ANTIOXIDANT AND CYTOPROTECTIVE PROPERTIES OF LONG CHAIN FATTY ACID ACYLATED DERIVATIVES OF QUERCETIN-*3*-*O*-GLUCOSIDE

by

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DEDICATION

To my friend Benisi

for her love, care, motivation & being with me throughout this journey….

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ABSTRACT

Quercetin-3-*O*-glucoside (Q3G), a glycosylated derivative of quercetin, is a polyphenolic compound known to possess diverse biological activities. Its moderately hydrophilic nature is a critical factor governing the accessibility to the active sites of oxidative damages *in vivo*. It was hypothesized that biological activities of Q3G can be further enhanced by regioselective acylation with fatty acids which gives more lipophilicity. Q3G was acylated with six selected long chain fatty acids: stearic acid, oleic acid, linoleic acid, α-linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), using *Candida antactica* lipase. The derivatives were evaluated for their potential in inhibiting lipid oxidation in food systems and human low density lipoprotein (LDL), and cytoprotection and anti-inflammatory effect in cell culture model systems. The fatty acid derivatives of Q3G possessed greater effectiveness in inhibiting lipid oxidation in oil-in-water emulsions, and better cytoprotective effect against H_2O_2- and cigarette smoke toxicant-induced cytotoxicity when compared to Q3G.

LIST OF ABBREVIATIONS USED

AA	Arachidonic acid
AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
AhR	Aryl hydrocarbon receptor
AIF	Apoptosis inducing factor
ALA	α-linolenic acid
ALEs	Advanced lipid oxidation end-products
AP-1	Activator protein -1
apo B	Apolipoprotein B100
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
CAD	Caspase-activated DNase
CAL B	*Candida antarctica* lipase B
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
COXIBs	COX-2 inhibitors
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid
EGCG	Epigallocatechin gallate
EGFR	Epidermal growth factor receptor
EMEM	Eagle's minimum essential medium
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-regulated kinase
FapGua	2, 6-diamino-4-hydroxy-5-formamidopyrimidine
FBS	Fetal bovine serum
FRAP	The ferric reducing antioxidant power
FTC	Ferric thiocyanate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSH	Glutathione
HIF-α	Hypoxia-inducible factor
H_2O_2	Hydrogen peroxide
HLB	Hydrophilic-lipophilic balance
HMW	High molecular weight
HNE	4-hydroxy-2-nonenal
HUVEC	Human umbilical vein endothelium cells
ICAD	Caspase-activated DNase inhibitor
IFN	Interferon
IL	Interleukin
JNK	c-jun N-terminal kinase

LDH	Lactose dehydrogenase
LDL	Low density lipoprotein
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MIP	Macrophage inflammatory protein
MRP	Multidrug resistance associated protein
MW	Molecular weight
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor κ-B
NNK	4-(methylnitrosoamino)-1-(3-pyridinyl)-1-butanone
NOS	NO synthase
NSAID	Non-steroidal anti-inflammatory drugs
ORAC	The oxygen radical absorbance capacity
PAH	Polyaromatic hydrocarbon
PBS	Phosphate buffered saline
PGE_2	Prostaglandin E_2
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PLA_2	Phospholipase A_2
PUFA	Polyunsaturated fatty acids
Q	Quercetin
Q3G	Quercetin-3-*O*-glucoside
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutases
TBA	2-thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TBHQ	Tertiary butylhydroquinone
TCA	Trichloroacetic acid
TH1	T helper-1
TH2	T helper-2
TLC	Thin layer chromatography
TMB	3,3',5,5'-Tetramethylbenzidine
TNFα	Tumor necrosis factor α
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UDP-sugar	Uridine 5′-diphosphosugar
UGT	Glycosyltransferase
UV	Ultra-violet
VEGF	Vascular endothelial growth factor
5-LOX	5-Lipoxygenase
7-AAD	7-Aminoactinomycin D
8-oxoGua	8-Oxo-7, 8-dihydroguanine

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CHAPTER 1 INTRODUCTION

Many epidemiological, as well as clinical studies, have revealed the role of polyunsaturated fatty acids (PUFA) in preventing several chronic pathological conditions, such as cardiovascular disease (CVD), brain disorders, inflammation and cancer (Warnakulasuriya and Rupasinghe, 2012; Siriwardhana et al., 2012; Rubio-Rodríguez et al., 2010). n-3 PUFA, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been extensively studied for their health promoting effects (Kathirvel and Rupasinghe, 2011). Therefore, fortifying foods with these PUFA is one of the current market trends in the food industry. However, PUFA are highly susceptible to oxidative deterioration due to their double bonds, and oxidized PUFA are unacceptable for consumption (Sun et al., 2011).

