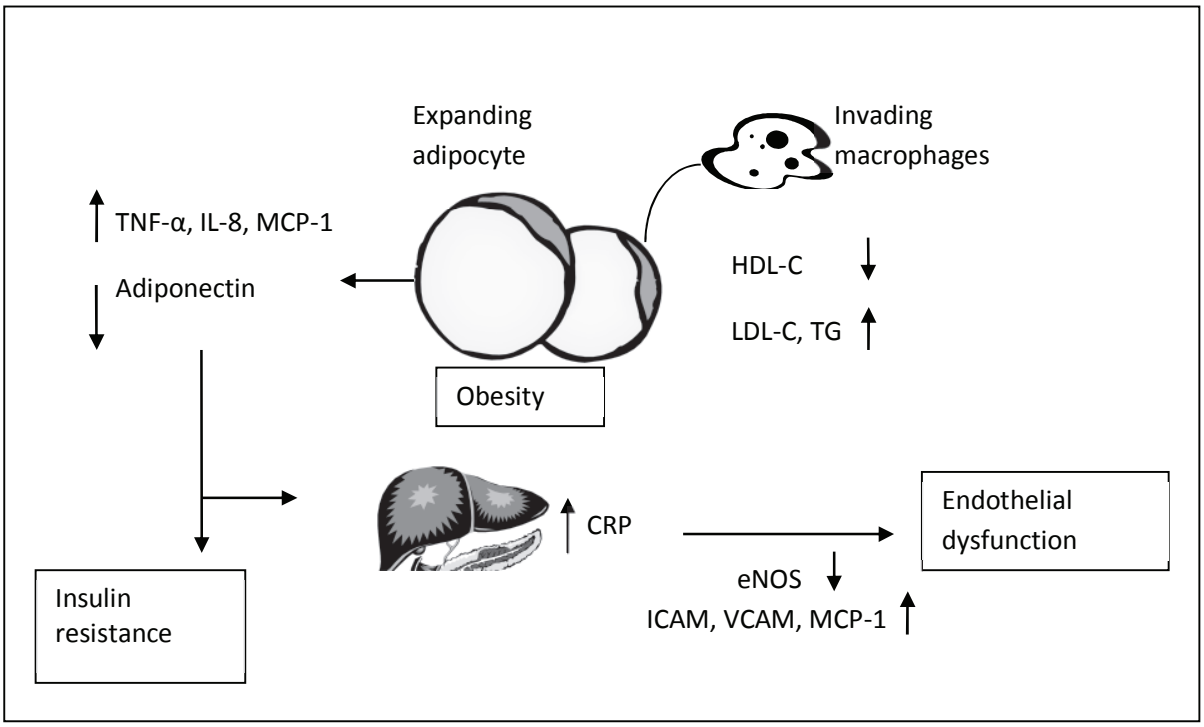


**Figure 2.1. Obesity induced inflammatory events (modified from Smith et al., 2007).**

**TNF-α, tumor necrosis factor-alpha; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; CRP, C-reactive protein; eNOS, endothelial nitric oxide synthase; ICAM, intercellular adhesion molecule-1; VCAM, vascular adhesion molecule-1; MCP-1, monocyte chemotactic protein-1; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triacylglycerols.**

Chronic inflammation has been found as the initial stage of chronic asthma, rheumatoid arthritis, diabetes, CVD and various types of cancers. Epidemiological studies have shown that inflammation is associated with 15-20% of cancer deaths (Mantovani and Pierotti, 2008). Inflammation cascade involves various factors and enzymes such as COX-1, COX-2, cytokines, TNF-α, interferon-gamma (IFN-γ) and various receptors.

### 3.1.1 Inflammatory Cytokines Involved in Endothelial Dysfunction

Interleukin-6 is mainly produced by adipose tissue. Interleukin-6 has a main role in elevating low-grade inflammation by induction of CRP in the liver. Previous studies have suggested its involvement in coronary heart disease (Bastard et al., 2002) and insulin resistance (Nonogaki et al., 1995). Due to recent evidences, increased CRP level, a predictive factor of coronary heart disease, is found to be directly associated with high body weight and visceral fat (Visser et al., 1999; Ridker et al., 2003). CRP directly down-regulates the activities of endothelial NO synthase (eNOS) which leads to lower NO production. Nitric oxide regulates the vasodilatory function of endothelium by inhibiting vasoconstrictors, leukocytes and platelet aggregation (Verma and Anderson, 2002). In the absence of NO, endothelium becomes permeable to leukocytes and leads to expression of endothelium adhesion molecules namely, vascular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and MCP-1. Monocytes form foam cells by phagocytosis of oxidized LDL causing fatty streaks and plaques (Lau et al., 2005). Low adiponectin level results in NO reduction in the vascular walls and causes leukocyte adhesion, promoting endothelial dysfunction. Recent evidences in humans confirmed that CRP levels are inversely correlated with adiponectin (Ouchi et al., 2003).

Adiponectin and leptin are adipokines released by adipose tissue which play a major role in inflammation-related events. The main role of leptin is to regulate food intake under normal conditions. The circulating levels of leptin are directly related to fat mass in the body. Similar to CRP, elevated leptin level can promote endothelial dysfunction by inducing MCP-1 expression and platelet aggregation (Yamagishi et al.,

2001). Leptin can lead to production of reactive oxygen species and promote angiogenesis (Sierra et al., 1998; Cooke and Oka, 2002; Konstantinides et al., 2001).

In contrast to leptin, adiponectin levels are inversely correlated to fat mass, insulin resistance and CRP levels (Higashiura et al., 2004). Adiponectin expresses anti-inflammatory properties by suppressing the VCAM-1 expression and transformation of macrophages to foam cells in endothelial dysfunction and TNF-α induced inflammatory events (Okamoto et al., 2002; Ouchi et al., 2000). Adiponectin has inhibitory properties against atherosclerosis and insulin resistance. Adiponectin also reduces the expression of TNF-α in macrophages, which explains most of the anti-inflammatory nature of adiponectin (Ouchi et al., 2000).

Among the factors involved at the initial stage of inflammation, TNF-α is a pro-inflammatory cytokine that interlinks various steps in the pathophysiology of atherosclerosis and insulin resistance (Hotamisligil et al., 1993; Antuna-Puente et al., 2008). Likewise, CRP and TNF-α can induce ICAM-1 and VCAM-1 and suppress NO synthase leading to endothelial dysfunction (Choy et al., 2001). The inducible nitric oxide synthase produced by macrophages plays a pro-inflammatory role, whereas e-NOS regulates vasodilation acting as an anti-inflammatory factor (Das, 2001). TNF-α can alter insulin signalling pathway through abnormal phosphorylation of insulin substrate receptor at serine residue (Antuna-Puente et al., 2008; Hermann et al., 2000).

### 3.1.2 Hyperlipidemia and Inflammation

High fat diet tends to increase endotoxemia by gut absorption of endotoxins during digestion of lipids and this absorption is affected by structure of the lipids in the

food (Cani et al. 2007, 2008; Laugerette et al., 2009). Obese patients and patients with type 2 diabetes contain low concentrations of lipopolysacchrides (LPS) in the plasma (Libby et al., 2002; Creely et al., 2007). Increased consumption of a high-fat diet leads to hyperlipidemia, characterized by elevated plasma levels of free fatty acids, triacylglycerols, LDL-cholesterol and reduced levels of HDL-cholesterol (Kolovou et al., 2005). Elevated concentrations of LDL-cholesterol are strongly related to the development of atherosclerotic plaque, susceptibility to peroxidation and coronary heart disease (Gordon and Kannel, 1971; Holvoet et al., 2006).

Other than pharmaceutical drugs (resins, statins, cholesterol inhibitors), various alternative therapies have been studied for controlling hyperlipidemia. Omega-3 polyunsaturated fatty acids have gained popularity for their anti-hyperlipidemic and anti-inflammatory properties (Micallef and Garg, 2009). EPA and DHA are two ω3 PUFAs which are derived from α-linolenic acid. Seafoods like mackerel, herring, tuna, salmon, sardines and trout are predominant sources of ω3 PUFA (Grundy and Denke. 1990). These fatty acids are beneficial for controlling coronary heart disease, type 2 diabetes, insulin resistance, hypertension and hyperlipidemia (Lombardo and Chicco, 2006). These two fatty acids are also found to drastically reduce serum free fatty acids and triacylglycerols (Hooper et al., 2006; Yokoyama et al., 2007).

Consumption of a typical high-fat Western diet increases the concentration of AA in human blood (James et al. 2000; Calder. 2002), whereas substituting saturated lipids in the diet with unsaturated ω3 PUFA decreases insulin resistance along with decrease in plasma triacylglycerols and LDL-cholesterol. After supplement of ω3 PUFA, patients

with type 2 diabetes and hypertriacylglycerolemia showed lower plasma levels of inflammatory cytokines (De Luis et al., 2009; Oliver et al., 2010; Rudkowska, 2010).

Most of the metabolic diseases are related to changes in life-style. The often recommended solution to avoid these diseases is to follow a healthy life style by getting rid of extra body fat and avoiding Western diet. However, it is hard to maintain these habits for life time (Jung et al., 2011). Various experimental studies have reported the effectiveness of natural compounds present in curcumin (Ling et al., 2012), onion peel (Jung et al., 2011), green tea (Rejia et al., 2011) and red wine (Della-Marte et al., 2009; Schmatz et al., 2009) in reducing the incidences of hyperlipidemia.

### 3.1.3 Non-Steroidal Anti-inflammatory Drugs

Several NSAIDs and corticosteroids are introduced by pharmaceutical companies to treat inflammatory diseases. These drugs selectively or non-selectively inhibit various steps of the inflammatory cascade. The main mechanism of action of NSAID is to block COX-2 but certain non-selective NSAIDs tend to block COX-1 as well, leading to inhibition of anti-inflammatory cytokines. Further, there are different effects related with these NSAIDs, mainly causing bleeding and ulceration in gastrointestinal tract and platelet dysfunction by blocking synthesis of COX-1 derived prostanoids (Jachak, 2006; Gautam and Jachak, 2009). The consumption of NSAIDs confers life-threatening complications like cardiovascular and liver failure, in particular elderly patients (Harirforoosh and Jamali, 2009; Roth, 2005). The imbalance of thromboxane and prostacyclin by NSAID confer cardiovascular risk (Chan and Primer, 2006). A study in patients has revealed that consumption of ibuprofen (iso-butyl-propanoic-phenolic acid)

has the highest chances of adverse cardiovascular effects (11.7 per 100 persons/years), followed by diclofenac (dichloranilino phenylacetic acid, 9.3 per 100/years) and naproxen (methoxynaphthylpropionic acid, 8.5 per 100 person/ years) (Solomon et al., 2008).

Natural products offer a great hope as an alternative therapy for inflammatory disorders. Polyphenols are a group of compounds, naturally occurring as plant secondary metabolites. The polyphenols can terminate free radical reactions by accepting an electron in various chain reactions in biological systems (Clifford, 2000). Polyphenols inhibit development of atherosclerotic plaque by reducing LDL oxidation (Aviram et al., 2000; Thilakarathna and Rupasinghe, 2012). A number of studies have demonstrated that consumption of polyphenols minimize the risk of coronary heart disease (Renaud and De Lorgeril, 1992; Dubick and Omaye, 2001; Nardini et al., 2007). Polyphenols confer cardio-protective effects by their antioxidant, anti-inflammatory and antiplatelet properties (Garcia-Lafuente et al., 2009).

### 3.1.4 Reactive Oxygen Species and Inflammation

Free radicals are produced as mediators in the metabolic processes like neurotransmission and inflammatory reactions. These free radicals either reactive oxygen species (ROS) or reactive nitrogen species (RNS) play vital roles in smooth muscle relaxation, respiratory burst and metabolism of xenobiotics (Moncada et al., 1991).

ROS and RNS have tendency to attack biomolecules like DNA, RNA, proteins and lipids (Diplock et al., 1998). The polyunsaturated fatty acids are the most likely target of these ROS and RNS due to the presence of double bonds (Halliwell, 1993). The lipid peroxidation produces 4-hydroxy-2-alkenals and malondialdehyde (MDA). MDA can

attack DNA leading to mutagenic lesions which may cause dangerous effects (Spiteller, 2001). The damage caused by ROS and RNS can vary from loss of enzyme function, increased cell permeability, cell signalling, apoptosis to initiate diabetes (Mehta et al., 2006), cancer (Valko et al., 2006) and inflammation (Antonicelli et al., 2000).

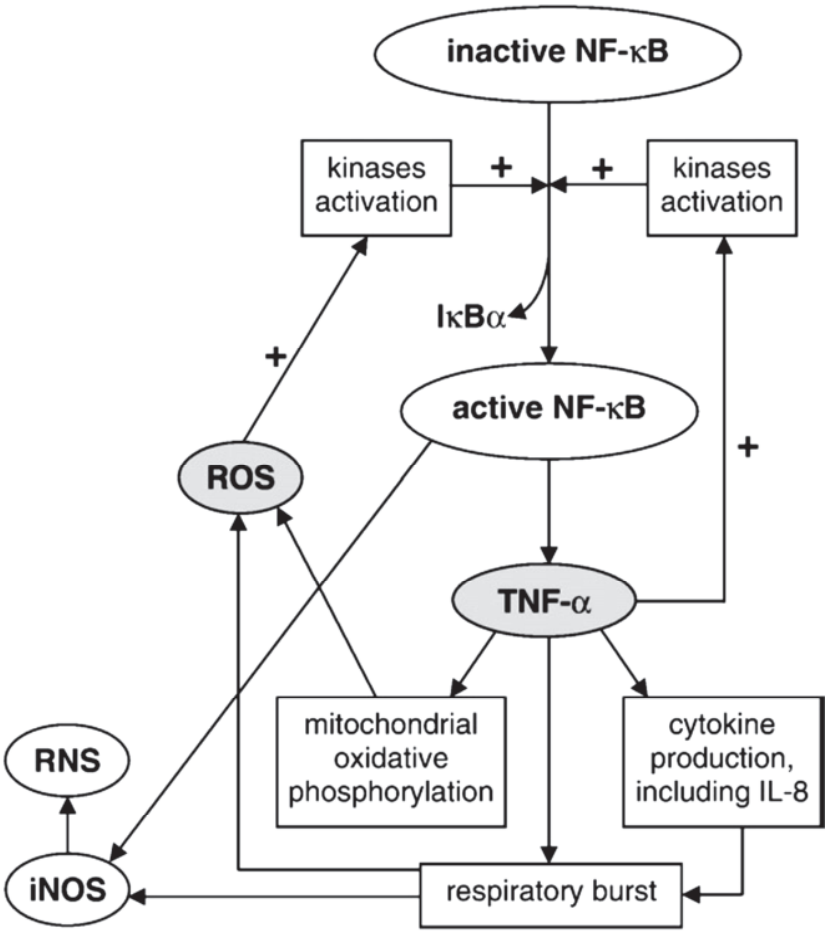


**Figure 2.2. Link between reactive oxygen species and inflammation (Boots et al., 2008).**

**ROS, reactive oxygen species; RNS, reactive nitrogen species; TNF-α, tumor necrosis factor alpha; NF- κB, necrosis factor-kappa B; IκBα, inhibitor of κBα. ; iNOS, inducible nitric oxide synthase.**

ROS can activate NF-κB by releasing inhibitor IκB-α activated NF-κB induces the production of various cytokines (Fig. 2.2) (Rahman et al., 2002). Under normal

conditions, human body has *de novo* defence system to protect the cells and biomolecules from free radicals. This antioxidants defence system comprises of antioxidant enzymes (peroxidase, superoxide dismutase, catalase) and antioxidants (α-tocopherol, carotenoid, ubiquinol-10, ascorbate, glutathione and uric acid) (Chandiere and Ferrasi-Iliou, 1999). This antioxidant system directly quenches free radicals. The process involves donating an electron or proton to the radical, resulting in a stable molecule and antioxidant itself gets oxidized in this reaction (Haenon and Bast, 2002).

## 3.2 POLYPHENOLICS-ANTIOXIDANTS AND ANTI-INFLAMMATORY PROPERTIES

Various epidemiological studies have reported that exogenous intake of polyphenolics-antioxidants from the natural sources such as fruits, vegetables, wine, can render beneficial effects on oxidative stress related diseases (Vertuani et al., 2004; Halliwell, 1996). The most studied compounds are plant secondary metabolites. Plant polyphenols are most ubiquitous group of secondary metabolites, containing more than 25,000 compounds (phenolic acids, flavonoids, stilbenes, lignans and lignins) (Romeir et al., 2009). In regular diet intake, the phytochemicals are present in lower concentrations and therefore, have less effect compared to pharmaceutical drugs. But daily consumption of phytochemicals can attribute significant health effects (Espin et al., 2007). The bioactive compounds like anthocyanins, carotenoids, lycopene, flavonols, stilbenes, coumarins, ellagic acids etc. are commercialized in the forms of pills, capsules, solutions, gels and granulates. The terminology ‘nutraceuticals’ has been coined for a product isolated or purified from foods that are generally sold in the medicinal forms not usually

associated with food. The phytochemicals have been also included in conventional foods or drinks to make them functional or fortified foods.

### 3.2.1 Bioactive Benefits of Polyphenolics

Phenolic compounds are widely studied plant bioactives since 1990s due to growing evidences of their health promoting and disease preventing effects in humans. Numerous studies have indicated various biological effects of phenolic compounds such as inhibition of cancer cell proliferation (Noratto et al., 2009), altering the activity of telomerase (Naasani et al., 2003), cyclo-oxygenase (Hussain et al., 2005), lipo-oxygenase (Sadik et al., 2003), modulate signal transduction pathway (Kong et al., 2000; Wiseman et al., 2001), platelet function (Murphy et al., 2003), prevent endothelial dysfunction (Carluccio et al., 2003) and cholesterol uptake (Leifert and Abeywardena, 2008).

Phenolic compounds are classified into different groups (phenolic acids and flavonoids) on the basis of their structure depending on the number of phenolic rings and the substitution pattern of those rings.

### 3.2.2 Flavonoids

Flavonoids contain a phenolic benzopyran structure (C6-C3-C6) where two aromatic rings A and B are attached to a heterocyclic ring C. As a function of hydroxylation pattern of ring C, flavonoids are further classified into anthocyanin, flavan-3-ols, flavones, flavanones and flavonols (Tsao, 2010). The sub group chalcones is also considered in flavonoid family, even though it lacks ring C. About 8000 flavonoids have

been discovered and there are many more to be identified yet (Harborne and Williams, 2000). In plants, flavonoids exist as either glycones or aglycones depending upon the glycosylation patterns. Like other phenolics, flavonoids are crucial for normal growth and development and defence system in plants. Some flavonoids are responsible for importing colour, flavour, odor to the flowers, fruits and leaves (Harborne, 1989; Gharras, 2009). Flavonoids, in themselves, structure a largest sub-group due to their glycosides, methoxides and various acylations on the three rings. The examples are quercetin and kaempferol which have 279 and 347 different glycosidic compounds, respectively (Tsao and McCallum, 2009; Williams, 2006).

### 3.2.3 Biochemical Facts of Flavonoids

The biochemical research on flavonoids started about 60 years ago in 1950's. At the beginning, some of the flavonoids were considered pro-carcinogenic due to their pro-oxidant properties. Later on, Watterberg (1985) reported the possibility of anti-mutagenic and anti-carcinogenic effect of flavonoids (Depeint et al., 2002). The various epidemiological studies have inversely correlated flavonoid consumption with incidences of stroke, CVD and cancer (Knekt et al., 2002; Goldbohm et al., 1995; Hertog and Hollman, 1996). Research on different members of flavonoid family postulates different anti-proliferative properties (Kuntz et al., 1995). In flavonoid structure, heterogeneous ring C play a crucial function in anti-proliferative activity and any change to its structure can have negative impact on this property (Agullo et al., 1996).

### 3.2.4 Relationship of Flavonols and Metabolic Diseases

A growing body of clinical trials has established polyphenolics as protective compounds against CVD, cancer and neurodegerative diseases (Stevenson and Hurst, 2007). Among flavonoid group, quercetin is a flavonol which is present in most fruits and vegetables. Quercetin has been reported for its benefits in diabetes (Dias et al., 2005; Coskun et al., 2005) and hyperglycemia (Jung et al., 2011). These properties are attributed to its ability to inhibit proinflammatory reaction cascade involving COX-2, NF-κB, IL-6, IL-1β and IL-18 (O'Leary et al., 2004; Comalada et al., 2005). However, quercetin has ability to inhibit production of IL-6 in *in vitro* and this might be by intervening the mitogen activated protein kinase cascade (MAPK) (Roos-Engstrand et al., 2005). Different studies have confirmed that quercetin can reduce serum level of CRP, iNOS and also inhibit the activation of the signal transducer and activator of transcription-1 (STAT-1) (Hamalainen et al., 2007; Hamalainen et al., 2011; Mothana et al., 2012).

Various cohort studies have proved that intake of flavonols lowers the risk of stroke incidence (Hollman et al., 2010). Quercetin exerts protective effect on endothelium dysfunction by inhibiting endothelin-1. Endothelin-1 is a major vasoconstrictor involved in inducing endothelial dysfunction (Romero et al., 2009). Other effect of quercetin in endothelium is increasing eNOS production in *in vivo* (Benito et al., 2002). Quercetin has depicted more positive effects than other flavonoids in reducing the progression of artherosclerosis (Loke et al., 2010), and has more contrasting effects on plasma HDL, LDL and triacylglycerols (Yamamoto and Oue, 2006; Kamada et al., 2005). Quercetin is a potent inhibitor of LDL oxidation and fatty streak formation (Frankel et al., 1993; Auger et al., 2005), and has been reported to inhibit ICAM-1, VCAM-1 and MCP-1

expression by down regulating TNF-α (Winterbone et al., 2009) and JNK/AP-1 pathway (Kobuchi et al., 1999).