Other than in the food systems, oxygen can become detrimental at the cellular level, when reactive oxygen species (ROS) are generated in excessive amounts. The generation of ROS takes place in the body, during regular metabolic processes and involvement of ROS in biological activities, such as signal transduction, is significant. Moreover, cellular antioxidant defense mechanisms play a pivotal role in maintaining homeostasis in the human body (Finkel, 2003). However, in addition to the endogenous production of ROS, human bodies are exposed daily to many exogenous ROS sources, such as xenobiotics, environmental pollutants, ultra-violet (UV) and ionizing radiation (Pizarro et al., 2009). Therefore, oxidative stress can result due to either exogenous or endogenous ROS which cause deleterious effects to cellular macromolecules; nucleic acids, lipids and proteins, leading to cellular dysfunction and cell death (Ryter et al., 2007). As a consequence of

oxidative damage, several pathological conditions, such as inflammatory, ischemic and neurological diseases have been reported (McCord, 2000).

Cigarette smoke, which contains a highly complex mixture of thousands of chemicals, including oxidants, carcinogens, mutagens and toxic substances, is a major exogenous source for cellular oxidative stress which can lead to the onset of airway inflammation (Lee et al., 2012; Stämpfli and Anderson, 2009; Toorn et al., 2007). Inflammation has a major role in maintaining the adaptive immune system of the body. However, chronic inflammation is associated with immunosuppression and leads to many pathological conditions; mainly, asthma and chronic obstructive pulmonary disease (COPD), which can further lead to tumor development and cancer (Rodriguez-Vita and Lawrence, 2010; Rahman and MacNee, 2000). Eicosanoids, produced via arachidonic acid (AA) metabolism, are the main pro-inflammatory lipid mediators. Among them, prostaglandin E_2 (PGE_2) is reported to be immunosuppressive, especially inhibiting macrophage and T cell activation (Rodriguez-Vita and Lawrence, 2010) and therefore, the up-regulation of the cyclooxygenase (COX) pathway is responsible for promoting inflammatory cascades (Greene et al., 2011). Non-steroidal anti-inflammatory drugs (NSAID) have been prescribed for inflammation associated pathological conditions, for decades. NSAID act as agents to block the COX pathway and inhibit the production of inflammatory prostaglandins. Therefore, inhibitors of the COX pathway can be considered as potential anti-inflammatory drugs (Tsatsanis et al., 2006).

Dietary antioxidants are widely used to overcome the detrimental effects from the oxidative damage in food systems, as well as in living cells (Viscupicova et al., 2010). Regular consumption of fruits and vegetables is recommended for a healthy life and

epidemiological studies have exhibited their ability to reduce the risk of chronic disease pathologies such as CVD and cancer (Chen et al., 2010; Katsoura et al., 2007). Flavonoids, ubiquitously present phytochemicals in fruits and vegetables, have taken a central role in current research into natural product chemistry, due to their great ability to promote diverse health benefits (Viscupicova et al., 2010). Importantly, many flavonoid compounds, isolated from different plant species, have been extensively studied for their antioxidant activity (Lue et al., 2010; Harborne and Williams, 2000). Aside from exhibiting antioxidant activity, extracts of many fruits and vegetables, such as apple peel, have been reported to influence other biological functions (Rupasinghe et al., 2010, Huber et al., 2009, Thilakarathna et al., 2013; Balasuriya and Rupasinghe, 2012).

Most of the flavonoids found naturally are present in their glycosylated forms (Park et al., 2011). Glycosylation is an important step in the biosynthesis of plant secondary metabolites since it improves solubility and biological activity, making them more potent as storage and disease resisting compounds (He et al., 2006). Quercetin is a plant flavonoid, abundantly found in glycosylated forms in nature (He et al., 2006). However, due to their moderately hydrophilic nature, glycosylated flavonoids are poorly miscible in lipophilic media and have less stability. This has become a major issue which limits their applicability, especially in lipophilic food systems. The high hydrophilic/lipophilic balance of these compounds impairs their functionality as antioxidants in oil-based food products. Moreover, lipophilicity has an important role in the cellular uptake of flavonoids, affecting penetration through cell plasma membranes which in turn, influences their bio-availability and their interactions with cellular targets such as proteins (Ardhaoui et al., 2004; Lorentz et al., 2010; Katsoura et al., 2007). The

hydrophilic or lipophilic nature of the flavonoid structure and its glycosylated form highly influences their incorporation in foods, pharmaceuticals and dermocosmetics. Since there is a growing demand for the various uses of flavonoids in these fields, it is necessary to identify possible methodologies to improve their incorporation in polar and non-polar food matrices.

In plants, enzymatic reactions convert the aglycone of the flavonoids into their glycosylated forms which when extracted, can be converted into additional conjugated forms to widen their use, as well as increase bioactivity. Presently, lipase-catalysed modifications via esterification or transesterification of flavonoids with aliphatic or aromatic groups in organic media are well-studied techniques for regioselective synthesis of structurally modified bio-actives. Among them, esterification of flavonoid molecules with fatty acids as acyl donors is widely cited in literature (Ardhaoui et al., 2004; Chebil et al., 2007; Ziaullah et al., 2013). This approach provides improved lipophilicity and biological activity in the studied compounds. Use of modified flavonoids in food and natural health products is still in the emerging state (Viskupicova et al., 2012).

CHAPTER 2 OBJECTIVES

2.1 RESEARCH HYPOTHESIS

Antioxidant and cytoprotective properties of naturally occurring flavonoid glycosides can be further enhanced by increasing their lipophilicity by acylation with long chain fatty acids.

2.2 OVERALL OBJECTIVE

The overall objective of this study was to evaluate six fatty acid derivatives of quercetin-3-*O*-glucoside (Q3G) as potential antioxidants to protect polyunsaturated fatty acids (PUFA) containing food systems from oxidation and *in vitro* cellular systems from the detrimental effects of oxidative damage.

2.3 SPECIFIC OBJECTIVES

The specific objectives of the research were to:

1. Structurally modify the plant flavonoid, Q3G by acylation with six selected saturated, monounsaturated and polyunsaturated fatty acids;
2. Evaluate the antioxidant properties of the fatty acid derivatives of Q3G, using standardized *in vitro* antioxidant capacity assays and lipid oxidation model systems: bulk oil system and oil-in-water emulsions;
3. Assess the cytoprotective properties of the fatty acid derivatives of Q3G under oxidative stress induced conditions, using a cell culture system of a human lung cell line (WI-38); and

4. Assess the cytoprotective properties of the fatty acid derivatives of Q3G and their effect on inflammatory biomarkers for cigarette smoke-induced cytotoxicity *in vitro.*

CHAPTER 3 LITERATURE REVIEW

3.1 FLAVONOIDS AS FOOD ANTIOXIDANTS

3.1.1 Flavonoids

Generally, flavonoids are phenylobenzo-γ-pirone derivatives, having a fifteen carbon skeleton with two phenyl rings (A and B) linked by a three carbon heterocyclic pyran/pyrone ring (C) (Pawlak et al., 2010; Iwashina, 2003) (Figure 3.2). A diverse group of phenolic compounds, with more than 7000 identified different types of structures, were reported under the category of flavonoids (Chebil et al., 2007). The variation in the C ring and hydroxylation pattern on the A and B rings define the major classes. Six major sub-groups of flavonoids are identified, based on their molecular structures; flavanol (catechins and epicatechins), flavone (luteonin, apigenin, tangeritin), flavonol (quercetin, kaemferol, myricetin, isorhamnetin, pachypodol), flavanone (hesteretin, naringenin, eriodictyol), isoflavone (genistein, daidzein, glycitein) and anthocyanidin (cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin) (Rice-Evans et al., 1996; Ghasemzadeh and Ghasemzadeh, 2011).