### 3.2.5 Apple as a Rich Source of Flavonols

Apples are a significant dietary source of flavonols in the North American population. Apples are ranked the second major source in terms of dietary phenolics (Sun et al., 2002). The more interesting aspect is that apple contains largest portion of free phenolics as compared to other fruits. Therefore, these bioactive compounds are more readily absorbed (Boyer and Liu, 2004). About 80% of the polyphenolics and 3 to 6-fold of total flavonols are distributed in apple peel (Leccese et al., 2009; Rupasinghe et al., 2010). The apple flesh contains catechins, procyanidins, phloridzin, caffeic acid and chlorogenic acid. The apple peel contains additional compounds, such as quercetin glycosides and cyanidin glycosides, which are not present in the flesh (Wolfe and Liu, 2003).

## 3.3 FAT INTAKE AND METABOLIC DISEASE

High fat intake is directly related to obesity which is further associated with high incidences of CVD, diabetes and other metabolic disease (Stone, 1990). Obesity has been described as a low- grade inflammatory condition (Das, 2001). The relationship between high fat intake and atherosclerosis has been under discussion since the early 1900's. Numerous epidemiological studies have demonstrated that high triacylglycerol and LDL-cholesterol levels in serum are associated with inflammation and endothelial dysfunction (Giugiano et al., 2006; Lau et al., 2005; He et al., 2009).

### 3.3.1 Dietary Fatty Acid Absorption and Distribution

Most fatty acids in the diet are present as triacylglycerols and after consumption they are digested in the small intestine and by breaking down into free fatty acids and 2-monoacyl-glycerols by pancreatic lipase-colipase complex before the uptake from intestine. These products are formed into micelles and the broken down products passively diffuse into enterocytes (Shen et al., 2001). In the enterocyte, free fatty acids and 2-monoacyl-glycerols combine again to form triacylglycerols. Then triacylglycerols, chloesteryl esters, phospholipids and apoproteins are synthesized to chylomicrons in the enterocytes and then released into the lymph system. In various body cells, fatty acids are stored as phospholipids in the cell membrane whereas as triacylglycerols in adipocytes (Small, 1991) (Figure 2.3). The liver acts as the main organ for processing chylomicron remanants and liposomes into lipoproteins (LDL, VLDL). Liver fatty acids are converted into triacylglycerols and then distributed as VLDL in blood. The VLDL in the peripheral tissue is converted into LDL by lipoprotein lipase. The liver controls the concentration of cholesterol in the circulation by removing LDL from blood. In contrast to LDL, HDL transports cholesterol and fatty acids from the blood circulation to the liver (Ramirez et al., 2001).

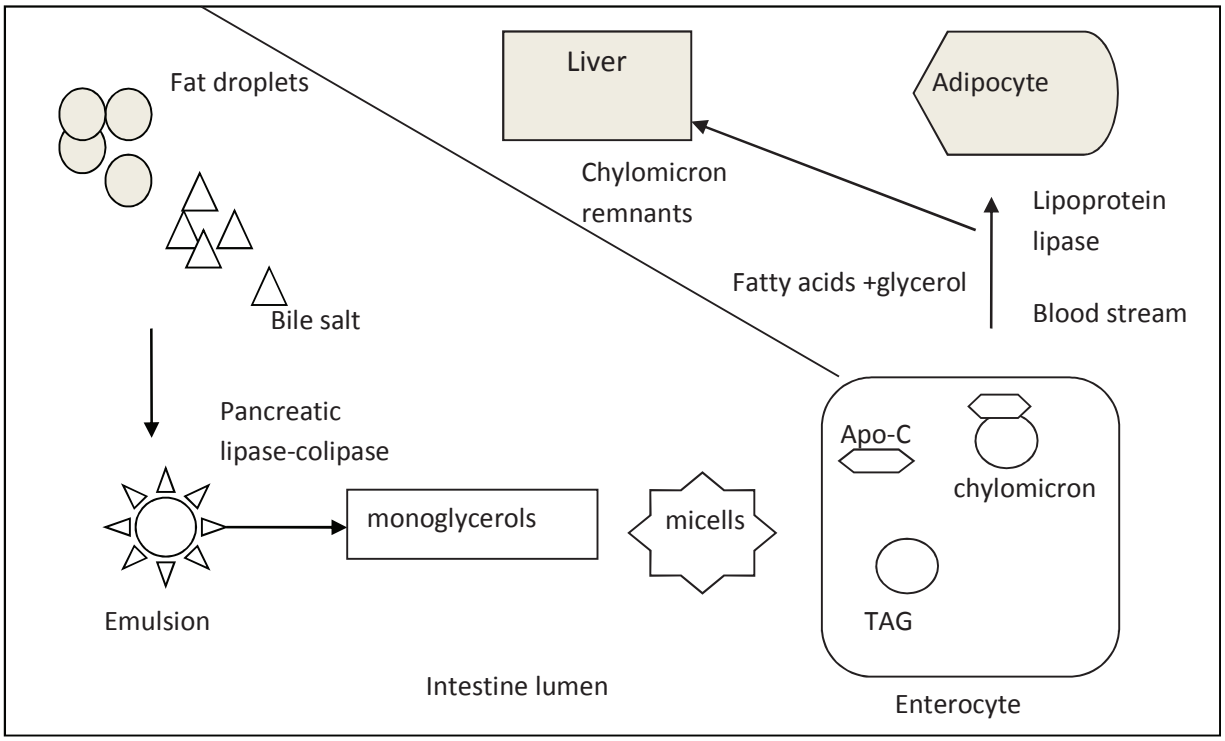


**Figure. 2.3. Fat digestion and absorption (modified from Benjamin Cummings, Addison Wesley Longman, Inc.).**

### 3.3.2 Fatty Acids and their Role in Inflammation

The dietary saturated fatty acids (SFA) (butyric acid, lauric acid, myristic acid, palmitic acid, stearic acid) are found in butter, coconut oil, cow milk products and meat. The consumption of SFA elevates blood triacylglycerols and LDL-cholesterol and therefore, increases the chances of CVD (Kris- Etherton et al., 2002; Oh et al., 2005; Hayes et al., 1991; Balk et al., 2006).

The polyunsaturated fatty acids (PUFA) contain multiple double bonds and are divided into two categories ω3 and ω6. The ω3 PUFA are characterized by the first double bond at the 3$^{rd}$ carbon from methyl group end and ω6 PUFA at the carbon-6

position. The common dietary ω3 PUFA is α-linolenic acid (ALA, 18:3) and is abundant in walnut and flaxseed. EPA and DHA are present in sea weeds, salmon, tuna, herring and mackerel. Linoleic acid is the common dietary ω6 PUFA which is found in sunflower and other main ω6 PUFA is AA abundant in meat. The high dietary intake of ALA increases its conversion to long chain EPA and DHA by elongation and desaturation (Norris and Spector, 2002; de Deckere et al., 1998). However, the main source of EPA and DHA is direct intake through diet as the conversion rate of ALA to DHA and EPA in the human body is very low (0.2-15%) (McKeigue, 1994; Francois et al., 2003). Linoleic acid and α-linolenic acid are essential fatty acids as human body lacks the enzymes to produce these fatty acids (Fig. 2.4).

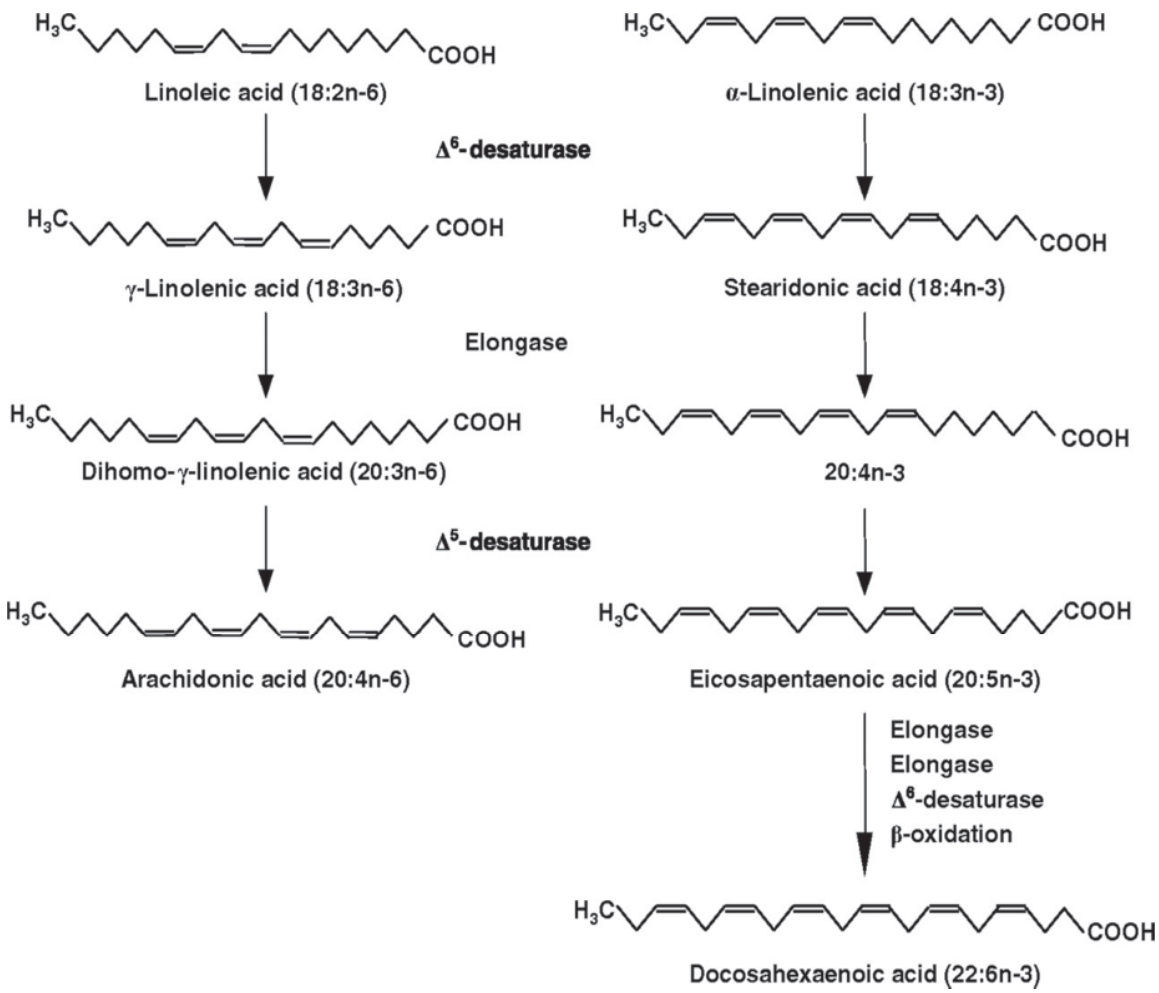


**Figure 2.4. Structural and metabolic synthesis of primary ω6 and ω3 PUFA by elongases and desaturases. (Wanton and Calder, 2007).**

The arachidonic acid has a critical role in the inflammatory diseases. The AA present in membrane phospholipids acts as substrate for the synthesis of pro-inflammatory cytokines. Arachidonic acid is released from phospholipid membrane by enzyme phospholipase 2. The released AA is broken down by COX-2 and lipooxygenase (LOX) to produce various prostaglandins, thromboxane $A_2$, leukotrienes (Fig. 2.5). However, intake of ω3 PUFA can increase the incorporation of ω3 PUFA in the membrane phospholipids and decrease the content of AA in the cell membranes (Rees et

al., 2006). The presence of ω3 PUFA leads to a competitive uptake by the enzymes COX and LOX and thus decreasing the AA substrate availability. The eicosanoids produced by EPA and DHA have less inflammatory potential and also produce E-series and D-series resolvins. These mediators are considered to have anti-inflammatory and immune-modulatory benefits (Serhan et al., 2000; Serhan et al., 2008).

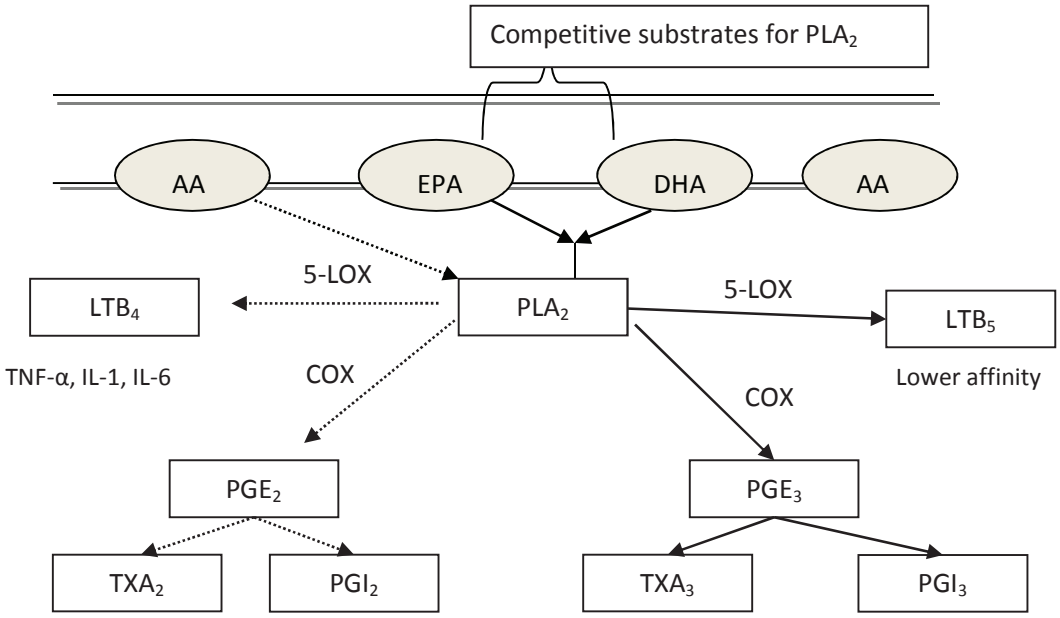


**Figure 2.5. Depiction of the cellular arachidonic acid and ω3 PUFA metabolism involved in inflammation (modified from Simopoulos, 2002).**
**AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PLA2, phospholipase 2; LOX, lipooxygenase; COX, cyclooxygenase; LTB, leukotriene B; PGE, prostaglandins E; TXA, thromboxanes A; PGI, prostacyclins.**

There are evidences that ω3 PUFA exert hypotriglyeridemic, antiarrhythmic, antithrombotic and anti-inflammatory effects (Kris-Etherton et al., 2002; Psota et al., 2006; Ferdinandusse et al., 2001).

## 3.4 ISOLATION AND PURIFICATION OF FLAVONOIDS

Phenolic compounds are secondary metabolites which occur ubiquitously in fruits and vegetables. They are synthesized are part of normal development in plants (Harborne, 1982; Pridham, 1960) and also induced by stress signal, such as wounding, drought, pathogens and/ or nutrient deficiency (Winkel-Shirley, 2002). The different types of phenolic compounds include phenolic acids, flavonoids, coumarins, tannins and lignin. Phenolics present in outer surface of plants are insoluble phenolics (lignins and hydroxycinnamic acids) and provide strength to the cell walls.

### 3.4.1 Extraction of Polyphenolics

The chemistry varies in different classes of phenolics and is one of the important factors influencing extraction of phenolics. Extraction of phenolics is also dependent on other factors such as sample particle size, solvent system, extraction method, storage time and presence of other substances (Naczk and Shahidi, 2004). The solvents used for phenolics extraction are methanol, ethanol, acetone, water, propanol, ethyl acetate and various combinations of these solvents. The extraction of phenolics can be improved by adjusting proper sample- to-solvent ratio (Naczk and Shahidi, 1991). Sample particle size significantly influence tannin recovery from dry beans (Deshpande, 1985). Optimization of polyphenolic extraction method is essential due to large variation in their polarity and biochemical modifications such as glycosylation, esterification affecting extraction output (Pellegrini et al., 2007). Michiels et al. (2012) evaluated various sample-solvent ratios and higher yield can be achieved at higher solvent-to-sample ratio. The proposed extraction conditions include using an extraction solvent, mixture of acetone: water: acetic acid

(70:28:2, v/v/v) at a solvent to solid ratio (20:1) and extraction for 1 h at 4$^{o}$C. Komes et al. (2011) demonstrated that hydrolysed extracts of medicinal plants (using 60% ethanol and 5 mL of 2 M hydrochloric acid) had higher total phenolic content than non-hydrolysed extracts.

### 3.4.2 Different Techniques for Purification of Flavonols

The extract of polyphenolics always contains a mixture of different classes of phenolics compounds and non-phenolic substances. Further purification may be needed to isolate desired phenolic compound from the crude extract. Several difficulties arise because no universal method can be used to isolate all phenolics. Flavonoids, the most prominent class of phenolics, contain flavones, flavonols, flavonones, flavanols, isoflavonoids, anthocyanins and all have the same basic structure. Numerous gas-solid and liquid-solid phase adsorption techniques have been employed to adsorb specific phenolics (Zagorodni, 2007; LeVav and Carta, 2007). Krammerer et al. (2010) evaluated non-polar adsorbent and ion-exchange resins to optimize recovery of different phenolics. In their study, phloridzin and rutin were successfully recovered using acidic resin cation-exchange chromatography.

Conventional methods used for purification of polyphenolic are ion-exchange resins and reverse-phase liquid chromatography. Counter-current chromatography (CCC) has been recently explored to be an excellent alternative for isolating various phenolic classes (Pauli et al., 2008). In CCC, separation of compounds is achieved on the basis of partition ratio between stationary liquid phase and mobile liquid phase. Modern commercial CCC includes high-speed counter-current chromatography, multilayer coil

counter-current chromatography and centrifugal partition chromatography. Anthocyanins from wine (Salas et al., 2005; Schwarz et al., 2003), flavanols and proanthocyanidins from green tea (Cao et al., 2000) were fractionated and isolated using CCC.

Purification and isolation using adsorption techniques is achieved on the basis of difference in polarity, molecular weight and affinity for the adsorbent (Bernardin, 1985). Owen et al. (2003) fractionated polyphenolics from carob fibre (*Ceratonia siliqua* L.) using silicic gel column chromatography using methanol elution gradient. Silva et al. (2007) optimized that water content in the extract influenced phenolic adsorption using different types of microporus resins. Liu et al. (2008) recovered polyphenolics from simulated tobacco polluted water sample employing a styrene-divinylbenzene resin (Amberlite XAD-4). Similar method was used by Scordino et al. (2004) for isolating hesperdin, anthocyanins and hydroxycinnamates. The purification methods using XAD-4 resin were effective due to its large specific surface area and therefore, showed high recovery of polyphenolics. Krammerer et al. (2011) demonstrated the interaction of apple polyphenolics with two anion exchangers containing polystyrene matrix and other having polyacrylamide backbone. The results indicated that tendency of individual phenolic compound was influenced by the combined presence of other compounds. The adsorption depending on intermolecular interactions and selection of adsorption resin should be based on the phenolic profile of the extract. Zessner et al. (2008) applied centrifugal partition chromatography to fractionate apple juice phenolics and then sub-fractionation was achieved by gel permeation chromatography (Sephadex LH-20). Normal phase chromatography was used for additional fractionation to increase procyanidin content. In another study, Cao et al. (2009) extracted and fractionated polyphenolics from apple

pomace employing combination of Sephadex LH-20 chromatography with high speed counter-current chromatography (HSCCC), and solvent extraction with HSCCC. The ethanolic extract of apple pomace was retained on Sephadex LH-20 and fractions were collected using aqueous ethanol gradient. The fractions with similar compounds were pooled and further purified using HSCCC. The complete separation of polyphenolics was achieved only by using combination of the two techniques.

High performance liquid chromatography (HPLC) is the most widely used analytical technique for the separation and characterization of polyphenolics (Carrasco-Pancorbo et al., 2007; Naczk and Shahidi, 2006; Valls et al., 2009). Reverse-phase columns with C18 stationary phase have enhanced the separation of different compounds (Gruz et al., 2008). Mostly, electrochemical, UV-visible, fluorescent, photodiode array detectors are used with HPLC methods for analysing food phenolics. The different classes of phenolic compounds have been successfully identified and quantified using mass spectrometry detectors coupled to HPLC. Mass spectrometry is a selective analytical technique which has improved the characterization and identification of phenolic compounds (Nicoletti et al., 2007; Buiarelli et al., 2007). Ion-trap mass spectrometry is an advanced and highly sensitive technique for identification of isomeric flavonoid glycosides which are not identified by mass spectrometry. It is based on the principle of sequential fragmentation of molecular ions (Prasain et al., 2004). The other techniques for polyphenolic analysis are electrospray ionisation (ESI), atmospheric-pressure chemical ionization, matrix-assisted laser desorption/ionization mass spectrometry (Valls et al., 2009). Liu et al. (2008) separated and quantified different polyphenolics from water samples using HPLC coupled with photodiode array detector. Owen et al. (2003)

elucidated the structures of polyphenolics present in carob fiber using nano ESI-MS and LC-ESI.

### 3.5 ESTERIFICATION OF FLAVONOLS

More than 8000 flavonoids have been reported and they exist in either aglycone or glycosylated form (Chebil et al., 2007). The antioxidant propensity of flavonoids is mainly associated with arrangement of substituents in the structure (Heim et al., 2002; Rice-Evans et al., 1996). Some flavonoid aglycone exhibits a higher antioxidant activity than its glycosylated form (Burda and Oleszek, 2001; Formica and Regelson, 1995; Ross et al. 2002). The presence of the C2-C3 double bond, the C-3 hydroxyl group, and the 3', 4' hydroxy in the B-ring of the flavonoid is the key structural characteristics for its biological activity (Ardhaoui et al., 2004) (Fig. 2.6).