Flavonoids are biosynthesized from phenylalanine (via shikimic acid pathway) and malonate (via acetate pathway) and they are observed mainly in plants, and also in a few fungi and marine corals (Iwashina, 2003). Among the approximately 70,000 phytochemicals identified, a large number are found in a glycosylated form, having higher water solubility and lower reactivity, which makes them more chemically stable compared to their aglycones. In plants, they become transportable storage compounds (Ford et al., 1998). In flavonoid chemistry, many different substitution patterns can be

seen in the aglycone structure via glycosylation, acylation, and methylation building a structural diversity (Masada et al., 2009). Among them, flavonoids are mostly found in plants as their glycosides, in which one or more sugar groups are attached to the hydroxyl groups, in the aglycon structure via a hemiacetal link (Park et al., 2011). Further, the addition of more sugar groups to the already bound sugar groups results in more variations. Glycosylation is catalysed by glycosyltransferase (UGT) that attaches a sugar molecule from a uridine 5′-diphosphosugar (UDP-sugar) to a lower molecular weight acceptor substrate (Masada et al., 2009). UGT-catalyzed addition of more sugar groups to the glycosylated flavonoids was reported to modify the physical attributes, such as color and taste of the plant parts in which they occur (Masada et al., 2009). The preferred glycosylation sites on the flavonoids are the C-3 position and less frequently, the C-7 position. Glucose has been the most common sugar residue observed in glycosylation, but other sugars include galactose, rhamnose, and xylose (Rice-Evans et al., 1996). Quercetin (3,3',4',5,7-pentahydroxy flavone) is a commonly found dietary flavonol aglycone in fruits and vegetables and has attracted much research interest due to its significance in biological activities (Park et al., 2011).

Table 3.1: Distribution of major flavonoids in food sources (Majewska et al., 2011)

Type of flavonoid	Food source
Flavonols	Onion, Broccoli, Curly kale, Leek, Lettuce, String bean, "Cherry" tomato, Broad bean, Apples, Cherries, Apricots, Blueberries, Strawberries, Redcurrants, Grapes, Red wine, Tomato juice
Flavones	Parsley, Celery, Carrot, Chicory, Lettuce
Flavanones	Citrus fruit (e.g. Oranges, Lemons and Grapefruits), Tomatoes, Aromatic plants (e.g. Mint)

Type of flavonoid	Food source
Isoflavones	Leguminous plants (e.g. Soy and its products)
Flavanols	Green tea, Red wine, Beer, Cider, Chocolate, Apricot, Grapes, Peaches, Persimmons, Apples
Anthocyanins	Blackcurrants, Blackberries, Blueberries, Plums, Cherries, Strawberries, Eggplants, Rhubarb, Cabbage, Beans, Radish, Onions, Red wine, Cereals

3.1.2 Flavonoids as bioactives

Flavonoids as plant polyphenols, demonstrate a broad spectrum of biological activities (Nijveldt et al., 2001; Thilakarathna and Rupasinghe, 2012; Jone et al., 2012; Balasuriya and Rupasinghe, 2011) and provide many physical and biological attributes to the plants themselves. Functions of flavonoids include plant–insect relationships such as pollinator attractants, oviposition stimulants, feeding attractants, as well as feeding deterrents, including phytoalexins, disease resistance as antifungal agents (Iwashina, 2003; Harborne and Williams, 2000) and finally, providing physical characteristics such as color, and taste. These non-nutrient phytochemicals biosynthesised in fruits and vegetables provide protection and cure for various diseases (Boyer and Liu, 2004).

Therefore, flavonoids have become the focus of research, in both the pharmaceutical and food industries, as health promoting agents. The French paradox has been documented, where low incidence of cardiovascular disease occurs among Mediterranean people, despite their high intake of saturated fat; flavonoid research has received great attention since flavonoid-rich red wine is another major component of Mediterranean diet (Tapas et al., 2008). Moreover, after identifying the flavonoid rutin, which was previously thought to be a vitamin (namely vitamin P) isolated from oranges, a boost in research was

instigated for identifying individual flavonoids and studying their activities (Nijveldt et al., 2001).