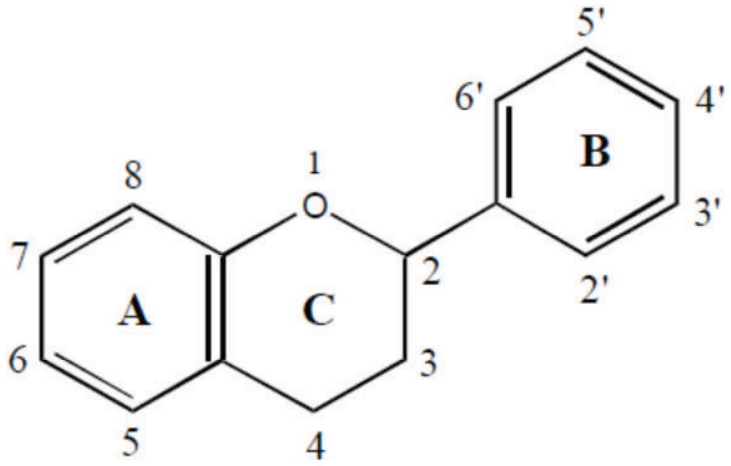


**Figure 2.6. Basic structure of flavonoid with three rings. The sub-groups: flavone, flavonol, flavanone, flavanonol contain carbonyl group at C-4 and flavonol and flavanonol also contain hydroxyl group at C-3 (Medjakovic et al., 2010).**

The variety of flavonoids rich extracts of berries, grapes, pine bark have been used in beverages and food in the market. The possible beneficial effects of flavonoids make them attractive to use in cosmetic and pharmaceutical formulations. However, the development of some of these compounds into commercial products based on lipid media is limited (Cazarolli et al., 2008, Figueroa and Villeneuve, 2005). Furthermore, the improved degree of lipophilicity of the compounds could increase their ability to cross plasma membrane-lipid bilayers to reach the site of free radical reaction and interact with target proteins and enzymes (Massaeli et al., 1999; Heim et al., 2002). Flavonoids can be effective in preventing oxidative degeneration of food, as they can quench lipidoxy and peroxy radicals. However, effectiveness of the compounds is limited due to their low partition coefficient. The flavonoid aglycone and methoxylated flavonoids can also be used in such foods, but they are often unstable and are not common in nature (Viskupicova et al., 2010). To achieve the maximum benefits from these compounds, flavonoid lipophilisation is one of the techniques. The motive is to produce selectively modified flavonoid analog via esterification of the hydroxyl groups with fatty acids. The structurally modified flavonoids have improved physico-chemical properties and can penetrate effectively across the plasma membrane (Ishihara and Nakajima, 2003; Suda et al., 2002; Viskupicova et al., 2010). The substitution positions are critical in flavonoid chemical structure. Therefore, while enhancing the beneficial effects, attention is required to preserve the basic antioxidant properties of flavonoid structure (Mellou et al., 2006; Chebil et al., 2007).

### 3.5.1 Factors Influencing Acylation Reaction

The acylation can be carried out by chemical, enzymatic or chemico-enzymatic methods. The conventional chemical acylation methods offer low regioselectivity. As flavonoid structure contains many hydroxyl groups; therefore, this method leads to various products with different degree of esterification (Bohm et al., 1998; Patti et al., 2000). However, lipase catalysed acylation is more productive and regioselective. The esterification reaction is affected by the type of enzyme, reaction media and operating conditions (Chebil et al., 2006). The choice of suitable acyl donor has an important impact on the sustainability of the esterification (Paravidino and Hanefeld, 2011). The different types of fatty acids with C2-C18 have been tested for flavonoid esterification (Viskupicova et al., 2010; Lue et al., 2010) and the highest flavonoid esterification were generally reported with shorter chain fatty acids (Ardhaoui et al., 2004). However, for rutin esters, the long chain fatty acid esters demonstrated higher antioxidant activity in lipid medium (Viskupicova et al., 2010). A certain degree of lipophilicity in flavonoids may have positive impact on its antioxidant nature and is an important determinant to estimate its biological activity (Viskupicova et al., 2010). Various flavonoid compounds, rutin, prunin, quercetin, isoquercitrin, naringenin, hesperetin and chrysin have been enzymatically esterified using different acyl donors such as, lauric acid, palmitic acid, vinyl laurate, vinyl acetate and vinyl butyrate (Lue et al., 2010; Celiz and Daz, 2011; Chebil et al., 2007).

In the literature, esterification of flavonoids has been reported using several enzymes such as acyl transferases, proteases, lipases and subtilisin (Chebil et al., 2007). Danieli and co-workers (1989) were the pioneers in acylation of isoquercitrin using

subtilisin as a biocatalyst and trifluoroethyl butanoate as acyl donor. However, among all the enzymes, lipases are most widely used for flavonoid esterification. In flavonoid esterfication, the regioselectivity may lead to multiple products. Danieli et al. (1997) reported that regioselectivity of the acylation is determined by the presence of primary and secondary hydroxyl groups. In the case of esterification of rutin, lipase preferred primary alcohol (C6”-OH), but in the absence of primary alcohol, it can acetylate rutin at secondary alcohol (C3”-OH, C4”-OH). Moreover, Chebil et al. (2007) reported that regioselectivity is based on high reactivity and availability of primary or secondary functional group. In quercetin, esterification of C4’-OH, C3’-OH, C7-OH groups occurs successively in case only phenolic hydroxyl groups are present. The regioselectivity also depends on nature and origin of the enzyme and presence of sugar moiety (Lambusta et al., 1993).

Reaction media is an important factor for esterification end product. Lambusta et al. (1993) tested various solvents such as, acetonitrile, tetrahydrofuran, tert-amyl alcohol and acetone in lipase catalysed catechin esterification. The highest yield was found with acetonitrile. Several researchers compared different solvents and reported the most suitable solvent is acetone or acetonitrile (Riva et al., 1996, Nakajima et al., 1999; Gao et al., 2001; Mellou et al., 2005). In acylation of glycones, isoquercitrin and rutin, the yield of esterified product varied with chain length of acyl donor (from 94% with ethyl laurate to 81% with ethyl butyrate for isorhamnetin) (Salem et al., 2010; Ardhaoui et al., 2004).

Addition of lipophilic moiety to isorhamnetin structure enhances its anti-proliferative activity on tumor cells and inhibits xanthine oxidase activity (Salem et al.,

2010). However, Celiz and Daz (2011) have reported contradictory results regarding the chain length of acyl donor on esterification of prunin (4′,5,7-trihydroxiflavanone-β-D-glucoside). In their study, esterification of prunin slightly enhanced radical scavenging activity and solubility in 1-octanol, the hydrophobic media which makes it more suitable for fat bearing food and pharmaceuticals. A higher conversion yield of flavonoid esters occurred with short and medium chain lengths (C4-C12) (Viskupicova et al., 2010; Salem et al., 2011; Ardhaoui et al., 2004).

Among flavonoids, quercetin is the most widely studied compound. The bioactivity of quercetin is well documented under *in vitro* conditions, however, the mode of action and its bioavailbility *in vivo* conditions are still under debate. After consumption, quercetin glycosides were not detected in plasma and it has been demonstrated that glycosides are cleaved from quercetin in the gut before absorption (Wu et al., 2002). Different studies in rats reported that quercetin glucuronides, sulphates and methylated quercetin derivatives occur in plasma after high intake of quercetin (Spencer et al., 1999; Crespy et al., 2001). However, in contrast, anthocyanin glycosides are absorbed by the intestine. This may be due to more stability of anthocyanin glycosides than quercetin glycosides (Felgines et al., 2003; Erlund et al., 2003).

Therefore, another reason behind acylation of flavonoids is to enhance stability of these compounds. The selection of suitable acyl donor like PUFA is a way to combine the beneficial attributes of compounds (Stamatis et al., 2001; Mellou et al., 2005). Mellou et al. (2006) have reported that rutin esters of linoleic acid significantly reduced the secretion of vascular endothelium growth factor release in tumor cells. Similarly, epigallocatechin gallate-docosapentaenoic acid esters also down-regulated the iNOS and

COX-2 biosynthesis at a transcriptional level in LPS-stimulated murine macrophages (Zhong et al., 2012).

### 3.6 RESEARCH ANIMAL MODEL

Various *in vitro* and *in vivo* research models have been used to investigate pathophysiology, immunology and genomics associated with metabolic diseases. However, the most of the animal study results cannot be correlated and applied to humans due to species differences in disease heterogeneity and pathophysiology (Wandler and Wehling, 2010). Nevertheless, at the early stage of disease development, the study of biomarkers can provide useful information. Therefore, a suitable animal model for a human disease can be used to identify the biomarkers. These biomarkers can be serum parameters, gene expression or inflammatory proteins. The selection of right biomarkers and quantitation system are the crucial steps in application of experimental animal study to human diseases and new drug developments (Wehling, 2009). The selection of a specific research model should be based on its relevance to humans metabolically and pathophysiologically. The body size should be appropriate for physical and metabolic analysis and should develop the disease biomarkers comparable to the humans (Russel and Proctor, 2006).

A wide variety of animal models have been used to study metabolic diseases in human. Ideally, human are most appropriate model for such research studies. However, there are safety restrictions testing new drugs in clinical studies for which animal is needed. Next to humans, primates such as chimpanzees and rhesus monkeys are most closely related in all aspects to human. However, their use is considered unethical and

they are expensive to maintain for small laboratory setting (Hansen and Bodkin, 1993). The most commonly used animals are rabbits, dogs, swine, mice, hamsters and rats.

Rat has been the most popular biomedical research model during the last 100 years. The present day laboratory rats derived from Norway rats are mostly albino. Various breeding efforts resulted in different rat sub types such as, Wistar, Sprague-Dawley, Evans, Fisher and many others. These rat subtypes show a variety of body metabolic and growing characterstics (Anderson et al., 2006). The small body size, high blood volume, ease to handle, resistant to stress and low maintainence cost make rat the most suitable animal model for metabolic disease studies (Hubner et al., 2005; Dillman, 2008).

Several inbred rat strains have been developed for particular diseases like spontaneously hypertensive rats for hypertension studies, salt resistant and salt sensitive strains, BB rats and Long/Evans rats for type 2 diabetes studies (Aitman et al., 1997; Rapp, 1982; Tirabassi et al., 2004). Rodent models do not tend to develop atherosclerosis easily, however, these animals because of their short life cycle are very suitable to study early development stages of this disease (Lind, 2009).

# CHAPTER 4.0 ANTIOXIDANT ABILITY OF FRACTIONATED APPLE PEEL PHENOLICS TO INHIBIT FISH OIL OXIDATION

## 4.1 ABSTRACT

Polyphenols isolated from frozen and dried apple peels were studied as potential natural antioxidants to stabilize omega-3 polyunsaturated fatty acid (ω3 PUFA) enriched fish oil. The ethanolic extracts of apple peels were fractionated by reverse phase chromatography using gradient elutions of 20-100% aqueous ethanol. The collected fractions were analysed by ultra pressure liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS). The total phenolic content and antioxidant capacity of each fraction were evaluated by Folin-Ciocalteu (FC), ferric reducing antioxidant power (FRAP) and 1, 1-diphenyl-2-picrylhydrazyl radical ($DPPH^{\cdot}$) scavenging assays. Inhibition of fish oil oxidation was studied using the thiobarbituric acid reactive substances (TBARS) assay. Polyphenols fractionated from the frozen peel extract and corresponding fractions had significantly higher FC, FRAP and $DPPH^{\cdot}$ scavenging values than from the dried apple peel ($p < 0.05$). The fractions abundant in flavonols, inhibited fish oil oxidation by 40 to 62% at a total phenolic concentration of 200 µg/mL. The fractionated polyphenols from both the dried and frozen apple peel showed a higher inhibition of lipid peroxidation compared to α-tocopherol, butylated hydroxytoluene and crude apple peel extracts.

**Keywords:** lipid peroxidation, polyphenols, fractionation, fish oil, antioxidant capacity, apple peel.

## 4.2 INTRODUCTION

Omega-3 polyunsaturated fatty acids (ω3 PUFA) have gained popularity due to their various heath promoting and disease preventing attributes. For example, ω 3 PUFA are reported to be highly effective against cardiovascular disease (Hu et al., 2002), cancer (Wolfe and Liu, 2003) and other metabolic diseases (Kolanowski, 2008). Fish oil, a major source of ω 3 PUFA, is composed of mainly all cis- 4, 7, 10, 13, 16, 19-docosahexanenoic acid (DHA) and all cis- 5, 8, 11, 14, 17-eicosapentaenoic acid (EPA), which are important for the growth and development of mammals (Singh et al., 2007).

However, the presence of unsaturated carbon chain makes ω3 PUFA vulnerable to lipid oxidation. The auoxidation of ω3 PUFA can occur due to free radicals generated by light, heat, metal ions and enzymes (Zuta et al., 2007). The products of oxidation, such as malondialdehyde and other aldehydes, alcohols as well as some ketones can deteriorate the flavour of food products containing ω3 PUFA (Rehman and Salariya, 2006). Moreover, the cytotoxic and genotoxic effects of fatty acid oxidation products have been reported (Fang et al., 1996). Therefore, synthetic and naturally sourced antioxidants are used to control lipid oxidation. However, some of the commercial antioxidants, such as butylated hydroxytoluene (BHT), have potential to promote DNA damage by binding to nucleic acids and, therefore, exert mutagenic, cancerous and cytotoxic effects (Saito et al., 2003; Dolatabadi and Kashanian, 2010).

Due to the above reasons and increased consumer health concerns, several plant-based antioxidants have attracted attention from the food industry (Kathirvel and Rupasinghe, 2011). In this regard, plant extracts such as rosemary (Wang et al., 2011), potato peel (Habeebullah et al., 2010), apple peel (Huber and Rupasinghe, 2009;

Rupasinghe et al., 2010), green tea (Wanasundara and Shahidi, 1998) and its omega-3 conjugates (Zhong and Shahidi, 2011; Zhong et al., 2012), oregano (Tsimidou et al., 1995), different oriental herbs (Kim et al., 1994) and seaweed extract (Kindleysides et al., 2012) have been reported for inhibiting lipid oxidation. In addition to the inhibition of lipid oxidation, apple peel phytochemicals have been reported to possess cardioprotective and anticancer properties (Wolfe et al., 2003; Knekt et al., 2002). These heath protective attributes of apple peel extract are associated with the presence of flavonols, anthocyanins, flavon-3-ols, phenolic acids and dihydrochalcones (Boyer and Liu, 2004). About 2-3 million kilograms of apple peel are wasted in apple processing plants every year in Nova Scotia (Huber and Rupasinghe, 2009). Previous studies have reported that about 80% of polyphenolics are concentrated in apple peel (Leccese et al., 2009). Moreover, the apple peel has five- to six-fold higher total antioxidant capacity than apple flesh (Vieira et al., 2011) and higher total flavonols (six- to seven-fold) than apple pomace (Rupasinghe and Kean, 2008).

The present study aimed to assess the antioxidant effectiveness of different phenolic fractions prepared from apple peel using heat-induced fish oil oxidation. The specific objectives were: (1) to compare the recovery and yield of phenolic compounds extracted and fractionated from frozen and dried apple peel; (2) to examine antioxidant capacity of apple peel extracts and fractions using $DPPH^{\cdot}$ scavenging ability and ferric reducing antioxidant power (FRAP) assays, and (3) to evaluate the ability of fractionated apple peel phenolics on preventing fish oil oxidation as compared to commercial antioxidants.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Chemicals and Reagents

Fish oil, composed of 22.9% monounsaturated fatty acids and 40.5% polyunsaturated fatty acids (containing relative proportion of 18.7% EPA and 76.6% DHA) was obtained from Ocean Nutrition Canada, Dartmouth, NS, Canada. 2-Thiobarbituric acid (TBA), trichloroacetic acid (TCA), 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 4,6-tripyridyl-S-triazine (TPTZ), ferric chloride ($FeCl_3$), sodium carbonate ($Na_2CO_3$), Folin-Ciocalteu (FC) reagent, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), chlorogenic acid, BHT, α-tocopherol were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2, 2′-Azobis (2-amidinopropane) dihydrochloride (AAPH) was obtained from Wako Chemicals (Richmond, VA, USA).

### 4.3.2 Materials

Apple peels (cv. Northern Spy) were collected from a commercial apple pie processing plant (Apple Valley Food Inc., Kentville, NS, Canada). The fresh apple peels were immediately blanched with 2% $CaCl_2$ in water at 55$^{o}$C±5 for 10 min to prevent oxidation of phenolic compounds. The excess water was drained and apple peels were transported in plastic containers to Nova Scotia Agricultural College. The required amounts of peels were dried at 60$^{o}$C±2 for 48 h using a Milner convection oven (Agincourt, ON, Canada) and ground using 1 mm sieve size of Willey mill (Model Laboratory Heavy Duty, Arthur Thomas Co., Philadelphia, PA, USA). For cold storage condition, the apple peels were frozen at -80$^{o}$C in plastic zip-lock bags.

### 4.3.3 Extraction of Apple Peel

For the first treatment, 100 g of dried apple peel powder were sonicated in 1L of absolute ethanol, 3 times for 15 min with 10 min intervals in between sonications (in triplicates). For the second treatment, 500 g of frozen apple peels were extracted in 2 L of absolute ethanol for 15 min with 10 min intervals three times. After the extraction, extracts were filtered separately using Whatman No. 1 filter papers under vacuum. The extracts were concentrated to 150-200 mL using a rotary evaporator (Rotavapor, R-200, Buchi, Flawil, Switzerland) at 37$^{o}$C and filtered using Whatman No. 1 filter papers under vacuum. The water to ethanol ratio was adjusted to 1:1 with load volume of 300-400 mL for the chromatographic column. Both procedures were conducted in triplicates.

### 4.3.4 Fractionation of Apple Peel Polyphenolics

The polyphenolic extract of apple peel (300 mL) was loaded on 400 g adsorbent resin (Sorbent SP-207-05 sepabead resin brominated, 250 µm, Sorbent Tech., Norcross, GA, USA) packed into a chromatography column (46 × 2.3 cm, length and internal diameter). The column was equilibrated with deionized water and maintained in 25% ethanol in water (Rupasinghe et al., 2010). The concentrated extract (as described in section 4.3.3: in 50% ethanol, 300-400 mL) was loaded onto the column. The column was washed with three bed volumes of water (425 mL each) to remove all water-soluble compounds, including sugars, until the Brix value of washed eluent reached < 0.01. The water wash eluent was collected for further analysis. The phenolic compounds were eluted with 20, 25, 30, 35, 40, 45, 50, 60, 70, 80 and 100% ethanol (425 mL each) to yield fractions F1, F2, F3, F4, F5, F6, F7, F8, F9 and F10, respectively. The column was

protected from light during the entire operation. All the fractions were stored at 4$^{o}$C for further analysis.

### 4.3.5 UPLC-MS/MS Analysis of Phenolic Fractions

Analyses of major individual phenolic compounds present in apple peel extract were performed according to the method reported by Rupasinghe et al. (2010). All analyses were conducted using an ultra pressure liquid chromatography (Waters, Milford, MA, USA) coupled with Micromass Quattro micro API MS/MS system and controlled with Mass lynx V4.0 data analysis system (Micromass, Cary, NC, USA). The column used was Aquity BEH $C_{18}$ (100 mm × 2.1 mm , 1.7 μm) (Waters, Milford, MA, USA). For the separation of the flavonol, flavan-3-ol, phenolic acid and dihydrochalcone, the mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). A linear gradient profile was used with the following proportions of Solvent A applied at time t (min); (t, A%): (0, 94%), (2, 83.5%), (2.61, 83%), (2.17, 82.5%), (3.63, 82.5%), (4.08, 81.5%), (4.76, 80%), (6.75, 20%), ( 8.75, 94%), (12, 94%). The analysis of cyanidin-3-*O*-galactoside was performed using the mobile phases of 5% formic acid in water (solvent A) and 5% formic acid in methanol (solvent B). The linear gradient profiles used were as follows: (t, A%): (4.76, 0%), (6, 70%), (6.57, 0%), (5.25, 0%), (12, 0%), (14, 48%), (17, 36%), (19, 10%), (22, 90%).

Electrospray ionization in negative ion mode (ESI-) was used for the analysis of the flavonol, flavan-3-ol, phenolic acid and dihydrochalcone. The following conditions were used: capillary voltage 3000V, nebulizer gas ($N_2$) temperature 375$^{o}$C at a flow rate of 0.35 mL/min. For the analysis of cyanidin-3-*O*-galactoside, electrospray ionization in

positive ion mode (ESI+) was used. The settings for positive ion experiments were as follows: capillary voltage (25-50V) was optimized for each individual compound. Multiple reaction-monitoring (MRM) mode using specific precursor/product ion transitions was employed for quantification in comparison with standards: m/z 301→105 for quercetin (Q), m/z 609→301 for Q-3-*O*-rutinoside, m/z 463→301 for Q-3-*O*-glucoside and Q-3-*O*-galactoside, m/z 448→301 for Q-3-*O*-rhamnoside, m/z 595→301 for Q-3-*O*-peltoside, m/z 273→167 for phloritin, m/z 435→273 for phloridzin, m/z 353→191 for chlorogenic acid, m/z 179→135 for caffeic acid, m/z 193→134 for ferulic acid and isoferulic acid, m/z 449→287 for cyanidin-3-*O*-galactoside, m/z 289→109 for catechin, m/z 290→109 for epicatechin, and m/z 305→125 for epigallocatechin. In MRM experiments, both quadrupoles were operated at unit resolution.

### 4.3.6 The Bulk Fish Oil Model System

The bulk fish oil model system was set according to a method described by Rupasinghe and Yasmine (2010) with slight modifications. For bulk oil model system, 200 μg/mL of total phenolics quantified by UPLC-MS/MS of the fractions, crude apple peel extract, BHT and α-tocopherol and four concentrations of following compounds: quercetin-3-*O*-glucoside, chlorogenic acid, epicatechin, cyanidin-3-*O*-galactoside and phloridzin in 95% ethanol (0.5 mM, 1 mM, 5 mM, 10 mM) were taken in 13 × 100 mm borosilicate glass tubes and evaporated to complete dryness under a stream of nitrogen. Then the dried compounds were dissolved in 20 μL of ethanol and mixed with 80 μL fish oil and the mixture was vortexed. The oxidation of fish oil containing antioxidants along with controls was induced by exposing to heat (50$^{o}$C) for 3 h using shaker oven at 150

rpm (Model Apollo HP50, CLP Tools, San Diego, CA, USA). Each treatment was conducted in triplicates and the whole experiment was repeated twice.

### 4.3.7 Total Phenolic Content by Folin-Ciocalteu (FC) Reagent Assay

Total phenolics were measured by FC reagent using the method described by Singleton et al. (1998). Chlorogenic acid was used as phenolic standard to calculate phenolic compounds present in the samples. Folin-Ciocalteu reagent (0.2 N) and 7.5% (w/v) sodium carbonate was prepared. At the first step, 20 µL of samples were added into each of a 96-well microplate, followed by 100 µL of FC reagent. After 5 min, 80 µL of $Na_2CO_3$ were added and the microplate was placed in the dark at room temperature for 2 h. The absorbance of resultant colour was read at 760 nm using a FLUOstar OPTIMA plate reader (BMG Labtech., Durham, NC, USA) and values were expressed as mg chlorogenic acid equivalents (CAE) per litre.

### 4.3.8 Thiobarbituric Acid Reactive Substances (TBARS) Assay

The products of oxidation of fish oil were quantified using TBARS assay as described by Rupasinghe et al. (2010). Briefly, the TBA reagent (1 mL), containing mixture of 15% (w/v) TCA and 0.375% (w/v) TBA in 0.25 M HCl, was added to the tubes and vortexed. The reaction mixture was placed at 80$^{o}$C for 45 min in a water bath. The pink coloured chromogen was extracted with 2 mL of 1-butanol. The reaction mixture was vortexed and centrifuge at 2000 g for 10 min. The fluorescence intensity of the chromogen was measured at an excitation/emission wavelength of 530/550 nm using 96-well microplate (COSTAR 9017) on the FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC, USA). The percentage inhibition of oxidation was calculated using the following equation:

% inhibition of oxidation= [1 - (sample absorbance/control absorbance)] × 100

### 4.3.9 Ferric Reducing Antioxidant Power (FRAP) Assay

Ferric reducing ability of apple polyphenolics was determined by method described by Benzie and Strain (1996) with some modifications. The reaction reagent was made freshly by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution, and 20 mM $FeCl_3$ solution in the ratio of 10:1:1. The TPTZ solution was prepared on the same day of analysis. The Trolox standard solution (1 mM) was prepared in ethanol. Samples (20 µL) were added to each of the 96-well microplate (COSTAR 9017), and then incubated at 37$^{o}$C for 15 min. The working reagent (180 µL) was added to each sample well by the plate reader programmed pump (BMG Labtech, Durham, NC, USA) using BMG Labtech software. The absorbance was read at 595 nm using FLUOstar OPTIMA plate reader. FRAP values were expressed as mg Trolox equivalents (TE) per litre.

### 4.3.10 DPPH$^{\cdot}$ Scavenging Activity

DPPH$^{\cdot}$ scavenging activity was determined by using the method of Shimada et al. (1992) with some modifications. DPPH solution was prepared in 95% ethanol, 100 µL of this solution were mixed with 100 µL of sample in 96-well microplate. Various concentrations (20, 40, 60, 80 and 100 µL) of the crude extract and fractions were used. The mixture was incubated for 30 min at room temperature. The color absorbance from the reaction was read at 517 nm in an Elx 800 universal microplate reader (Bio-Tek Instruments,Winooski, VT, USA). The DPPH$^{\cdot}$ scavenging activity was expressed as IC50 value (the concentration of the antioxidant required to scavenge 50% of DPPH present in the test solution).

### 4.3.11 Statistical Analysis

All measurements were taken in triplicates and expressed as mean±standard deviation. The extraction and fractionation procedures for both dried and frozen peel experiments were conducted independently in triplicates. The significant difference between dried and frozen apple peel TBARS, FRAP and FC-total phenolic values were tested using one-way analysis of variance (ANOVA) and the multiple mean comparison was performed in General Linear Model (SAS V8, Cary, NC, USA) using Tukey's test. The FC-total phenolic and FRAP values were log transformed for ANOVA and multiple means comparison. To evaluate the relationship between the antioxidant activity and polyphenolic contents, the Pearson correlation coefficient analyses were performed using MINITAB 15 (State College, PA, USA). The differences at the 5% level ($p < 0.05$) were considered statistically significant.

## 4.4 RESULTS

### 4.4.1 Distribution of Phenolic Compounds

The UPLC-MS/MS data revealed that, based on dry matter (DM), frozen peel extract had nearly three-fold higher total phenolics than dried peel powder (Table 4.11). Originally, the total phenolics concentration was calculated to be 238.4 mg/500 g frozen peel weight, for easier comparison, it was converted to 357.6 mg total phenolics/100 g DM (frozen peel contained 13.3% dry matter). Similar conversions were applied to the dried peel phenolic values which contained 95% dry matter. Among the fractions, the

highest phenolic concentrations were detected in F6, F7 and F8 (72.4, 159.8, 148.8 mg total phenolics/L, respectively) in frozen peel fractions (Table 1), whereas F8 contained the highest (71.7 mg total phenolics/L) in dried peel (Table 4.2). Among all the phenolic compounds, flavonols were present in highest concentration. The frozen and dried peel extracts had 45 (114.7 mg/L) and 54% (58.8 mg/L) flavonols, respectively. Fractions F7 and F8 were, particularly rich in quercetin-3-*O*-glycosides, having 114.7 and 91.6 mg flavonols/L in frozen peels (Table. 4.1). However, only 36.0 mg and 48.7 mg/L flavonol concentrations were detected in fractions F7 and F8, respectively, in the dried peels (Table 4.2). Therefore, the flavonol contents were two to three-fold higher in the fractions derived from the frozen peel than in their dried counterparts.

**Table 4.1. Concentration of major polyphenolics (mg/L) recovered after fractionation of crude extract from frozen apple peels using UPLC- MS/MS*.**

| Fraction | Ethanol (%) | Volume (mL) | Q-3-G | Phloridzin | Cy-3-gal | Chl. Acid | EC | Total (mg/L) | Total (mg) |
|---|---|---|---|---|---|---|---|---|---|
| **Crude** | 100 | 2075 | 79.5 ±5.3 | 30.8 ±2.9 | 23.5 ±4.1 | 12.1 ±0.5 | 26.4 ±0.4 | 172.9 | 357.6 |
| **Wash** | 0 | | - | - | - | 0.2 ±0.1 | - | 0.2 | 0.1 |
| **F1** | 20 | 425 | - | - | - | 0.2 ±0.1 | - | 0.2 | 0.1 |
| **F2** | 25 | 425 | - | - | - | 4.4 ±1.1 | - | 4.4 | 1.8 |
| **F3** | 35 | 425 | - | - | - | 15.4 ±2.1 | 13.2 ±1.4 | 28.6 | 12.1 |
| **F4** | 40 | 425 | - | - | - | 8.7 ±1.4 | 45.2 ±3.3 | 53.9 | 24.1 |
| **F5** | 45 | 425 | 6.9 ±2.6 | 0.2 ±0.1 | 5.2 ±0.2 | 1.5 ±0.5 | 13.3 ±0.9 | 27.1 | 11.5 |
| **F6** | 50 | 425 | 47.8 ±16.6 | 14.6 ±4.9 | 6.9 ±0.6 | 0.4 ±0.1 | 2.7 ±0.3 | 72.4 | 31.7 |
| **F7** | 60 | 425 | 114.7 ±10.7 | 40.1 ±1.9 | 5.0 ±0.7 | - | | 159.8 | 68.1 |
| **F8** | 70 | 425 | 91.6 ±15.2 | 41.9 ±4.2 | - | - | - | 148.8 | 63.2 |
| **F9** | 80 | 425 | 7.5 ±3.2 | 3.9 ±2.3 | - | - | - | 11.5 | 4.9 |
| **Total Yield (mg/500g FW)** | | | 76.1 | 28.6 | 4.8 | 8.7 | 26.7 | | 144.9 |
| **Total Yield (mg/100g DM)** | | | 114.1 | 42.9 | 7.3 | 13.1 | 40.0 | | 217.3 |
| **% Recovery (DM)** | | | 69.7 ±1.1 | 64.0 ±3.4 | 12.6 ±3.9 | 51.8 ±7.3 | 72.5 ±3.5 | | 60.7 ±1.66 |

*Data are presented as mean ±SD. Q-3-G, Quercetin-3-*O*-glycosides; Cy-3-gal, Cyanidin-3-*O*-galactoside; Chl. Acid, Chlorogenic acid; EC, Epicatechin.

**Table 4.2. Concentration of major polyphenolics (mg/L) recovered after fractionation of crude extract from dried apple peels using UPLC- MS/MS*.**

| Fraction | Ethanol (%) | Volume (mL) | Q-3-G | Phloridzin | Cy-3-gal | Chl. Acid | EC | Total (mg/L) | Total (mg) |
|---|---|---|---|---|---|---|---|---|---|
| Crude | 100 | 960 | 58.8 ±4.7 | 28.1 ±0.5 | 4.9 ±0.7 | 6.4 ±0.2 | 10.4 ±1.8 | 108.5 | 104.2 |
| Wash | 0 | 425 | - | - | 6.8 ±0.3 | 0.5 ±0.9 | - | 7.3 | 3.1 |
| F1 | 20 | 425 | - | - | - | 0.4 ±0.4 | - | 0.4 | 0.2 |
| `F2 | 25 | 425 | - | - | - | 1.0 ±0.1 | - | 1.0 | 0.4 |
| F3 | 30 | 425 | - | - | - | 4.6 ±0.2 | - | 4.6 | 2.0 |
| F4 | 35 | 425 | - | - | - | 0.8 ±0.1 | - | 0.8 | 0.3 |
| F5 | 40 | 425 | - | - | - | - | 4.3 ±1.2 | 4.3 | 1.8 |
| F6 | 45 | 425 | 3.3 ±0.3 | 1.4 ±0.1 | - | - | 1.0 ±0.1 | 5.7 | 2.4 |
| F7 | 50 | 425 | 36.0 ±0.1 | 7.8 ±0.7 | - | - | - | 45.3 | 19.3 |
| F8 | 60 | 425 | 48.7 ±0.8 | 23.1 ±1.5 | - | - | - | 71.7 | 29.8 |
| F9 | 70 | 425 | 17.5 ±1.0 | 14.4 ±1.4 | - | - | - | 32.0 | 13.6 |
| Total Yield (mg/100g DW) | | | 47.0 | 20.8 | 3.0 | 3.2 | 2.4 | - | 76.5 |
| Total Yield (mg/100g DM) | | | 44.8 | 19.8 | 2.9 | 3.1 | 2.3 | - | 72.8 |
| % Recovery (DM) | | | 76.1 ±2.2 | 73.6 ±4.6 | 61.8 ±0.3 | 50.7 ±6.4 | 22.5 ±7.6 | - | 69.8 ±4.0 |

*Data are presented as mean ±SD. Q-3-G, Quercetin-3-*O*-glycosides; Cy-3-gal, Cyanidin-3-*O*-galactoside; Chl. Acid, Chlorogenic acid; EC, Epicatechin.

The percent recovery of different phenolic compounds, namely flavonols, phloridzin, cyanidin-3-*O*-galactoside, chlorogenic acid and epicatechin was calculated for both extraction sources on a DM basis. The percent recovery of flavonols was higher in dried apple peels (76%) than frozen peels (70%). In addition, a similar trend was observed for phloridzin in dried and frozen peels (74 and 64%, respectively). Cyanidin-3-*O*-galactoside was detected only in the frozen peel fractions, whereas it was eluted only in the water wash step in the dried peels. However, recoveries of chlorogenic acid (52%) and epicatechin (73%) were higher in the frozen peel fractions than dried peel fractions. Overall, total phenolics recovery was 70% for dried peels and 61% for frozen peels (Tables 4.1, 4.2).

Overall, the yield of phenolic compounds on DM basis was higher in the frozen peels, compared with oven-dried peels. When frozen peels were used, the yield of flavonol and phloridzin was two-fold, chlorogenic acid four-fold and epicatechin seventeen-fold higher than the dried peels. The yield of total phenolics was approximately three-times higher in the frozen apple peels than dried apple peels (Tables 4.1, 4.2)

### 4.4.2 Total Phenolics and Antioxidant Capacity of Apple Peel Extract and Fractions

Total phenolic content, as chlorogenic acid equivalents, was determined using FC assay (Table 4.3). Both crude extracts prepared from dried and frozen peels showed high phenolic content at 830.9 and 3370.1 mg CAE/L, respectively. The frozen peel extract contained almost four-times higher FC-total phenolics compared to the dried peels ($p < 0.05$). Regardless of the starting material, fraction F8 had the highest concentration of FC-total phenolics in both dried (55.10 mg CAE/L) and frozen (1727.4 mg CAE/L) peels

(Table 4.3). Similarly, FRAP values of frozen peel extract was about five-times higher than dried peel extract on the basis of mg TE/L Meanwhile, fractions F8 exhibited the greatest reducing capacity in both frozen peels (553.4 mg TE/L) and dried peels (1567.5 mg TE/L) ($p < 0.05$). The fraction F8 of frozen peels exhibited about three-fold higher FRAP value than dried peels (Table 4.3).

The phenolic compounds present in dried and frozen apple peel crude extracts (IC50 = 1.65 µg/mL and IC50 = 2.52 µg/mL, respectively) showed strong $DPPH^{\cdot}$ scavenging activity (Table 4.3). The IC50 values of dried peel fractions F7 and F8 were comparable to the crude extract of dried peel ($p < 0.05$). In case of frozen peel fractions, the greatest scavenging activity was observed in F6 (IC50 = 2.33 µg/mL).

**Table 4.3. Total phenolic content (FC) and antioxidant capacity (FRAP and DPPH) of crude extracts and corresponding fractions prepared from dried (DP), frozen apple peel (FP) and corresponding fractions*.**

| Fraction | Total Phenolic Content (mg CAE/L)[x] | | FRAP (mg TE/L)[y] | | DPPH (IC50 µg/mL)[z] | |
|---|---|---|---|---|---|---|
| | **DP** | **FP** | **DP** | **FP** | **DP** | **FP** |
| Crude | 830.9 ±23.4$^{c}$ | 3370.1 ±232.0$^{a}$ | 802.0 ±33.3$^{c}$ | 3976.4 ±66.6$^{a}$ | 1.7 ±0.1$^{hi}$ | 2.5 ±0.1$^{ghi}$ |
| F3 | 11.5 ±0.8$^{h}$ | 42.0 ±1.6$^{g}$ | 11.7 ±0.1$^{k}$ | 371.3 ±9.9$^{ef}$ | 8.5 ±0.9$^{d}$ | 18.9 ±0.1$^{a}$ |
| F4 | 8.3 ±0.3$^{h}$ | 56.4 ±10.1$^{efg}$ | 14.6 ±0.1$^{jk}$ | 391.4 ±10.1$^{e}$ | - | 13.3 ±0.3$^{c}$ |
| F5 | 12.2 ±3.6$^{h}$ | 50.0 ±1.5$^{efg}$ | 13.3 ±0.6$^{j}$ | 321.4 ±17.5$^{fg}$ | 4.2 ±0.1$^{ef}$ | 17.9 ±0.1$^{b}$ |
| F6 | 1.7 ±0.3$^{i}$ | 89.2 ±7.1$^{d}$ | 4.6 ±0.4$^{l}$ | 279.8 ±0.0$^{g}$ | 3.4 ±0.2$^{fg}$ | 2.3 ±0.1$^{hi}$ |
| F7 | 10.6 ±0.1$^{h}$ | 71.5 ±9.3$^{de}$ | 69.9 ±0.9$^{h}$ | 339.0 ±1.8$^{efg}$ | 2.6 ±0.1$^{gh}$ | 4.2 ±0.2$^{ef}$ |
| F8 | 55.1 ±3.9$^{efg}$ | 1727.4 ±96.7$^{b}$ | 553.4 ±9.3$^{d}$ | 1567.5 ±5.2$^{b}$ | 2.2 ±0.2$^{hi}$ | 4.5 ±0.1$^{e}$ |
| F9 | 44.1 ±1.7$^{gh}$ | 62.2 ±0.4$^{fg}$ | 34.8 ±0.6$^{i}$ | 12.1 ±2.6$^{k}$ | - | - |

*Data are presented as mean ±SD. [a–l]Different superscript letters between fractions denote significant differences (Tukey's test, $p < 0.05$). [x]CAE, Chlorogenic Acid Equivalents; [y]TE, Trolox Equivalents;[z] IC50, 50% inhibitory concentration; FRAP, Ferric Reducing Antioxidant Power; DPPH, 1, 1- diphenyl-β-picrylhydrazyl radical scavenging activity; DP, Dried peel; FP, Fresh peel.

### 4.4.3 Inhibition of Fish Oil Oxidation by Apple Polyphenolics

Apple phenolics were incorporated at 200 µg/mL phenolic concentration into bulk fish oil. TBARS values revealed that dried apple peel polyphenolics in fractions F5, F6 and F7 inhibited fish oil oxidation by 58, 62 and 39%, respectively ($p < 0.05$). Crude extract of dried apple peels prevented fish oil oxidation better than α-tocopherol and BHT (Fig. 4.1). However, fractions F3 (31%) and F7 (37%) of forzen peel extracts also effectively controlled fish oil oxidation and the percent inhibition by fraction F8 was comparable to that of BHT. Crude extract of frozen apple peel was as effective as BHT and was significantly better than α-tocopherol ($p < 0.05$) (Fig. 4.1).

The TBARS values of the major phenolic compounds present in apple peel were also compared individually at different concentrations (0.5, 1.0, 5.0 and 10 mM) (Fig.4.2). In general, the percentage inhibition of fish oil oxidation was concentration dependent for all tested compounds. Among the phenolic compounds, cyanidin-3-*O*-galactoside (82%), chlorogenic acid (76%) and phloridzin (73%) rendered the highest inhibition of fish oil oxidation at a 10 mM concentration ($p < 0.05$). Furthermore, the TBARS value of epicatechin treated sample (58%) was significantly higher than quercetin-3-*O*-galactoside (46%) at 10 mM concentration ($p < 0.05$).

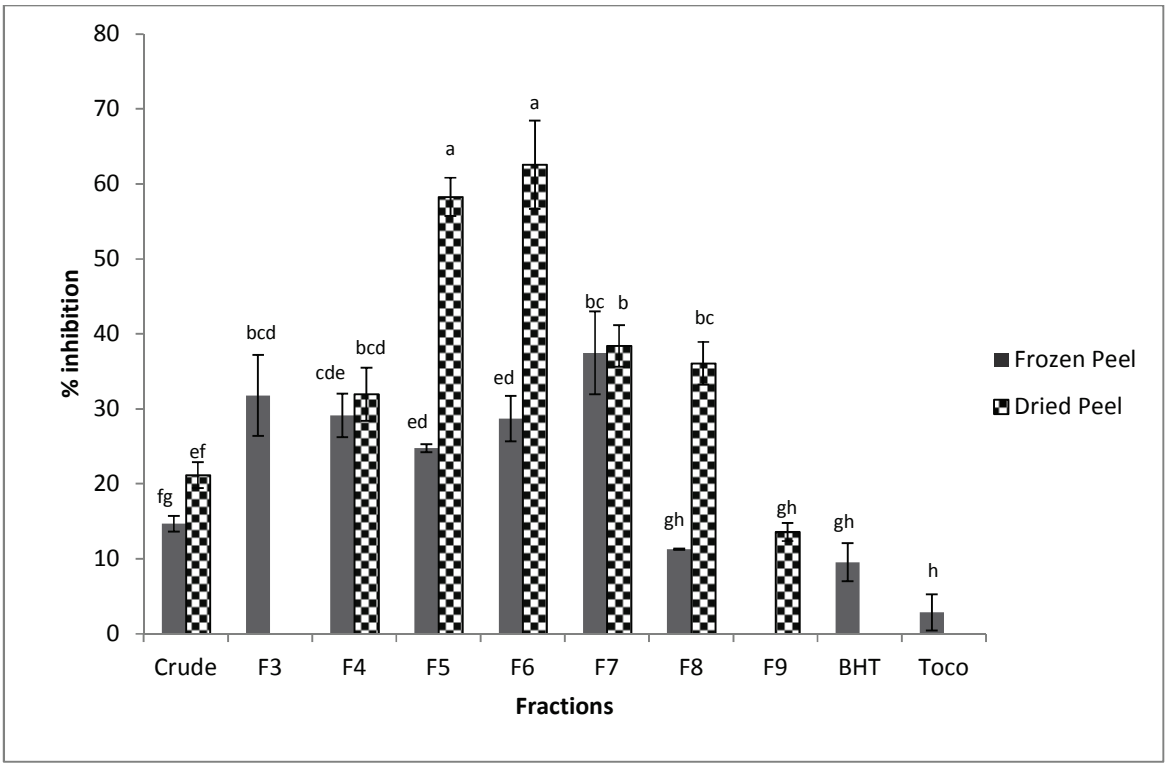


**Figure 4.1. Percent inhibition of fish oil oxidation by polyphenolics in dried and frozen apple peel fractions, BHT and α-tocopherol at concentration of 200 µg total phenolics/mL in fish oil using TBARS assay. Error bars represent standard deviation (n=3). $^{a–h}$Different superscript letters between fractions denote significant differences (Tukey's test, $p < 0.05$).**

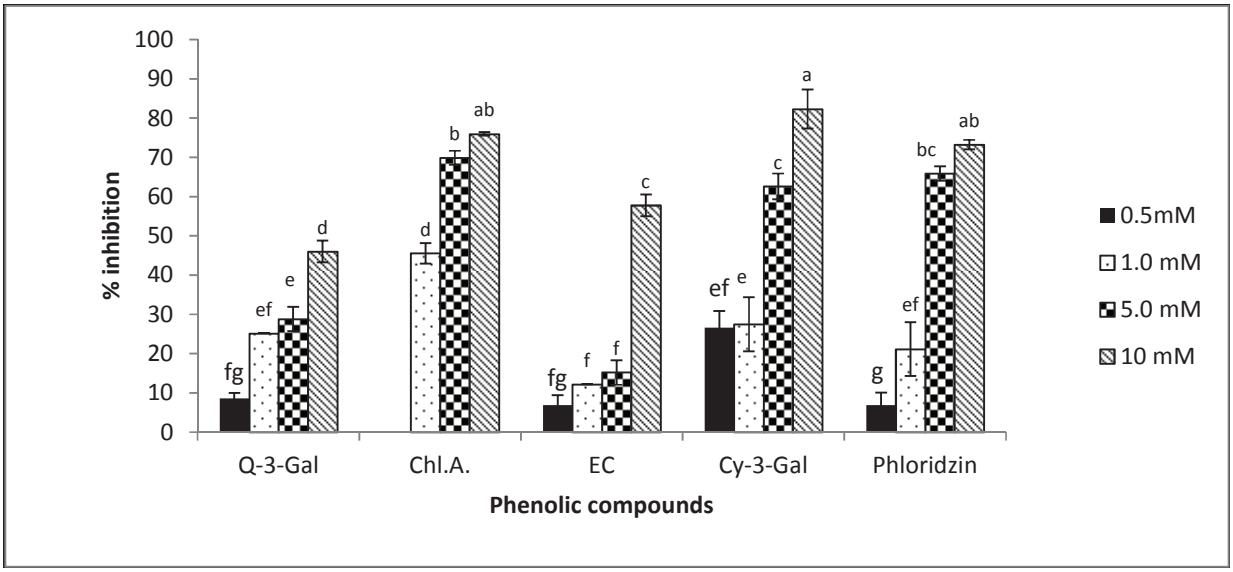


**Figure 4.2. Concentration dependent inhibition of fish oil oxidation by major phenolic compounds found in apple peel using TBARS assay. Error bars represent standard deviation (n=3). [a–g] Different superscript letters denote significant differences (Tukey's test, $p < 0.05$). Q-3-Gal, Quercetin-3-*O*-galactoside; Chl. Acid, Chlorogenic acid; EC, Epicatechin; Cy-3-Gal, Cyanidin-3-*O*-galactoside.**

### 4.4.4 Correlation Analysis of Total Phenolic Content and Antioxidant Activity

Pearson correlation was applied to FC-total phenolic content, UPLC-MS/MS phenolic content, FRAP and $DPPH^{\cdot}$ scavenging activity. Pearson correlation coefficient showed a significant linear relationship between FC-total phenolics and FRAP values in the dried ($r = 0.719$, $p = 0.045$) and frozen apple peels ($r = 0.985$, $p = 0.000$). The total phenolic content measured by UPLC-MS/MS also correlated with the FRAP values($r = 0.938$, $p = 0.001$; $r = 0.691$, $p = 0.058$) in both dried and frozen peel fractions, respectively. A similar correlation also existed between $DPPH^{\cdot}$ scavenging activity and total phenolics measured by UPLC-MS/MS ($r = -0.832$, $p = 0.020$) values in frozen peel fractions. The $DPPH^{\cdot}$ scavenging activity values (IC50) were significantly correlated with flavonols ($r = -0.980$, $p = 0.020$) and phloridzin ($r = -0.976$, $p = 0.024$) contents in dried peels. The flavonol ($r = 0.888$, $p = 0.044$; $r = 0.947$, $p = 0.001$) and phloridzin ($r = 0.913$, $p = 0.030$; $r = 0.963$, $p = 0.002$) contents were also linearly correlated with FRAP values in the dried and frozen peels, respectively. There was a significant correlation between TBARS values with IC50 values ($r = 0.919$, $p = 0.027$) in dried peel fractions. A correlation also existed between TBARS and FC-total phenolic values ($r = -0.805$, $p = 0.029$) in the frozen peels (Tables 4.4, 4.5).