The biological functions of flavonoids extend via various metabolic pathways. As a major role, flavonoids are reported to protect biological systems from oxidative damage; therefore, they are helpful in overcoming many pathological conditions in the human body (Boyer and Liu, 2004). Having excellent antioxidative properties, flavonoids exert anti-atherosclerotic effects by inhibiting low density lipoprotein (LDL) oxidation (Thilakarathna et al., 2013). Some phenolic compounds are reported to inhibit the release of arachidonic acid in the cells and also, inhibit the cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) pathways, thereby preventing the activation of the inflammatory cascades. Deoxyribonucleic acid (DNA) damage, due to reactive oxygen species (ROS), can lead to mutations which may cause the initiation or progression of tumorigenesis. Flavonoids are reported to have anti-tumorogenic effects and act as anti-carcinogenic agents. Moreover, many *in vitro* studies have demonstrated that flavonoids possess diverse biological activities, such as being cardiotonic, lipid lowering, antiulceractive, hepatoprotective, anti-inflammatory, antineoplastic, antimicrobial (antibacterial, antiviral, antifungal), vasorelaxant, anti-thrombogenic and anti-osteoporotic (Kenekt et al., 1997; Nijveldt et al., 2001; Tapas et al., 2008). Therefore, fruit and vegetable rich-diet provides a solution for many chronic diseases prevalent in the western world, such as coronary heart diseases and different cancers (Boyer and Liu, 2004; DuPont et al., 2000; Thilakarathna and Rupasinghe, 2012).