**Table 4.4. Pearson correlation coefficients to exhibit linear relationship among the antioxidant capacity measures (FRAP, DPPH and TBARS values) and the phenolic compounds analyzed by FC and UPLC-MS/MS in the different fractions of frozen apple peels.**

| | Total phenolics | | Antioxidant Capacity | | |
|---|---|---|---|---|---|
| **Compounds** | **FC** | **UPLC-MS/MS** | **FRAP** | **DPPH** | **TBARS** |
| Total phenolics (FC) | - | 0.687* | 0.985* | -0.520 | -0.805* |
| Total Phenolics (UPLC-MS/MS) | 0.687* | - | 0.691* | -0.832* | -0.395 |
| FRAP | 0.985* | 0.691* | - | -0.474 | -0.726 |
| DPPH | 0.487 | -0.832* | -0.474 | - | 0.306 |
| Total flavonols | 0.900* | 0.745 | 0.947* | -0.502 | -0.407 |
| Phloridzin | 0.927* | 0.751 | 0.963* | -0.504 | -0.467 |
| Chl. Acid | 0.930* | 0.860 | 0.939* | -0.413 | -0.892 |
| Cy-3-gal | 1.000* | 0.612 | 1.000* | -0.384 | -0.829 |
| Epicatechin | 0.470 | 0.201 | 0.952* | -0.281 | -0.942* |

*Significant correlations are shown ($P \leq 0.05$). Chl.Acid, Chlorogenic acid; Cy-3-gal, Cyanidin-3-*O*-galactoside; FRAP, Ferric Reducing Antioxidant Power; DPPH, 1, 1-diphenyl-2-picrylhydrazyl radical scavenging activity; TBARS, Thiobarbituric Acid Reactive Substances; FC, Folin-Ciocalteu.

**Table 4.5. Pearson correlation coefficients to exhibit linear relationship among the antioxidant capacity measures (FRAP, DPPH and TBARS values) and the phenolic compounds analyzed by FC and UPLC-MS/MS in the different fractions of dried apple peels.**

| | Total Phenolics | | Antioxidant Capacity | | |
|---|---|---|---|---|---|
| **Compounds** | **FC** | **UPLC-MS/MS** | **FRAP** | **DPPH** | **TBARS** |
| Total phenolics (FC) | - | 0.659* | 0.719* | -0.044 | -0.436 |
| Total Phenolics (UPLC-MS/MS) | 0.659* | - | 0.938* | -0.680 | -0.542 |
| FRAP | 0.719* | 0.938* | - | -0.592 | -0.406 |
| DPPH | -0.044 | -0.680 | -0.592 | - | 0.919* |
| Total flavonols | 0.669 | 0.967* | 0.888* | -0.980* | -0.481 |
| Phloridzin | 0.716 | 0.943* | 0.913* | -0.976* | -0.689 |
| Chl. Acid | 0.951 | 0.762 | 0.739 | -1.000 | -1.000 |
| Epicatechin | 0.596 | 0.578 | 0.595 | -0.301 | -0.661 |

*Significant correlations are shown ($P \leq 0.05$). Chl. Acid, Chlorogenic acid; FRAP, Ferric Reducing Antioxidant Power; DPPH, 1, 1-diphenyl-2-picrylhydrazyl radical scavenging activity; TBARS, Thiobarbituric Acid Reactive Substances; FC, Folin-Ciocalteu.

## 4.5 DISCUSSION

Apples, especially apple peels, have been considered as one of the significant sources of dietary phenolic compounds. Apple peel and flesh contain epicatechin, phloridzin, procyanidins, caffeic acid and chlorogenic acid, but flavonols (quercetin glycosides) and anthocyanins (cyanidin glycosides) are only present in peel tissues (Wolfe and Liu, 2003; Rupasinghe et al., 2010). Apple is ranked the second highest in terms of its dietary phenolic content among all the fruits. Most of the phenolic compounds in apple are not bound to the cell wall and are, therefore, more available for absorption after intake (Sun et al., 2002). Previous studies have reported that total phenolic concentration in apple peel may vary from 150 to 700 mg/100g DW, depending on the cultivar (Huber and Rupasinghe, 2009). It has also been demonstrated that processing techniques and temperature influence phenolic yield and that oven-drying decreases the total phenolic contents of apple peels (Rupasinghe et al., 2008). Likewise, the flavonols and catechin contents were also negatively affected by oven-drying (Wolfe and Liu, 2003).

Apples with peels (i.e. whole apple) are found to have high antioxidant capacity compared to most other fruits (Sun et al., 2002). Generally, the antioxidant activity of fruits and vegetables increases with the increase in the concentration of total phenolics and total flavonoids (Ghasemi et al., 2009). Similarly, Habeebullah et al. (2010) reported that phenolic rich fractions and crude extracts of potato peels showed high $DPPH^{\cdot}$ scavenging activity. The measurement of $DPPH^{\cdot}$ scavenging activity depicts the intrinsic ability of a compound to donate a hydrogen or an electron to the free radicals and ROS (Paixao et al., 2007). The lower IC50 value indicates higher radical scavenging ability of

the plant extract (Maisuthisakul et al., 2007) and this also explains the negative correlation between total phenolics and radical scavenging activity. This property of phenolics has been explained by a fast electron transfer process (Foti et al., 2004); however, only phenolics with a certain structure and with hydroxyl groups at specific positions show radical scavenging activity (Nguelefack et al., 2011). A linear correlation between phenolic compounds and radical scavenging ability has been reported in previous studies (Tsao et al., 2005; Huber and Rupasinghe, 2009). Even though, no direct correlation was observed between flavonol content and radical scavenging activity, the high radical scavenging activity in fractions F6, F7 and F8 might be due to the abundance of flavonols. Chinnici et al. (2004) reported that quercetin glycosides exhibited the highest $DPPH^{\cdot}$ scavenging activity when compared with many other individual phenolic compounds; however, flavonols made a major contribution to antioxidant capacity in crude apple pulp samples. Similar observations with lack of correlations between flavonols and antioxidant capacity have already been reported in the literature (Anagnostopoulou et al., 2006; Nickavar et al., 2007; Ghasemi et al., 2009).

Tsao et al. (2005) observed that the antioxidant activity of apple polyphenolics followed the following trend: cyanidin-3-*O*-galactoside > procyanidins > quercetin > chlorogenic acid > phloridzin and also reported a strong correlation between FRAP values and flavan-3-ols. Previous literature has reported a direct correlation between FRAP and total phenolic content (Kanatt et al., 2005; Huber and Rupasinghe, 2009). deGraft-Johnson et al. (2007) found that purified apple quercetin glycosides in combination with other polyphenolics exhibited a strong ferric reducing ability compared to apple extract

under *in vitro* conditions, and also suggested the involvement of catechol –OH group of B-ring in ferric reduction.

TBARS values depicted that both crude apple extracts were comparable to the synthetic antioxidant BHT in controlling bulk oil oxidation. Similar findings were reported using potato peel extract (Habeebullah et al., 2010) and crude apple peel extract (Rupasinghe et al., 2010). However, crude extract of frozen and dried apple peel was more effective than α-tocopherol. In earlier studies, quercetin glycosides depicted similar effects on lipid peroxidation (Becker et al., 2007; Huber et al., 2009). In the present study, the $DPPH^{\cdot}$ scavenging values showed a significant correlation with TBARS values in dried apple peels. According to Jacobsen (2010a), scavenging of free lipid radicals, peroxyl and alkoxyl radicals is the most important mechanism for preventing lipid oxidation. Some antioxidants are able to act via more than one antioxidant mechanism like metal chelation and scavenging of reactive oxygen species (Tehrany et al., 2011).

The polar antioxidants express their antioxidant function more effectively in bulk oil systems as explained by polar paradox theory (Frankel et al., 1994; Zhong and Shahidi, 2012). The activity of the antioxidants may affected by the location of the polar antioxidants at the air-oil interface where oxidation is most possible, whereas the non-polar antioxidants will mix in the oil phase creating low concentrations and their antioxidant activity is dimnished (Jacobsen, 2010a).

The effectiveness of an antioxidant also depends on its concentration as high concentration of antioxidants may render a pro-oxidant effect (Farvin and Jacobsen, 2012). The non-polar γ-tocopherol at concentration of 220 μg/g fish oil resulted in pro-oxidant effect (Horn et al., 2009). This may be due to the synergistic effect of endogenous

antioxidants with γ-tocopherol. The efficacy of different antioxidants may be related to the combined effect of their localization and mechanism of action (Jacobsen, 2010b).

The fractions F3 and F7 of frozen peel extract exhibited strong inhibition of fish oil oxidation. The principle components of fraction F3 were chlorogenic acid and epicatechin, whereas fraction F7 contained mainly Q-3-*O*-glycosides and lower amounts of phloridzin and cyanidin-3-*O*-galactoside. However, in the case of dried apple peels, fractions F5 and F6 controlled fish oil oxidation more effectively than the frozen apple peel fractions. The principle component identified in frozen peel fraction F5 was epicatechin along with some possible undetected phenolics. The most active frozen peel fraction, F6 contained mainly Q-3-*O*-glycosides along with smaller amounts of phloridzin and epicatechin. The difference between inhibitory activities of fish oil oxidation in dried and frozen apple peel fractions indicated that epicatechin and its interaction with Q-3-*O*-glycosides, phloridzin at lower concentration can better control the oxidation of fish oil.

In our study, quercetin-3-*O*-galactoside and epicatechin, when tested individually, could not exhibit strong effect as compared to apple peel fractions. Vanzani et al. (2005) found that apple proanthocyanidins exhibited strong inhibition of lipid peroxidation. However, the total phenolic concentrations showed no direct correlation with percent inhibition values and there was no significant correlation with individual phenolic compounds. Although the reason behind this is not clear, this may be due to a synergistic effect of more than one compound, as described by Breinholt et al. (2003). They have suggested that quercetin alone is not responsible for such inhibition of lipid peroxidation and the effects from epicatechin, quercetin and other polyphenolics may contribute as well. The higher antioxidant activity, in terms of controlling lipid oxidation, might

primarily be due to free radical scavenging effect of phenolic compounds present in fractions F5, F6 and F7.

In conclusion, the yield of total phenolics and flavonols on DM basis was higher in the fractionated frozen peel indicating that the drying process can impact some polyphenolics. The fractionated apple peel extract displayed a stronger inhibitory effect on fish oil oxidation than BHT, α-tocopherol and the crude apple peel extract itself. The fractions containing quercetin glycosides and epicatechin in combination with other phenolic compounds, such as phloridzin and cyanidin-3-*O*-galactoside, showed the greatest antioxidant capacity. Further investigations using pure phenoliccompounds are needed to fully reveal the synergistic effect of different combinations of active phenolic compounds in the inhibition of fish oil oxidation.

# CHAPTER 5.0 MODULATION OF LIPID PROFILE AND INFLAMMATORY CYTOKINES BY APPLE FLAVONOLS, ω3 PUFA, THEIR COMBINATION AND ISQUERCITRIN-EICOSAPENTAENOIC ACID ESTER IN HYPERLIDEMIC RATS

## 5.1 ABSTRACT

This study investigated the effects of apple flavonols (AF) on the lipid profiles and lipopolysacchride (LPS)-induced inflammation in rats fed a high fat-diet for 4-weeks. Seventy-two male Wistar rats (6-weeks old) were randomly divided into six groups (n=12) and fed a high-fat diet (HFC); HF + LPS (HFL); HFL + AF; HFL + eicosapentaenoic acid (EPA); HFL + AF + EPA, and HFL + isoquercitrin-ester (QE), respectively. LPS (50 mg/kg body weight) was injected intraperitonially five hours prior to the sacrifice, with the HFC rats being injected with the vehicle. At the end, rats were killed following overnight fasting and blood samples were collected for the analysis of serum lipids and inflammatory cytokines. The results demonstrated that serum triacylglycerol concentrations were significantly lowered by AF, EPA and AF + EPA groups and the high density lipoprotein (HDL)-cholesterol levels were increased in all the four treatment groups compared to HFL or HFC control group. Serum total cholesterol levels were not affected by either of the treatments. Hepatic total cholesterol, but not the triacylglycerol concentration was reduced in all the four treatment groups compared to both HFC and HFL. The serum concentrations of CRP and IL-6 were lower in AF, EPA, AF + EPA and QE treatment groups ($p < 0.05$) than HFL. QE treatment significantly elevated circulating adiponectin and decreased IFN-γ concentrations in hyperlipidemic rats compared to HFL. The results indicate that AF and EPA reduced the inflammatory biomarkers and a synergistic effect was observed when used in combination. The structurally modified flavonol, QE affected the regulation of inflammatory biomarkers and lipid metabolism in a rat model of hyperlipidemia and inflammation.

**Key words**: apple flavonols, cytokines, hyperlipidemia, lipids, rats.

## 5.2 INTRODUCTION

Nowadays, the increasing prevalence of obesity in Western population is a major risk factor behind metabolic disorders such as type 2 diabetes, cardiovascular disease (CVD), hyperlipidemia, hypertension and cancer (Abete et al., 2011). These metabolic disorders are characterized by the presence of subclinical inflammation, further causing the development of insulin resistance and endothelial dysfunction (Raymond et al., 2006; Kuipers et al., 2011). Recent evidences suggest that a chronic high-fat diet could contribute to metabolic endotoxemia and low-grade inflammation (Cani et al., 2007; Amar et al., 2008; Laugerette et al., 2011). At the initial stage of metabolic disorders, circulating concentrations of C-reactive protein (CRP), tumor nuclear factor-α (TNF-α), interleukin-6 (IL-6), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C) and triacylglycerols are altered and this response may cause development of CVD and insulin resistance (Abete et al., 2011; Salas-Salvado et al., 2011).

A healthy life style with nutritional intervention is a key to achieve and maintain normal lipid profile (Rudkowska, 2010). Polyphenolics are micronutrients abundantly distributed in the fruits and vegetables. The evidences have suggested their beneficial effects of polyphenolics on the risk of degenerative diseases like CVD and cancer (Manach et al., 2004). Apples are known as a major source of dietary polyphenolics (Boyer and Liu, 2004). Along with other health promoting effects (Graziani et al., 2005; Tsao, 2010), flavonoids also have anti-inflammatory properties (Lauren et al., 2009; D'Argenio et al., 2012). The major flavonol found in apple peel is quercetin glycosides which represent approximately 70% of all flavonols in the North American diet (Hertog et

al., 1993). Quercetin is well documented for its antioxidant and anti-inflammatory effects (Perez-Vizciano and Duarte, 2010). However, bioavailability of quercetin glycosides has been reported to be low (D'Archivio et al., 2010**)**. Apart from flavonoids, omega-3 polyunsaturated fatty acids (ω3 PUFA) are well recognized as biologically active molecules for cardiovascular protection (Rudkowska, 2010). However, the synergistic effect of dietary intervention of flavonoids and ω3 PUFA is not well documented. Therefore, it was hypothesized that the beneficial properties of quercetin glycosides can be enhanced by structurally modifying them with ω3 PUFA by acylation process. The suggested mechanism of action of ω3 PUFA action includes its anti-arrhythmic effects (Connor, 2000), anti-thrombotic effects (Geleijnse et al., 2002), anti-inflammatory effects (Calder, 2009) and hypotriacylglycerolemic effects (Harris et al., 2008).

In the present study, flavonol-rich concentrate was prepared from apple peels using reverse-phase chromatography. Quercetin-3-*O*-glucoside (isoquercitrin) ester (QE) was synthesized using eicosapentaenoic acid (EPA) as acyl donor. The aim of this study was to evaluate the potential of apple flavonols (AF) and EPA alone and in combination, as well as their ester form QE in modulating serum and hepatic lipid profiles and inflammatory responses in a rat model with diet-induced hyperlipidemia and lipopolysacchride (LPS)-induced inflammation.

## 5.3 MATERIALS AND METHODS

### 5.3.1 Chemicals and Apparatus

All dietary ingredients for animal feed except apple flavonols, EPA and quercetin esters were purchased from Dyets Inc., Bethlehem, PA, USA. EPA rich fish oil was provided by Ocean Nutrition Canada, Dartmouth, NS, Canada. The composition of the oil was 75.7% total polyunsaturates (ω3 and ω6) and 70.6% of polyunsaturates were ω3 PUFA (containing relative proportion of 82% EPA and 14% DHA). Quercetin-3-*O*-glucoside was purchased from Indofine Chemical Company Inc., Hillsborough, NJ, USA. Liver lipid quantification kit was obtained from BioVision, Milpitas, CA, USA and serum lipid quantification kit was supplied by BioPacific Diagnostic Inc., North Vancouver, BC, Canada. Rat cytokine flex kits were purchased from BD Biosciences, Mississauga, ON, Canada and rat ELISA kits (IL-6 and adiponectin) were obtained from Invitogen, Burlington, ON, Canada. The lyophilized powder of lipopolysacchride (*E.coli*) was purchased from Sigma-Aldrich, Oakville, ON, Canada. QE was synthesized using a previously described method by Ziaullah et al. (2012).

### 5.3.2 Animal Model and Experimental Design

The animal study was conducted in the animal facility of the Atlantic Veterinarian College, University of Prince Edward Island (UPEI), Charlottetown, with the approval of the Animal Care and Use Committee at UPEI.

Six-week old male Wistar rats (Charles River, Montreal, QC, Canada), weighing 200 - 250 g at the beginning of the study, were housed individually in cages with a 12-

hour dark: light cycle. During one week adaptation period, the animals were fed the standard laboratory rodent chow with free access to the diet and drinking water. Then, animals were weighed and randomly divided into six groups (n= 12/group). Two groups were used as controls. One was fed a high-fat diet (HFC) to induce hyperlipidemia or HFC diet with LPS-induced inflammation (HFL) by injecting LPS 5 hr prior to sacrifice. The composition of the high-fat diet is given in Table 5.1. The four treatment groups were fed with the HF.

The first treatment group (HFL+AF) was given apple flavonols (AF, 25 mg/kg BW/day) mixed with HFC diet; the second treatment group (HFL+EPA) was fed with HF diet containing EPA rich fish oil (1 g/kg BW/day) The combination of AF (25 mg/kg BW/day) and EPA rich fish oil (1 g/kg BW/day) was given to third treatment group (HFL+AF+EPA); the fourth treatment group (HFL+QE) was fed with HFC diet containing quercetin-3-*O*-glucoside-EPA ester (QE, 25 mg/kg BW/day) The required amounts of treatment compounds were calculated on the basis of the body weight and feed intake of the animals. The diets were prepared weekly and stored in the refrigerator. The fasting weight of the rats was recorded and also at five hours prior to sacrifice, LPS (50 mg/kg BW) was injected intraperitonially to the five groups except HFC.

**Table 5.1. Composition of the high fat diet**

| *Ingredients* | *Composition (%)* |
|---|---|
| **Casein** | 22.90 |
| **Corn starch** | 26.98 |
| **Sucrose** | 12.14 |
| **Fat** [a] | 22.90 |
| **Cellulose** | 6.35 |
| **DL-methionine** | 0.38 |
| **Mineral mix** [b] | 4.43 |
| **Vitamin mix** [c] | 1.26 |
| **Choline bitartrate** | 0.25 |
| **Butylated hydroxytoluene** | 0.023 |
| **Cholesterol** | 1.91 |
| **Cholic acid** | 0.477 |

[a] 96% of lard and 4% of sunflower oil.
[b] Mineral mix contained: 5000 mg Ca, 1561 mg P, 3600 mg K, 1019 mg Na, 1571 mg Cl, 300 mg S, 507 mg Mg, 35 mg Fe, 6 mg Cu, 10 mg Mn, 30 mg Zn, 1 mg Cr, 0.2 mg I, 0.15 mg Se, 1 mg F, 0.5 mg B, 0.15 mg Si, 0.5 mg Ni, 0.1 mg Li and 0.1 mg V per kilogram of the mineral mix.
[c] The composition of vitamin mix was 20 mg thiamine HCl, 15 mg riboflavin, 7 mg pyridoxine HCl, 90 mg niacin, 40 mg calcium pentothenate, 2 mg folic acid, 0.6 mg biotin, 10 mg cyanocobalamin (B12, 0.1%), 4 mg menadione sodium bisulfate, 5000 IU vitamin A palmitate, 50 IU vitamin E acetate, 2400 IU vitamin D3, 100 mg inositol per kilogram of the vitamin mix.

### 5.3.3 Collection and Storage of Blood and Tissue Samples

Food intake and behavior of the animals were recorded daily and animals were weighed weekly in the 4-week dietary intervention period. Five hours prior to sacrifice, all the animals (except HFC group) were given 50 mg/kg BW of LPS by intraperitoneal injection. The animals were anaesthetized by isoflurane inhalation and blood was collected by cardiac puncture in the serum tubes (BD Vacutainers, NJ, USA). Serum was separated by centrifugation at 2,500 rpm for 15 minutes using Allegra 25R (Beckman Coulter, Mississauga, ON, Canada) and stored at -80$^{o}$C. Liver, spleen and adipose tissue were dissected, washed with saline solution, frozen in liquid nitrogen and stored at -80$^{o}$C.

### 5.3.4 Lipid Profile Analysis in Rat Serum

Analysis of serum triacylglycerols, total cholesterol and HDL-C was done using Pointe 180 chemistry analyzer (Pointe Scientific Inc., Canton, MI, USA.), as described by Wang et al. (2010). The triacylglycerols concentration was detected by the enzymatic determination of glycerol using glycerol phosphate oxidase after hydrolysis by lipoprotein lipase. Serum triacylglycerols were hydrolyzed to glycerol and free fatty acids by lipase. The glycerol was converted to glycerol-1-phosphate by glycerol kinase, which was oxidized by glycerol phosphate oxidase to yield hydrogen peroxide. The triacylglycerol reagent (1 mL) containing 4-chlorophenol (3.5 mM), ATP (0.5 mM), 4-aminophenazone (0.3 mM), glycerol kinase (250 U/L), glycerol phosphate oxidase (4500 U/L), peroxidase (2000 U/L), lipase (200,000 U/L) was incubated at 37$^{o}$C for five minutes and 10 µL of serum was added. After incubating for five minutes at 37$^{o}$C, the concentration of red quinonimine dye, produced by hydrogen peroxide and 4-chlorophenol and 4-aminophenazone in the presence of peroxidase, was measured at 500 nm. This procedure measured the total triacylglycerols in serum including the mono- and di-glycerols and the free glycerol fraction.

The total cholesterol was measured using cholesterol esterase and cholesterol oxidase. Cholesterol reagent, containing cholesterol esterase, oxidase, 0.3 mM 4-aminoantipyrine, 15 mM phenol, phosphate buffer pH 6.8, was incubated at 37$^{o}$C for five min. The red color was developed by adding 10 µL serum samples at 37$^{o}$C and absorbance was read at 500 nm.

The HDL-C was separated by precipitating the LDL and very low density lipoprotein (VLDL) after adding dextran sulphate magnesium ions (20 µL) in the serum

(200 µL) at room temperature. After five minute of incubation at room temperature, the HDL-C fraction was separated by centrifuging at 5,600 g. The supernatant (50 µL) was used for HDL-C quantification, following the same steps as total cholesterol analysis.

### 5.3.5 Quantification of Liver Cholesterol and Cholesteryl Ester

The hepatic total cholesterol, free cholesterol and cholesteryl esters were determined using quantification kit supplied by BioVision, Milpitas, CA, USA (Wang et al., 2010). The rat liver tissue was homogenized in 1 mL of chloroform/ isopropanol/ nonidet P-40 (octylphenoxypolyethoxyethanol) (7:11:01, v/v/v) and 200 µL of centrifuged supernatant was dried under nitrogen. The dried samples were dissolved in 200 µL of assay buffer by sonicating and vortexing. The samples were diluted 10-time and 2 µL of samples were added to each wells of a 96-well plate, containing 48 µL of assay buffer. The reaction mixture was prepared for total cholesterol, containing 45.6 µL of cholesterol assay buffer, 0.4 µL of cholesterol probe, 2 µL of enzyme mix and 2 µL cholesterol esterase per well. The cholesterol esterase was omitted from reaction mixture while analysing free cholesterol and 2 µL of assay buffer was added instead of cholesterol esterase. The reaction mixture (50 µL) was added to each well and incubated at 37$^{o}$C for 1 h in dark. The fluorescence was measured at Ex/Em 535/590 using Varioskan Flash plate reader, Thermofisher Scientific, Hudson, NH, USA.

### 5.3.6 Quantification of Liver Triacylglycerols

Triacylglycerols from rat liver tissue were quantified using triacylglycerols quantification kit supplied by BioVision, Milpitas, CA, USA (Wang et al., 2010). The liver tissue (100 mg) was homogenized in 5% Nonidet P-40 in water. The samples were

heated for five minutes in the water bath at 90$^{o}$C and cooled down to room temperature. The heating step was repeated to ensure all triacylglycerols were in solution. The samples were centrifuged at 12,600 g for three minutes. The samples were diluted 20-time with deionized water and standard triacylglycerol was diluted to 0.1 mM. The standards were diluted to generate concentration of 0, 2, 4, 6, 8 and 10 nmol/well. Lipase (2 µL) was added to each well containing sample or standard and the plate was incubated for 20 min at room temperature. The triacylglycerol reaction mixture was prepared containing 47.6 µL of assay buffer, 0.4 µL triacylglycerol probe, and 2 µL of triacylglycerol enzyme mix. After incubation, 50 µL of triacylglycerol reaction mixture was added to each well and reaction was incubated further for 1 h at room temperature in the dark. The fluorescence was measured at Ex/Em 535/590 using Varioskan Flash plate reader, Thermofisher Scientific, Nepean, ON, Canada.

### 5.3.7 Cytokines Analysis using Cytometric Bead Array

Five cytokines, IL-2, IL-4, IL-10, TNF-α and IFN-γ, were quantified in rat serum samples using BD Rat flex sets (BD Biosciences, Mississauga, ON, Canada). The analysis was done as described in BD cytometric bead array instruction manual. The cytokines were determined using FCAP Array software on BD FACSArray Bioanalyzer, Mississauga, ON, Canada. The kit employed beads with discrete fluorescence intensities to detect cytokines at 40-10,000 pg/mL in rat serum. For the assay, 6 µL of capture beads (1 µL/cytokine) were mixed with 44 µL of bead dilutent and transferred into each microplate well. Similarly, 6 µL of phycoerthyrine detection reagent (1 µL/cytokine) were added to 44 µL of dilutent for each well. A set of 10 standards was prepared according to

the instructions in the kit. After preparation, all the samples and standards (50 µL) were transferred to the plate, followed by adding 50 µL of the capture beads per well. The plate was incubated for one hour at room temperature. After the incubation, 50 µL of diluted detection reagent were added to each well. After incubating the plate for two hours, the plate was centrifuged at 500 rpm for five minutes and the supernatant were removed. The pellet was resuspended in 150 µL of wash buffer. The samples were acquired on the flow cytometer and data analyzed using FCAP Array software.

### 5.3.8 Determination of CRP in Serum

CRP was detected and quantified using solid phase sandwich ELISA (enzyme-linked immunosorbent assay) kit supplied by BD Biosciences, Mississauga, ON, Canada. The rat serum samples were diluted (1:8000) with wash buffer. Different standard dilutions were made from stock standard according to the manual instructions. The serum samples and serially diluted standards (100 µL) were added to designated wells and incubated at room temperature for 30 min. The unbound compounds were removed by washing the plate 4-5 times with wash buffer and decanting on stack of paper towels. The detection antibody/enzyme conjugate was diluted to 1X working concentration and 100 µL were added to each well. The plate was incubated at room temperature for 30 min and washed with buffer to remove unbound conjugate. Then, 100 µL of 3, 3', 5, 5'-tetramethylbenzidine (TMB) were added to each well and plate was incubated for 10 minutes at room temperature. The reaction was stopped by adding 100 µL stop solution. The concentration of CRP in serum samples was detected by reading the absorbance at 460 nm using BioTek Powerwave, Winooski, VT, USA.

### 5.3.9 Determination of Serum IL-6

The rat IL-6 ELISA kit supplied by Invitrogen, Burlington, ON, Canada, was used for quantification of IL-6 in serum. The standards were prepared by serially diluting stock standard (7500 pg/mL). The serum samples were diluted by adding 2 µL of serum to 398 µL of standard dilutent buffer. The incubation buffer (50 µL) was added to all microplate wells. Then, 100 µL of standards and diluted samples were added to the appropriate wells. The samples were mixed and the covered plate was incubated for 2 h at 37$^{o}$C. The wells were decanted and washed four-times with wash buffer. The biotinylated rat IL-6 Biotin conjugate (100 µL) was added to each well except the chromogen blank. The plate was incubated for another 90 min at room temperature. The wells were decanted and washed four-times with washing buffer. The streptavidin-HRP solution (100 µL) was added to all appropriate wells except chromogen blank. And the plate was incubated for 30 min at room temperature. All the unbound compounds were removed by washing and 100 µL of chromogen were added to each well. The plate was incubated for another 20 min and 100 µL of stop solution were added to stop the reaction. The optical density of IL-6 complex was read at 450 nm using Varioskan Flash plate reader, Thermofisher Scientific, Nepean, ON, Canada.

### 5.3.10 Determination of Serum Adiponectin

The adiponectin in rat serum was quantitatively determined using rat adiponectin ELISA kit, Invitrogen, Burlington, ON, Canada. The stock standard (48 ng/mL) was serially diluted to 24, 12, 6, 3, 1.5, 0.75, 0.375 ng/mL and the rat serum samples were diluted to 1:4000. All the standards and serum samples (100 µL) were added to

designated antibody-coated wells. The plate was incubated for 1 h at 37$^{o}$C. The unbound residuals were removed by washing and decanting the wells. The secondary antibody (100 µL) was added to each well and plate was incubated for 1 h at 37$^{o}$C. The wells were washed five times with wash buffer and 100 µL of 1X detector solution were added to the wells. After incubating at 37$^{o}$C for 1 h, the residual solution was decanted and wells were washed properly with washing buffer. The substrate solution (100 µL) was added to each well and incubated for 20 minutes at room temperature. The reaction was stopped by adding stop solution and absorbance was read at 450 nm using Varioskan Flash plate reader, Thermofisher Scientific, Nepean, ON, Canada. The blank reading was subtracted from the readings of samples and standards.

### 5.3.11 UPLC-MS/MS analysis of apple peel phenolic compounds

Analyses of major individual phenolic compounds present in apple peel concentrate were performed according to the method described in Section 4.3.5.

### 5.3.12 Statistical Analysis

All measurements were expressed as mean ± standard deviation (SD) (n=12). The significant differences among different groups were tested using one-way ANOVA and the multiple mean comparison was performed in General Linear Model (SAS V8, Cary, NC, USA) using least significant differences (LSD) test. The differences at the 5% level ($p < 0.05$) were considered statistically significant.

## 5.4 RESULTS

### 5.4.1 Composition of Flavonol rich-Apple Peel Concentrate

The total phenolic composition of apple peel concentrate is shown in Table (5.2). The total polyphenolics content of apple peel concentrate analyzed by ULPC-MS/MS was 350 mg/g dry weight of concentrate. The apple peel concentrate consisted of 63% Q-3-glycosides that was 221.9 mg/g dry weight of concentrate.

**Table 5.2. Concentrations of phenolic compounds of Northern Spy apple peel concentrate (AF) prepared using reverse- phase chromatography.**

| Phenolic Compounds | Polyphenolic Content [a] (mg/g DW) |
|---|---|
| **Quercetin-3-*O*-galactoside** | 104.6± 30.4 |
| **Quercetin-3-*O*-rhamnoside** | 99.98± 28.9 |
| **Quercetin-3-*O*-glucoside** | 10.89± 2.6 |
| **Quercetin-3-*O*-rutinoside** | 3.66± 1.7 |
| **Quercetin** | 2.88± 1.0 |
| **Phloridzin** | 66.78± 13.1 |
| **Phloritin** | 0.69± 0.1 |
| **Chlorogenic acid** | 16.55± 6.6 |
| **Caffeic acid** | 0.77± 0.1 |
| **Catechin** | 6.12± 1.4 |
| **Epicatechin** | 37.86± 12.6 |
| **Total Phenolics by UPLC-MS/MS** | 350.7± 97.6 |

[a] Data presented as mean ±SD, n=3.
The polyphenolics extracted in absolute ethanol were fractioned using 20-100% ethanol gradient by reverse phase chromatography. The fractions were analyzed using UPLC-MS/MS and the fractions rich in flavonols were concentrated and dried into powder.

### 5.4.2. Synthesis of EPA Esters of Isoquercitrin (Quercetin-3-*O*-glucoside)

The isoquercitrin-EPA esters were synthesized by the method described by Salem et al. (2010) and Ziaullah et al. (2012). Novozyme lipase 435® (2.0 g) was added to a flame dried round bottom flask having 3Å molecular sieves, followed by the addition of isoquercitrin (0.5 g, 1.077 moles), dry acetone (5 mL) and EPA (1.73 mL, 5.38 moles) under inert conditions. The mixture was stirred and heated at 45°C for 12-24 h. The completion of reaction was confirmed using thin layer chromatography (6 acetone: 4 toluene). The reaction mixture was filtered, evaporated and passed through a silica gel column chromatography (40 cm x 3.5 cm) (acetone: toluene; 40:60 to 50:50) to get pure esters. The yield of pure esters of isoquercitrin was approximately 81 % (Figure 5.1, 5.2).

**(6-{[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-chromen-3-yl]oxy}-3,4,5-trihydroxy-tetrahydro-2*H*-pyran-2-yl)methyl (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-5,8,11,14,17-eicosapentaenoate (13)**

Yield: 81 %; greenish yellow spongy solid; $R_f$: 0.57 (Acetone:Toluene; 1:1: few drops of AcOH); IR (KBr) $cm^{-1}$: 3346, 2946, 2835, 2490, 2180, 2044, 1893, 1768, 1647, 1591, 1466, 1446, 1231, 1027, 941, 737, 706; **$^1$HNMR** (DMSO-$d_6$, 300 MHz): δ 12.67 (br.s, 1H, ArOH), 10.03 (br.s, 1H, ArOH), 7.55 (br d, 2H, *J* = 8.5 Hz, H-2′, H-6′), 6.87 (d, 1H, *J* = 8.5 Hz, H-5′), 6.42 (s, 1H, H-8), 6.23 (s, 1H, H-6), 5.49-5.17 (m, 13H, H-5", H-6‴, H-8‴, H-9‴, H-11‴, H-12‴, H-14‴, H-15‴, H-17‴, H-18‴, H-1", 2OH), 4.20 (br.d, 1H, *J* = 11.4 Hz, H-6a"), 3.99 (dd, 1H, *J* = 11.4 Hz, *J* = 7.2 Hz, H-6b"), 3.49-3.14 (m, 6H, H-2", H-3", H-4", 2xOH), 2.82-2.71 (m, 8H, 2xH-7‴, 2xH-10‴, 2xH-13‴, 2xH-16‴), 2.14-1.85 (m, 6H, 2xH-2‴, 2xH-4‴, 2xH-19‴), 1.39-1.24 (m, 3H, 2xH-3‴, OH), 0.92 (br t, 3H, *J* = 7.8 Hz, $CH_3$).

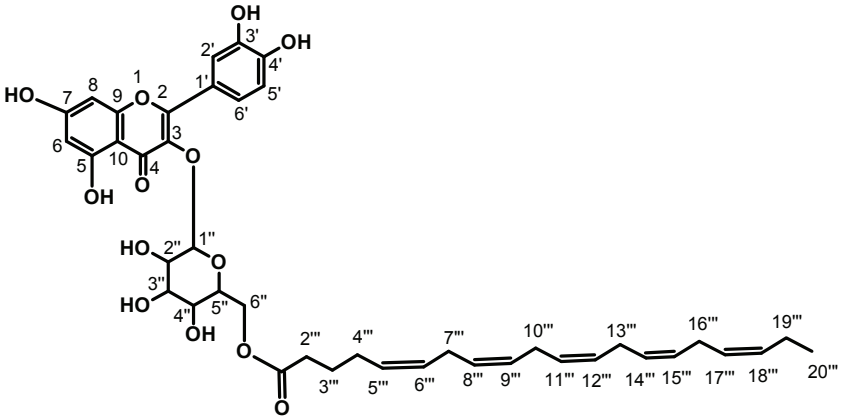

**Figure 5.1. Structure of the synthesized isoquercitrin-eicosapentaenoic acid ester compound; (6-{3,5-dihydroxy-2-[3-(4-hydroxyphenyl)propanoyl]phenoxy}-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)methyl (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-5,8,11,14,17-eicosapentaenoate.**

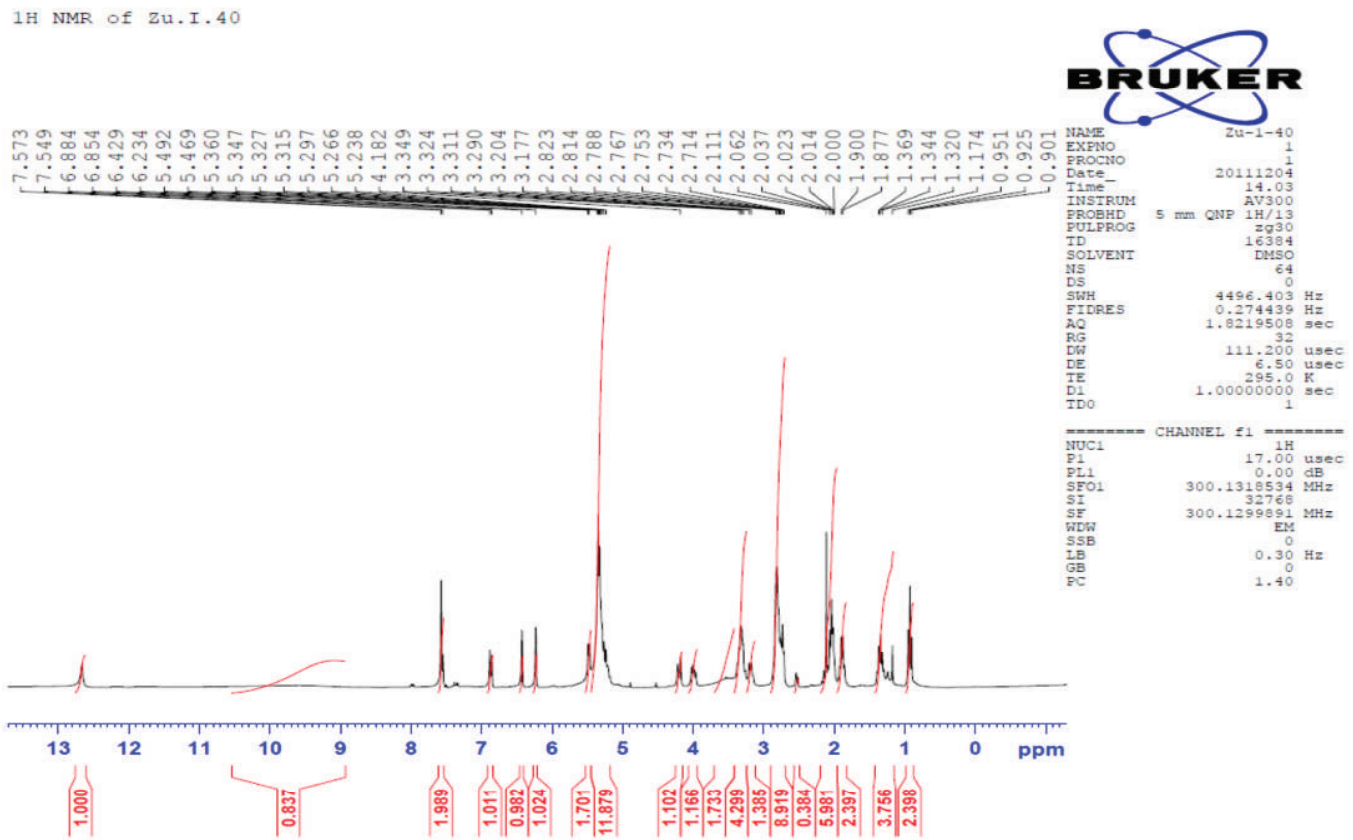


**Figure 5.2. NMR spectrum of (6-{3,5-dihydroxy-2-[3-(4-hydroxyphenyl)propanoyl]phenoxy}-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)methyl (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-5,8,11,14,17-eicosapentaenoate.**

### 5.4.3 Effect of Different Dietary Supplements on Food Intake, Weight of Body, Liver and Spleen

There was no significant difference in the final body weight and food intake (Table 5.3) among the six groups after 4-week study ($p > 0.05$). Similarly, the treatments had no effect on the food intake. No effect was observed on the liver and spleen weights of the animals among the six treatment groups ($p > 0.05$) (Table 5.3).