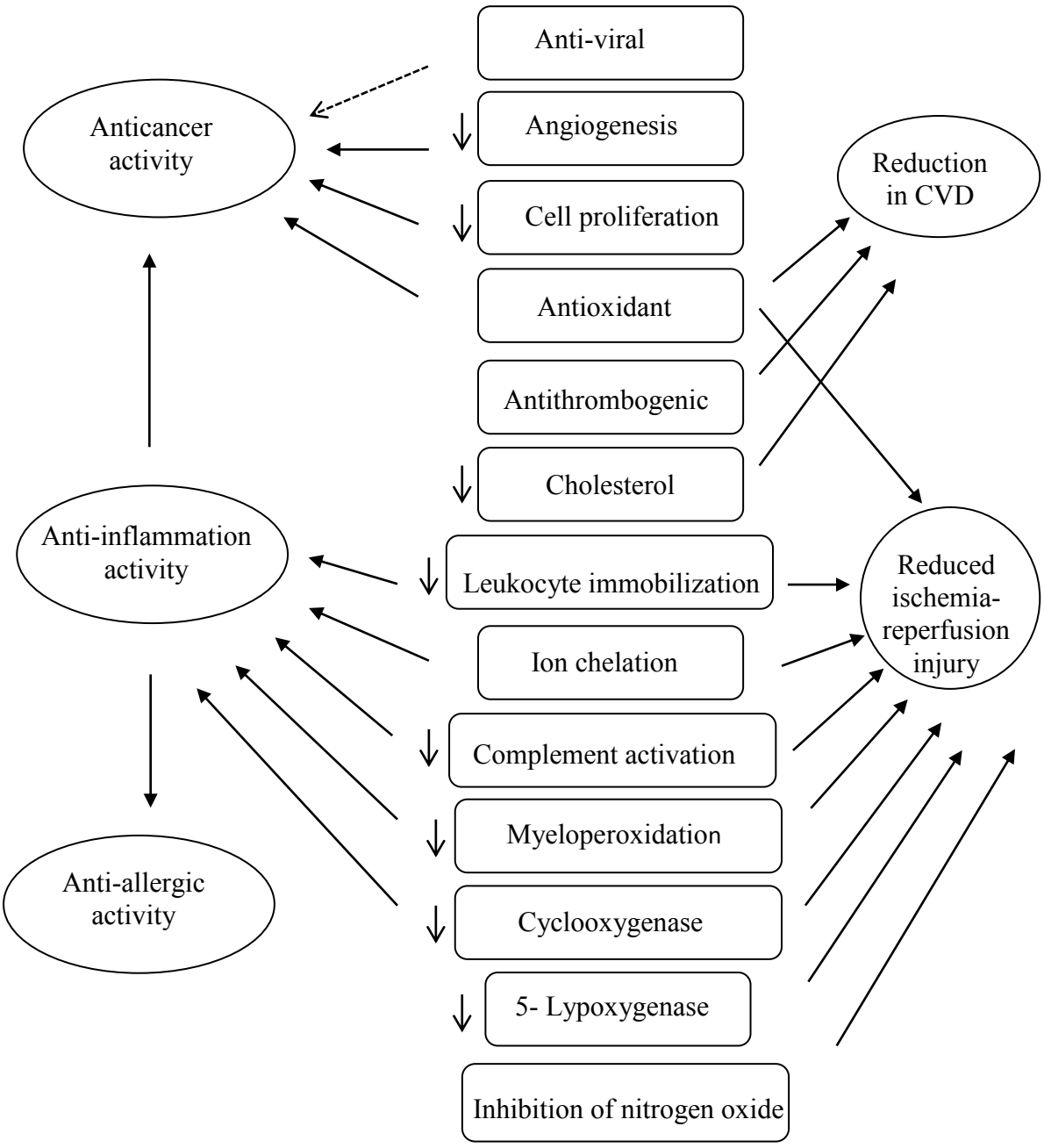


Figure 3.1: Biological effects of flavonoids in relation to disease conditions (Nijveldt et al., 2001)

3.1.2.1 Antioxidant activity of flavonoids

"For a polyphenol to be defined as an antioxidant, it must satisfy two basic conditions: first, when present in low concentration relative to the substrate to be oxidized, it can delay, retard, or prevent the autoxidation or free radical-mediated oxidation; second, the resulting radical formed after scavenging must be stable through intramolecular hydrogen bonding on further oxidation" (Rice-Evans et al., 1996). Antioxidants are non-enzymatic defence compounds of organisms against ROS and reactive nitrogen species (RNS). Most antioxidant compounds are introduced through diet (Berker et al., 2007). Due to their ability to act as hydrogen or electron donors, flavonoids are known to possess strong free radical scavenging activities and dietary flavonoids have been shown to exert significant antioxidant effects (Lue et al., 2010; Gupta et al., 2010). The role of flavonoids as antioxidants can be explained by their various functions: scavenging free radicals, quenching singlet oxygen, disconnecting radical reactions, chelating metals and inhibiting certain enzymes (Majewska et al., 2011; Hendrich, 2006). Another possible contributory mechanism to the antioxidant activity of flavonoids is their ability to stabilise membranes by decreasing membrane fluidity. Many studies have reported that flavonoids are promising antioxidants, as their conjugated ring structure and hydroxyl groups are capable of scavenging superoxide anions ($O_2^{-\bullet}$), singlet oxygen, lipid peroxy radicals and hydroxyl radicals (Gupta et al., 2010; Harborne and Williams, 2000). Furthermore, flavonoids enhance the production of antioxidant enzymes and also inhibit the enzymes, such as xanthine oxidase, protein kinase C, which are responsible for superoxide anion production, and other enzymes involved in ROS generation, such as COX, LOX, microsomal monooxygenase, glutathione s-transferase, and mitochondrial

succinoxidase, nicotinamide adenine dinucleotide (NADH) oxidase (Gupta et al., 2010). Flavonoids are reported to decrease the number of immobilized leukocytes, which is a protective measure for inflammation-like conditions such as reperfusion injury (Nijveldt et al., 2001).

Biological and pharmacological attributes of flavonoids are mainly due to their activity as antioxidants (Majewska et al., 2011). The interference of flavonoids with the free radical producing system can be described in several ways. The direct scavenging of radicals makes the radical a non-radical and the flavonoid itself becomes a stable radical (Nijveldt et al., 2001).

$$\text{Flavonoid(OH)} + \text{R}\bullet \longrightarrow \text{Flavonoid(O}\bullet\text{)} + \text{RH}$$