**Table 5.3. Food intake, body weight gain, liver weight and spleen weight of the six treatment groups of hyperlipidemic rats[a].**

| *Group[b]* | *Food intake (g/day)* | *Body weight gain (g)* | *Liver weight (g)* | *Spleen weight (g)* |
|---|---|---|---|---|
| **HFC[x]** | 23.89 ±2.7 | 286.4 ±33.4 | 39.28 ±5.0 | 1.54 ±0.4 |
| **HFL** | 23.71 ±2.7 | 297.4 ±35.3 | 41.10 ±4.9 | 1.68 ±0.2 |
| **HFL+AF** | 24.20 ±2.9 | 287.9 ±28.4 | 41.41 ±4.4 | 1.92 ±0.4 |
| **HFL+EPA** | 23.81 ±2.4 | 287.7 ±29.0 | 38.51 ±2.2 | 1.68 ±0.3 |
| **HFL+AF+EPA** | 24.24 ±2.6 | 298.2 ±31.1 | 41.90 ±6.2 | 1.7 ±0.3 |
| **HFL+QE** | 23.76 ±2.9 | 287.6 ±36.4 | 38.13 ±4.2 | 1.74 ±0.3 |

[a] Values are expressed as mean ±SD, (n=12).
[b] For description of the treatment groups, refer to the section 5.3.2.
There was no significant difference in food intake, body weight gain, liver and spleen weight among the treatment groups ($p > 0.05$).
[x] HFC, high fat control; HFL, high fat + LPS; HFL + AF, high fat + LPS + apple flavonols; HFL + EPA, high fat + LPS + eicosapentaenoic acid; HFL + QE, high fat + LPS + quercetin-EPA ester; HFL + AF + EPA, high fat + LPS + apple flavonols + eicosapentaenoic acid.

### 5.4.4 Serum and Liver Lipid Profiles

***Serum Lipid Profile***

The effects of six treatments on serum lipid profiles are summarized in Figure 5.3. Average serum triacylglycerol concentrations were not different in both control groups, HFC (160.9 mg/dL) and HFL (147.3 mg/dL) ($p > 0.05$).Addition of AF, EPA and their combination to the HFL diet caused significant decreases of approximately 33-35% in serum triacylglycerols concentrations ($p < 0.05$), but no significant effect was observed in HFL+QE treatment. Interestingly, no significant effect was observed on the serum total cholesterol concentration in all the six groups and the serum lipid profile was not significantly altered by LPS injection ($p > 0.05$). Compared to the HFC and HFL, serum HDL-C concentration was increased significantly by 58% in HFL+AF , by 44% in HFL+EPA, by 41% in HFL+AF+EPA and by 42% in the HFL+QE ($p < 0.05$). The AF and combined treatment of AF and EPA effectively reduced serum non-HDL-C concentrations by 53% and 49%, respectively ($p < 0.05$), whereas HFL+EPA and HFL+QE groups were not different from HFC and HFL groups ($p > 0.05$).

***Liver Lipid Profile***

The liver lipid profiles of the six groups of animals are shown in Figure 5.4. There was no significant difference in liver triacylglycerols among the six groups ($p > 0.05$). When compared to HFL, AF, EPA, AF+ EPA, and QE treatments showed significant reduction of 12-18% in liver total cholesterol ($p < 0.05$). The free cholesterol was significantly lowered by 36-38% in all the dietary supplements as compared to HFL ($p < 0.05$). There was no significant effect on liver triacylglycerols and cholesteryl ester concentration among all the six treatment groups ($p > 0.05$).

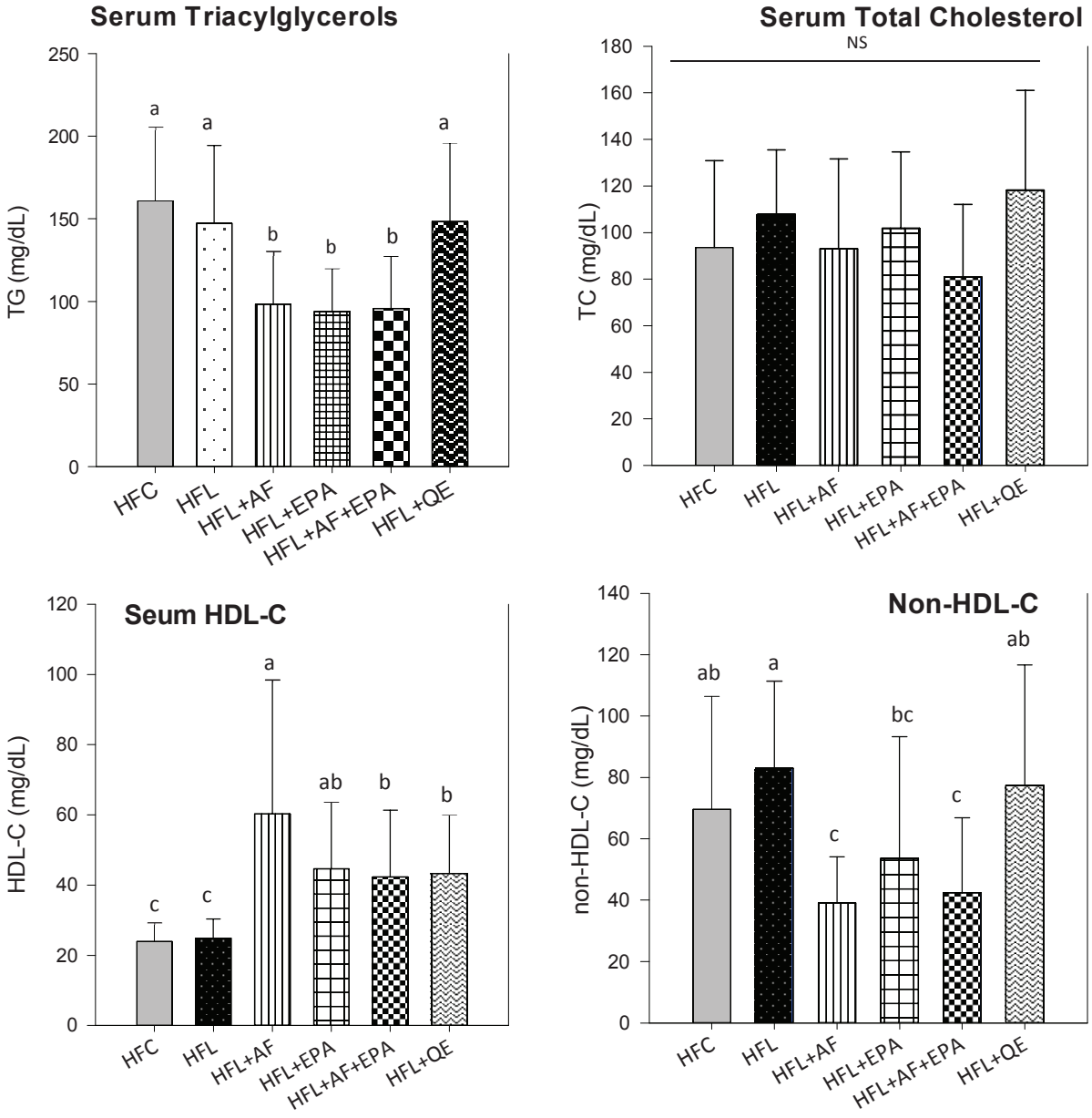


Figure 5.3. Serum triacylglycerols (TG), HDL-cholesterol (HDL-C) and non-HDL-cholesterol levels in six groups of high-fat diet fed rats. Except control 1 (HFC), LPS (5mg/ kg) was injected in control 2 (HFL) and the four treatment groups, 5 hr prior to sacrifice. Results were expressed as mean ±SD (n=12). Data were analyzed by ANOVA and statistical significance by LSD test using General Linear Model. The values with different letters are significantly different *P <0.05 $^{NS}$ not significant.

HFC, high fat control; HFL, high fat with lipopolysacchride; LPS, lipopolysacchride; AF, apple flavonols; EPA, eicosapentaenoic acid; QE, quercetin-EPA ester.

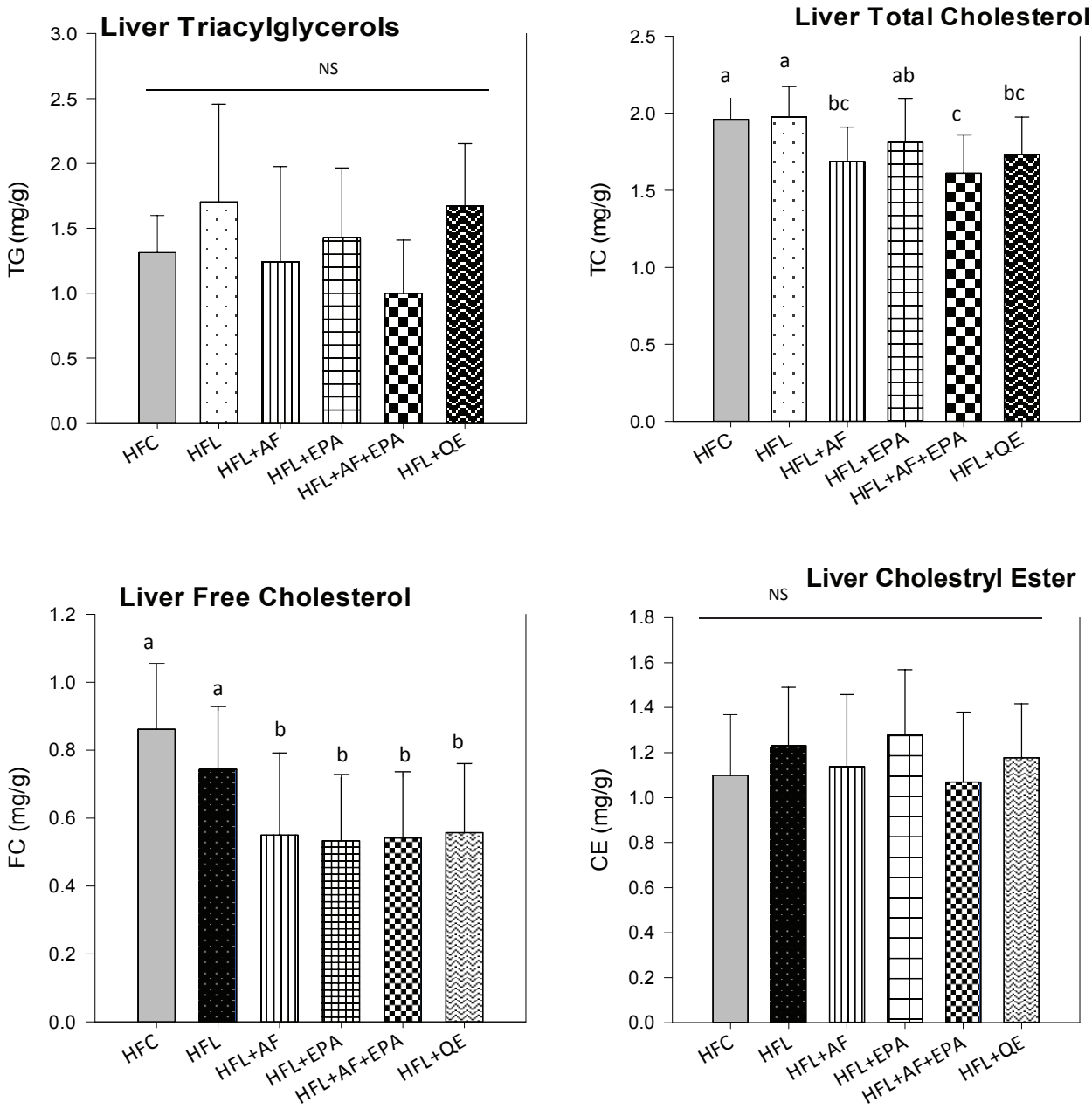


Figure 5.4. Liver total cholesterol (TC), free cholesterol (FC), triacylglycerols (TG) and cholesteryl ester (CE) levels in six groups of high-fat diet fed rats. Except control 1 (HFC), LPS (5mg/ kg) was injected in control 2 (HFL) and the four treatment groups, 5 hr prior to sacrifice. Results were expressed as mean ±SD (n=12). Data were analyzed by ANOVA and statistical significance by LSD test using General Linear Model. The values with different letters are significantly different *P <0.05 $^{NS}$ not significant.

HFC, high fat control; HFL, high fat with lipopolysacchride; LPS, lipopolysacchride; AF, apple flavonols; EPA, eicosapentaenoic acid; QE, quercetin-EPA ester.

### 5.4.5 Serum Inflammatory Cytokines

Serum concentrations of CRP, adiponectin, TNF-α and IFN-γ in rats fed with six different diets are shown in Figure. 5.5. There was no significant difference in CRP concentration between HFC and HFL ($p > 0.05$). A significant decrease of 11% with HFL+AF, 27% with HFL+EPA, and 29% with HFL+AF+EPA compared to HFL was observed in the CRP concentration. Additionally, QE was most effective which lowered serum CRP by 48% relative to the HFL group ($p < 0.05$).

An increase of approximately 39% in adiponectin concentration was observed in HFL+QE treatment group as compared to HFL ($p < 0.05$). However, no significant changes were observed among HFC, HFL, HFL+AF, HFL+EPA and HFL+AF+EPA treatments ($p > 0.05$) (Figure 5.5).Serum TNF-α concentration was increased by 25-fold after LPS injection in HFL compared to HFC ($p < 0.05$). However, there was no significant difference observed among HFL and other treatment groups (HFL+AF, HFL+EPA, HFL+QE, HFL+AF+EPA; $p > 0.05$).

The IFN-γ concentration in HFL group was increased by 99-fold compared to HFC group ($p < 0.05$). The EPA treatment (3095 pg/mL) lowered the IFN-γ concentration, however, it did not achieve significance. There was no significant effect of AF and AF+EPA treatments on IFN-γ concentration ($p > 0.05$). However, QE treatment depicted drastic decrease of 71% in IFN-γ concentration and it almost abolished the effect of LPS as there was no significant difference between HFC and HFL+QE groups ($p > 0.05$).

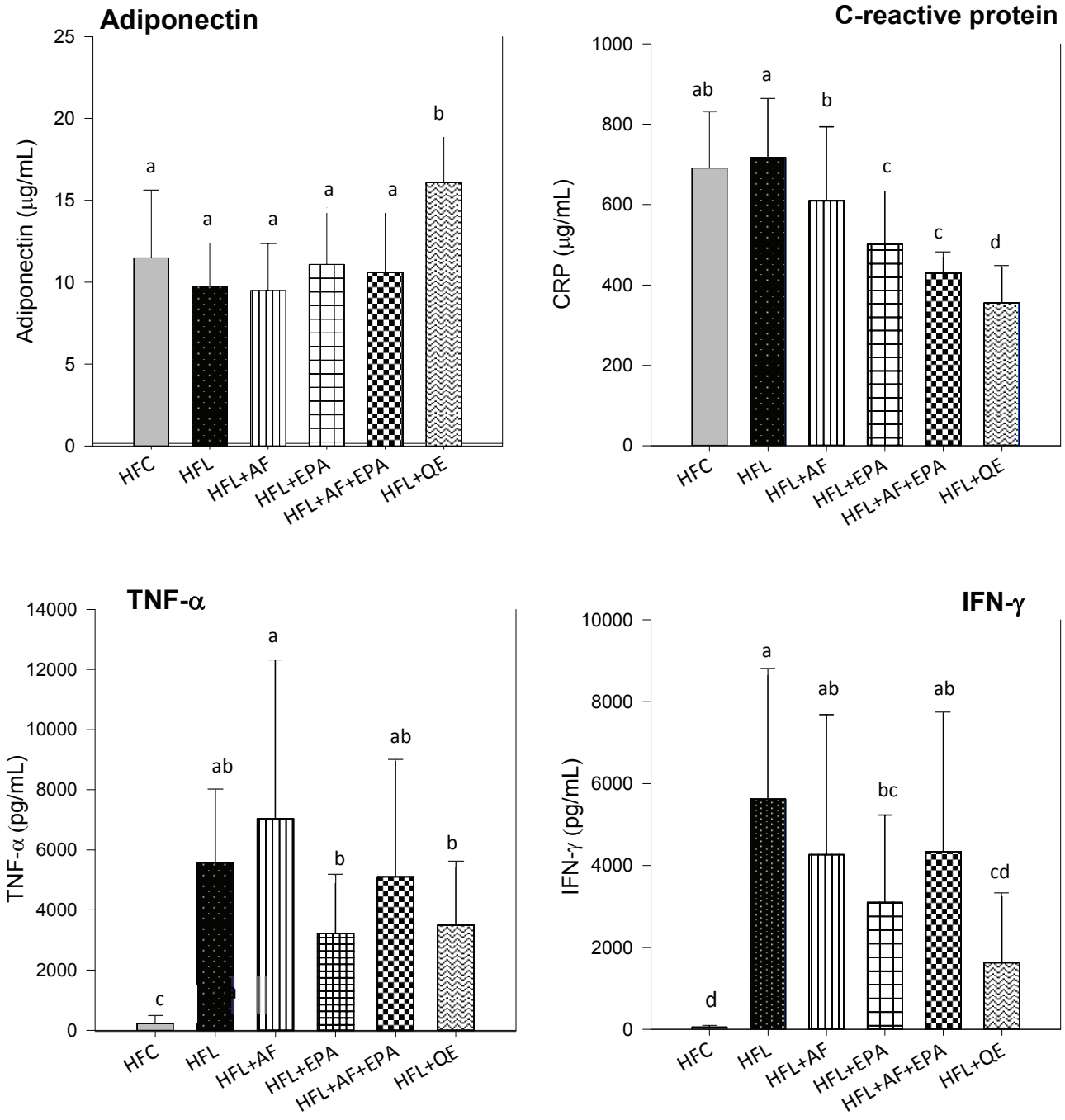


Figure 5.5. Serum concentrations of adiponectin, C-reactive protein, TNF-α and IFN-γ in six groups of high fat fed rats. Except control 1 (HFC), LPS (50 mg/ kg) was injected in control 2 (HFL) along with four treatment groups, 5 hr prior to sacrifice. Results were expressed as mean ±SD (n=12). Data were analyzed by ANOVA and statistical significance by LSD test using General Linear Model. The values with different letters are significantly different *P <0.05, $^{NS}$ not significant. HFC, high fat control; HFL, high fat with lipopolysacchride; LPS, lipopolysacchride; AF, apple flavonols; EPA, eicosapentaenoic acid; QE, quercetin-EPA ester.

The concentrations of IL-6, IL-10, IL-1α, IL-2 and IL-4 are depicted in Figure 5.6. Mean IL-6 concentration was significantly raised by nearly two-fold after LPS injection in HFL compared with no inflammation HFC group ($p < 0.05$). Whereas mean IL-6 concentrations tended to decrease significantly by 41% in HFL+AF (41%), by 25% in HFL+EPA, by 44% in HFL+QE and by 35% HFL+AF+EPA compared to HFL ($p < 0.05$).

Mean IL-10 concentration in the HFL and HFL+QE was significantly higher (19-fold) than HFC group ($p < 0.05$). The HFL+AF and HFL+AF+EPA treatments further increased IL-10 concentration by 30-fold, compared to HFC ($p < 0.05$). Similarly, mean IL-1α concentration in HFL group were significantly increased by 21-fold compared to HFC ($p < 0.05$) and none of the dietary supplements showed significant effect on suppression of IL-1α concentrations. In the case of IL-4 and IL-2, no significant differences were observed between any treatment groups ($p > 0.05$).

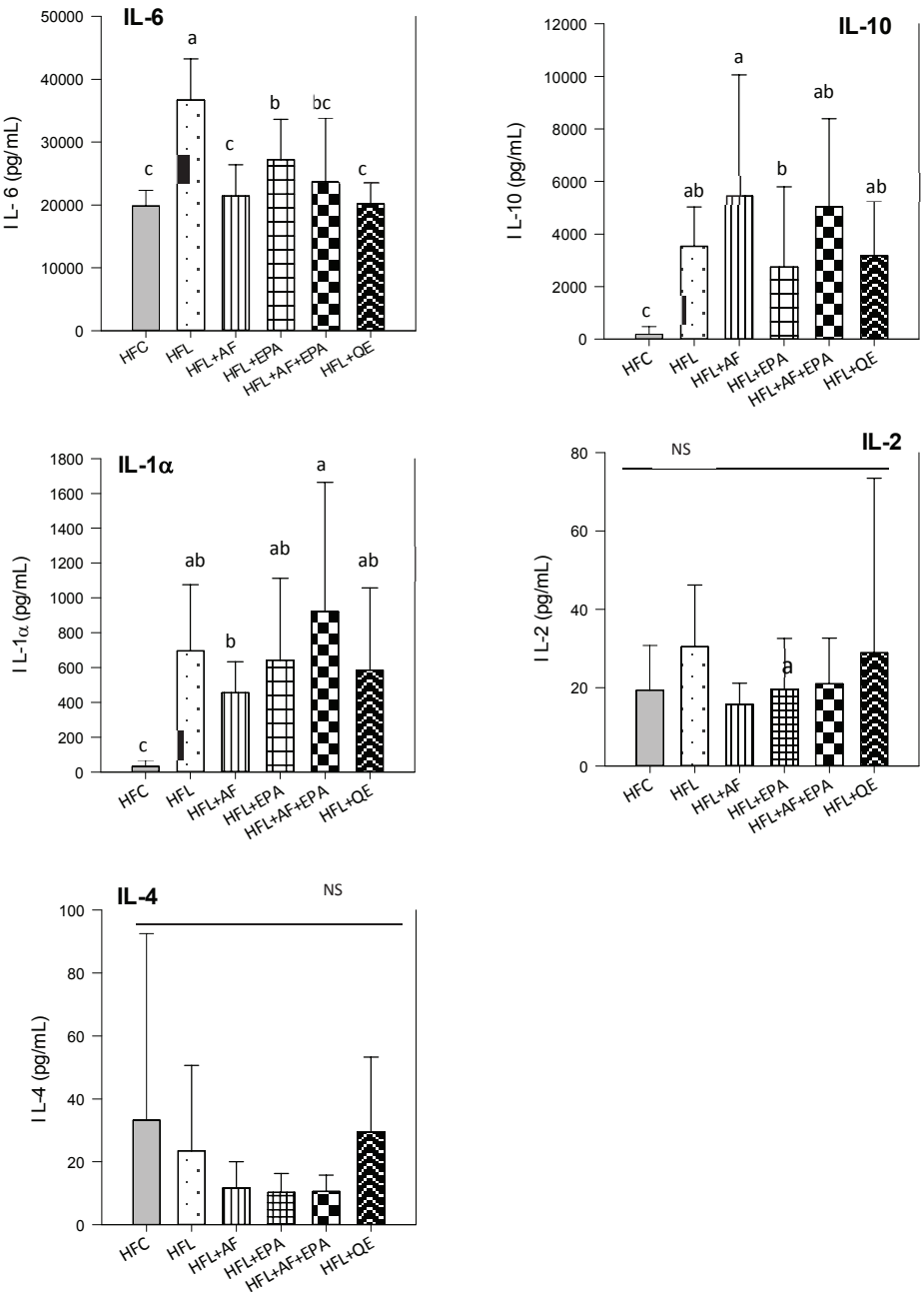


Figure 5.6. Serum concentrations of IL-6, IL-10, IL-1α, IL-2 and IL-4 in six groups of high fat fed rats. Except control 1 (HFC), LPS (50 mg/ kg) was injected in control 2 (HFL) along with four treatment groups, 5 hr prior to sacrifice. Results were expressed as mean ±SD (n=12). Data were analyzed by ANOVA and statistical significance by LSD test using General Linear Model. The values with different letters are significantly different *P <0.05, $^{NS}$ not significant.

HFC, high fat control; HFL, high fat with lipopolysacchride; LPS, lipopolysacchride; AF, apple flavonols; EPA, eicosapentaenoic acid; QE, quercetin-EPA ester.

## 5.5 DISCUSSION

A moderate dose of LPS is known to trigger signal transduction associated with inflammation and mimick the natural response of the body under inflammatory conditions. High fat-induced obesity in combination with LPS-induced inflammation produce typical characteristics ( increased levels of triacylglycerols and LDL-cholesterol and inflammatory cytokines) similar to metabolic diseases such as CVD and insulin resistance (Cani et al., 2007). Taking into account these antecedents, the present study investigated the alterations in serum and liver lipid profile along with pro-inflammatory cytokines after injecting LPS in rats fed with a high-fat diet and treated with AF, EPA, QE and AF + EPA.

Numerous studies have reported *in vitro* and *in vivo* anti-inflammatory (Lauren et al., 2009; Jung et al., 2009; Pastene et al., 2010) and antioxidant effects (Carrasco-Pozo et al., 2011) of apple peel extract. Quercetin glycoside is a major flavonol group present in apple peel. Quercetin has been reported to inhibit the production of pro-inflammatory cytokines by suppressing nuclear factor-κB (NF-κB) (Kim et al., 2007; Egert et al., 2009). In the present study, AF supplementation showed a significant decrease in serum IL-6 and CRP concentrations in hyperlipidemic rats with inflammation compared to HFL control group. Similar *in vitro* and *in vivo* experiments have reported that quercetin attenuates the expression of IL-6 and TNF-α (Liu et al., 2005; Carvalho et al., 2010; Chuang et al., 2010; Jung et al., 2011). Apple peels also contain a high concentration of phloridzin in addition to quercetin glycosides. Phloridzin shows similar biological properties as the flavonols (Balazs et al., 2012; Lauren et al., 2009).

Among all pro-inflammatory cytokines, TNF-α and IL-6 have a major role in inducing endothelial dysfunction and insulin resistance (Bhagar and Vallace, 1997; Lau et al., 2005). TNF-α increases expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1), monocyte chemotactic protein-1 (MCP-1) and reduce nitric oxide (NO) bioavailability, triggering endothelial dysfunction (Ouchi et al., 2003). Circulating concentrations of IL-6 and TNF-α together regulate the mRNA expression of CRP in liver (Yudkin et al., 1999).

Serum CRP concentration is an important biomarker for CVD (Verma et al., 2006) which mediates the process of endothelial dysfunction by amplifying the pro-inflammatory cytokines and their activities (Pasceri et al., 2000). Chun et al. (2008) has reported that flavonoid intake is inversely associated with serum CRP concentrations. In the present study, there was no significant difference in serum CRP between HFC and HFL, indicating that a subclinical inflammation might be present in the obese rats even before LPS injection. This further confirms the finding that obesity in itself is a pro-inflammatory state (Yudkin et al., 1999; Antuna-Puente, 2008). However, the previous observations related to CRP from intervention studies of quercetin glycosides have been inconsistent (Arts and Hollman, 2005; Williamson and Manach, 2005; Floegel et al., 2011). Larmo et al. (2009) suggested a possible synergistic effect lowering CRP level by several polyphenols in sea buckthorn berry extract. In the present study, we have also shows AF, EPA and their combination significantly reduced serum CRP concentrations in hyperlipidemic rats with inflammation. The possible mechanism suggested to reduce CRP expression may be through blocking NF-κB activation by flavonoids (Wanner and Metzger, 2002).

Pro-inflammatory cytokines, TNF-α, IFN-γ, IL-6, IL-10 and IL-1α, increased significantly after LPS injection in all five groups compared to HFC. This inflammatory response after LPS was in accordance with the previous studies (Lauren et al., 2009; Jung et al., 2009). However, no significant effect was observed on TNF-α levels in the treatment groups with AF, EPA, their combination and QE. This observation was in contrast with the previous studies using flavonols (Park et al., 2008; Kobori et al., 2010; Ciftci and Ozdemir, 2011). Although there was a tendency to decrease TNF-α concentration in some of the animals in AF-treated group, but this effect was not significant due to large variation within the group. As there was a significant increase in anti-inflammatory cytokines in the present study, it suggests that TNF-α expression might be up-regulated by an alternative pathway, not by NF-κB (Lira et al., 2010; Kim et al., 2011).

Mean serum IL-6 concentrations were remarkably reduced in AF, EPA, QE and AF + EPA treated hyperlipidemic rats compared to HFL in the present study. The flavonols may reduce IL-6 production by inhibiting its gene expression of the transcription factors including NF-κB (Park et al., 2008), and quercetin might inhibit the adhesion and accumulation of the neutrophils to endothelial cells (Liu et al., 2005). De Luis et al. (2009) found that ω3 PUFA supplementation decreased plasma CRP and TNF-α levels in a clinical study, however, IL-6 concentrations were not affected.

The results of present study showed no significant effects of AF and its combination with EPA on IFN-γ concentrations. Interferon- γ is a pro-inflammatory cytokine and previous studies have reported significant reduction of IFN-γ concentrations by quercetin in pancreatic β-cell line (Kim et al., 2007) and in diet induced obese mouse

model (Stewart et al., 2008). These results might be due to the presence of various flavonol glycosides which have different biological activities from the flavonol aglycones (Larmo et al., 2009). However, Bravo et al. (2006) found that there was no significant effect of ω3 PUFA on IFN-γ plasma concentrations in hypercholesterolaemic rats whereas it significantly reduced TNF-α and IL-6 concentrations. Moreover, in our study, QE and EPA treatments rendered a significant reduction in IFN-γ concentrations. The drastic reduction by QE might be attributed to the combined properties of two compounds (i.e. isoquercitrin and EPA), although the detailed mechanism involved is still unclear.

Circulating concentrations of adiponectin and CRP are considered predictive markers of CVD and diabetes and are strongly correlated with the visceral fat accumulation (Kolberg et al., 2009; Salas-Salvado et al., 2011). Adiponectin is a highly expressed cytokine by adipose tissue which exerts anti-inflammatory, anti-diabetic and anti-atherogenic effects (Yamauchi et al., 2003; Ohashi et al., 2006). In the present study, there was no significant alteration in adiponectin concentrations in AF, EPA and their combined treatment groups. The results from flavonol treatment were contrasting to the previous reports (Kobori et al., 2010; Yen et al., 2011; Kim et al., 2012). However, according to Matsunaga et al. (2008), ω3 PUFA depleted adiponectin concentrations in dextran sulphate sodium induced colitis in mice. Interestingly, QE treatment significantly elevated the adiponectin concentrations in the present study.

With regard to the effect on IL-10, its concentration increased significantly with LPS injection in HFL group and appeared to increase further after flavonol treatment. There was no significant difference in IL-10 concentrations among all LPS treated groups. This observation is in contrast with the reports that IL-10 exerts anti-

inflammatory effects (Iyer et al., 2010). However, LPS has found to stimulate IL-10 expression in the previous studies (Deitschel et al., 2010; Kim et al., 2011). Similar increase in IL-10 production with quercetin treatment has been reported in other studies (Marina et al., 2010; Carvalho et al., 2010). This might be due to inhibition of arachidonic acid pathway by blocking the cyclooxygenase or lipooxygenase and affecting the balance of pro-inflammatory and anti-inflammatory cytokines expression (Seeram et al., 2003). Quercetin and catechins have been reported to increase IL-10 expression by inhibiting release of TNF-α and IL-1β (Lyu and Park, 2005). The exact mechanism behind expression of IL-10 under inflammations still needs to be elucidated.

IL-4 is a Th-2 derived cytokine which promote growth of T cells, activate B cells, and inhibit macrophage activation and Th-1 cells (Paludan, 1998). We did not observe a significant change in IL-4 and IL-2 concentrations with LPS treatment and bioactive treatments. However, in the previous studies, quercetin exhibited an inhibitory effect on IL-4 gene expression in mononuclear cells (Nair et al., 2002). The anti-inflammatory effect observed in our study could result from different interactions between the compounds present along with flavonols which is expected to be different from the previous reports which use single pure flavonol (Lauren et al., 2009).

In the present study, IL-1α concentration was significantly elevated in all treatment groups with LPS injection compared to HFC. However, there was no significant difference among the treatment groups and HFL. In the literature, EPA has been reported to inhibit synthesis of IL-1 and IL-2 both *in vitro* and *in vivo* conditions (Purasiri et al., 1994). The role of IL-1α in progression of inflammation is not well known. IL-1 is a pro-inflammatory cytokine and IL-1α has a distinct role in inducing inflammation. Dube et al.

(2001) reported that the absence of IL-1α expression greatly reduced the severity of inflammation.

Hyperlipidemia or dyslipidemia is characterized by elevated concentrations of circulating triacylglycerols, LDL-cholesterol and free fatty which is a hallmark for progression to atherosclerosis and diabetes (De Backer et al., 2003). Rodents can develop diet-induced hyperlipidemia and obesity which mimick human metabolic syndrome (Buettner et al., 2007; Gallou-Kabani et al., 2007). The consumption of ω3 PUFA could induce lipid lowering effects by reducing mRNA expression of the lipogenic enzymes (Siddiqui et al., 2008) and mediating transcriptional factors related to gene expression of lipid oxidation events (Abete et al., 2011). The ω3 PUFA mediated mitochondrial fatty acid oxidation may increase the leakage of ROS from the electron transport chain (Liu et al., 2002). Therefore, the presence of antioxidants like flavonols could counteract the negative effects at the site of oxidation. Furthermore, there are evidences for beneficial effects of ω3 PUFA and polyphenolics of hyperlipidemia and inflammation, but little is known about the combined effects of ω3 PUFA and AF. In this study, we found that AF, EPA and their combined treatment significantly reduced serum triacylglycerols. Uchiumi et al., (2004) reported an increase in serum triacylglycerols after LPS injection. However, there was no significant change in serum triacylglycerols and overall lipid profile, following the LPS injection in the present study. There was no effect on serum lipid profile when the LPS was injected 18 hours prior to sacrifice the rats (Kim et al., 2011). Based on the literature evidences, this effect might be due to the short time interval between the LPS injection and sacrifice of the animals

Radler et al. (2011) reported that ω3 PUFA in combination with polyphenolics and carnitine reduced plasma concentration of fatty acids and triacylglycerols. Quercetin has been reported for its hypocholesterolaemic and hypotriacylglycerolemic influences (Rivera et al., 2008; Kamada et al., 2005). The dietary intake of polyphenolics may reduce the lymphatic absorption of cholesterol in rats by interfering with the micellar solubility of lipids (Ikeda et al., 1992) and increase the faecal excretion of steroids (Aprikian et al., 2001).

Several epidemiological and experimental evidences have demonstrated that ω3 PUFA reduce plasma triacylglycerols and elevate HDL-C (Hooper et al., 2006; Yokoyama et al., 2007; Bravo et al., 2006). Harris et al. (2008) explained the hypotriglycerdemic effect of ω3 PUFA in terms of its ability to reduce hepatic synthesis of triacylglycerols and removing them from circulation. In previous studies, ω3 PUFA containing diet significantly reduced plasma cholesterol in the rats (Adan et al., 1999; Bravo et al., 2006). In our study, no treatment had a significant effect on serum total cholesterol concentrations, however, they significantly elevated serum HDL-C concentrations.

In this study, the dietary treatments with AF, EPA, AF+ EPA and QE did not reduce hepatic triacylglycerols, however, hepatic total cholesterol was markedly lowered compared to both control groups. These results are consistent with the previous findings that apple polyphenolics reduced total hepatic cholesterol in hypercholesterolaemic Sprague-Dawley rats (Osada et al., 2006). The flavonoids have been studied for reducing cholesterol synthesis by inhibiting 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase and inhibiting triacylglycerol secretion (Casaschi et al., 2004; Ogawa et al., 2005).

Phloridzin has been reported to be absorbed in the small intestine as phloretin which reduces absorption of glucose and fatty acids attributing to the beneficial effects in dyslipidemia (Najafian et al., 2011).

Omega-3 PUFA treated diet effectively reduced hepatic triacylglycerols and total cholesterol in obese Long-Evans rats (Shirouchi et al., 2007). These effects of ω3 PUFA may be encountered by suppressing the sterol regulatory element-binding protein-1 (SREBP-1) mRNA expression and enhancing the expression of lipolytic enzymes (Fujiwara et al., 2003). SREBP-1 is a membrane-bound transcription factor that regulates the expression of several genes (including fatty acid synthase and acetyl-CoA carboxylase) involved in cellular fatty acid synthesis in liver and other tissue (Okamato et al., 2006).

AF supplementation modulates serum and hepatic lipid profile in hyperlipidemic rats. This finding elucidates lipid lowering effects of apple flavonols under obesity. Although, AF contained a high percentage (63%) of quercetin glycosides, the effect of other phenolic compounds could not be excluded. Furthermore, AF showed high efficacy in combination with EPA to alter inflammation and lipid profile. This study further confirmed the role of ω3 PUFA to reduce serum triacylglycerols and hepatic cholesterol. The combined supplementation of AF and ω3 PUFA has rendered a synergistic effect on serum and hepatic lipid profiles without any adverse effects.

Moreover, the present study has reported for the first time that the effects of quercetin-EPA esters on inflammatory biomarkers. The QE have offered complementary anti-inflammatory effects compared to their parent compounds. QE treatment lowered the serum CRP concentration by two-fold as compared to its parent compounds (quercetin

glycoside and EPA). Additionally, QE treatment maintained a higher adiponectin concentration in obese rats under inflammation than those obese without inflammation. In literature, there are evidences that esterified phytosterols enhance their solubility, efficacy in lipid medium and improve their incorporation in food matrices (Mattson et al., 1977; Micallef and Garg, 2009).

In conclusion, the present study has demonstrated that AF, EPA, alone or in combination exert anti-inflammatory and hypolipidemic effects in the experimental animal model of rat. The QE offered a comprehensive strategy to provide additional anti-inflammatory effects. However, quercetin-EPA ester may not have a different mode of action from the parent compounds, but it exerts a different effect on inflammatory cytokines compared to AF and EPA. These findings can be beneficial in the prevention and early stage treatment of obesity and inflammation associated metabolic disorders.

# CHAPTER 6.0 CONCLUSIONS

## 6.1 ANTIOXIDANT, ANTI-INFLAMMATORY AND HYPOLIPIDEMIC ACTIVITIES OF APPLE FLAVONOLS

Habitual high fat intake stimulates chronic low-grade inflammation which promotes insulin resistance and CVD. The inflammatory response is characterized by elevated serum pro-inflammatory cytokines (TNF-α, IL-1, IL-6, CRP). The prolonged use of non-steroidal anti-inflammatory drugs (NSAID) render several health risks. However, natural antioxidants and bioactives have great potential to benefit these conditions.

Apple peel phenolics were fractionated to investigate their ability to act as a natural antioxidant to inhibit fish oil oxidation. The crude extracts of both dried and frozen apple peel depicted the highest FRAP and $DPPH^{\cdot}$ values which correspond to high phenolic content. A strong correlation was observed between FRAP and total phenolic content. $DPPH^{\cdot}$ values also showed a significant correlation with total flavonol and phloridzin content. The yield of total phenolics and flavonols was significantly higher in the frozen peel when compared to dried apple peel. Both crude extracts showed inhibition of bulk fish oil oxidation comparable to the commercial antioxidants, BHT and α-tocopherol. However, among the fractions, the dried apple peel fractions containing epicatechin, quercetin glycosides and phloridzin, the frozen peel fractions containing mainly quercetin glycosides showed the greatest TBARS values. The fractionated apple phenolics have synergistic and higher ability to control fish oil oxidation compared to the crude apple extract. Even though flavonols have strong antioxidant activities *in vitro*, it shows very low biological activity *in vivo* as little or none of the compound gets to the target tissues. The low effectiveness of flavonols *in vivo* conditions is because the

flavonols are reported to have poor bioavailability (D'Archivio et al., 2010). Taking in account the poor bioavailability of the flavonols, isoquercitrin-EPA ester was synthesized to compare its anti-inflammatory activity with apple flavonols and EPA. To further evaluate the effect of apple flavonols on *in vivo* inflammation conditions, a rat model was used. Our results demonstrated that the inflammation and obesity conditions were successfully achieved in the experimental model. QE treatment exhibited strong anti-inflammatory activity by inhibiting serum CRP, IL-6, IFN-γ and elevating circulating adiponectin level. Serum adiponectin and IFN-γ levels were only altered by QE treatment and it also produced the highest reduction in serum CRP level compared with all treatment groups. Since long-term intake of NSAIDs has many unwanted side effects, the anti-inflammatory effects of QE are worth of further evaluation in development of new natural anti-inflammatory drugs.

## 6.2 RECOMMENDATIONS FOR FUTURE RESEARCH

As a result of this research project, many additional questions evolved which remained to be elucidated. The crude apple peel which contained the highest amount of total phenolics exhibited relatively lower antioxidant protection on fish oil oxidation than the fractioned phenolic compounds. The inhibition of fish oil oxidation by individual phenolic compounds was different from the phenolics present in apple peel fractions, this could be due to the interaction of the different phenolic compounds present in the apple peel. According to our observation in the fish oil oxidation experiment, the best possible combination for controlling fish oil oxidation was quercetin-3-*O*-glycoside and epicatechin. However, to have a better understanding of the interaction between different phenolic compounds and their potency, further research using pure flavonols is needed.

In animal model study, certain findings with AF are not consistent with the previous reports for quercetin. AF showed no significant effect on TNF-α, IFN-γ, IL-1α, IL-2, IL-4 and IL-10 activity. This might be due to the presence of various quercetin glycosides with combination of other phenolics. However, similar results were observed with EPA and its combination with apple flavonols. The effects of EPA and AF were evident on certain inflammatory biomarkers like IL-6 and CRP. This could be due to the variations between the animals within each group and this might be avoided by further extending the duration of the animal study. Therefore, it is necessary to find out the mechanism of action of these supplements. However, QE depicted strong anti-inflammatory effects by controlling the adiponectin and IFN-γ levels in addition to the effects rendered by AF and EPA. These results showed some interesting biological properties of QE, however, further research on mechanism of action of QE and its

pharmakinetics in the body in comparison to quercetin glycosides and EPA is required to understand its unique effects. It could be assumed that these effects are manifested through multiple mechanisms. The the glycosylated flavonols are considerably more hydrophilic which may have difficulty to penetrate gut wall by passive diffusion. It is important to evaluate (i) the absorption mechanism involved for AF and QE, (ii) the bio-transformation of AF and QE in the digestive track including small intestine and large intestine, (iii) hepatic metabolism, and (iv) plasma concentrations of AF and QE and their metabolites after consumptions.

As AF markedly increased serum HDL-C and reduced serum triacylglycerols along with liver total cholesterol in rats, it is also worth to investigate mechanism of action of apple peel flavonols on cholesterol metabolism.

In conclusion, results of the present study have indicated that apple peel flavonols have potential antioxidant ability as demonstrated by protection against oxidation of ω3 PUFA, as well as anti-inflammatory and hypolipidemic properties as depicted in the rat model study. The structurally modified isoquercitrin may have future implication for treatment of obesity related inflammation.

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## APPENDIX: Supplementary Data

**Fish oil composition used as a supplement treatment in animal study.**

| Fish oil content | Fatty acid composition (g/100g) |
|---|---|
| **Fat** | 83.0 |
| **cis-Polyunsaturated fatty acids** | 62.9 |
| **Omega-3 polyunsaturated fatty acids** | 58.6 |
| **Eicosapentaenoic acid** | 48.3 |
| **Docosahexaenoic acid** | 8.62 |
| **Omega-6 polyunsaturated fatty acids** | 4.30 |
| **Cis-monounsaturated fatty acids** | 10.9 |
| **Saturated fatty acids** | 4.34 |
| **Trans fatty acids** | 0.877 |

**Fish oil composition used in heat-induced oxidation treatment.**

| Fish oil content | Fatty acid composition (g/100g) |
|---|---|
| **Fat** | 83.2 |
| **cis-Polyunsaturated fatty acids** | 33.7 |
| **Omega-3 polyunsaturated fatty acids** | 29.9 |
| **Eicosapentaenoic acid** | 5.59 |
| **Docosahexaenoic acid** | 22.9 |
| **Omega-6 polyunsaturated fatty acids** | 3.72 |
| **Cis-monounsaturated fatty acids** | 19.1 |
| **Saturated fatty acids** | 25.6 |
| **Trans fatty acids** | 1.16 |