The structure of the flavonoid molecule has a great influence on its biological activity. The number and location of hydroxyl groups on the flavonoid structure have a significant impact on its antioxidant activity (Pawlak et al., 2010) and the number of hydroxyl groups has a linear relationship with the peroxyl radical absorbing activity (Cao et al., 1997). The hydroxyl configuration of the B-ring provides significant support for scavenging ROS (Heim et al., 2002). *O*-Dihydroxy structure (3′, 4′-catechol structure) in the B ring and the presence of 3-hydroxyl group are highly determinative for the radical scavenging activity (Tournaire et al., 1993; Heim et al., 2002). Further, the 2,3-double bond, in conjugation with the 4-oxo function in the C ring, helps to delocalize electrons from the B ring. The C2-C3 double bond of the C ring stabilizes the resulting phenoxyl radical. The 3-OH group on the C-ring makes the molecule a highly active scavenger. The 5-OH and 7-OH groups may also help in the scavenging potential in certain cases

(Tapas et al., 2008; Pawlak et al., 2010). It has been proposed that the 3,4-dihydroxy moiety of the catechol rings, the 3-hydroxy-4-keto or the 5-hydroxy-4-keto groups function as possible binding sites for chelating metals, which is an important role in antioxidant activity (Harborne & Williams, 2000).

The methods most commonly used in the determination of total antioxidant capacity are found in two major groups. The assays based on a single electron transfer reaction are monitored through a change in colour as the oxidant is reduced. Secondly, the assays are based on a hydrogen atom transfer reaction, where the antioxidant and the substrate compete for free radicals (Huang et al., 2005; Prior et al., 2005). There is no single method adequate for evaluating the antioxidant capacity of foods, since different methods can yield widely diverging results. Therefore, various methods, based on different mechanisms, must be used to get more accurate evaluation (Tabart et al., 2009). Furthermore, flavonoids present in a mixture can interact and their interactions can affect the total antioxidant capacity of a solution. Thus, to obtain a synergistic effect in designing new dietary supplements or fortified foods, choosing the best combinations of flavonoids is important (Hidalgo et al., 2010; Sun et al., 2011).

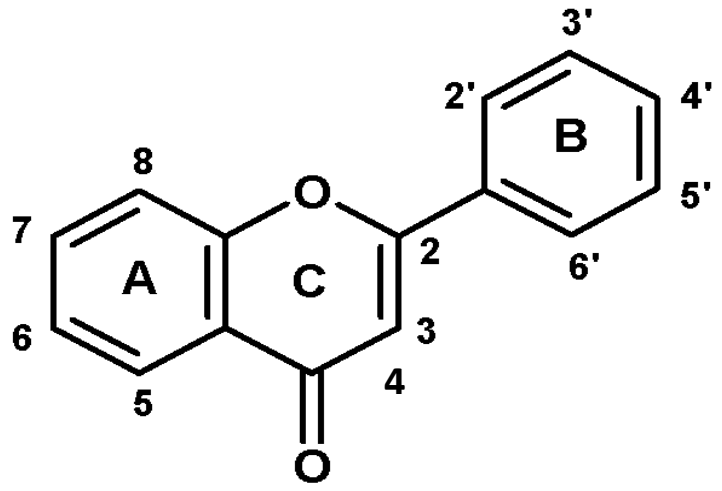


Figure 3.2: Basic structure of flavonoid (Pawlak et al., 2010)

3.2 LIPID OXIDATION IN FOOD SYSTEMS

3.2.1 Importance in dietary lipids

Nowadays, consumption of dietary lipids, especially polyunsaturated fatty acids (PUFA), has been received a great focus in nutritional recommendations due to their importance in clinical benefits, preventing chronic diseases such as cardiovascular disease (CVD) and cancer (Russo, 2009; Sun et al., 2011). Although fatty acids can be synthesised in the human body, there are essential fatty acids, linoleic acid (n-6) and α-linolenic acid (ALA, n-3), which cannot be synthesised and therefore, have to be obtained through the diet (Russo, 2009). Linoleic acid is the parent fatty acid of the n-6 series of PUFA and leads to the synthesis of other n-6 fatty acids, such as AA (C-20). ALA is the parent fatty acid of the n-3 series of fatty acids and eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) are other important n-3 fatty acids, derived from ALA. Whereas the synthesis of those n-3 fatty acids in the human body is low and

not adequate to fulfil bodily requirements, it is necessary to obtain them through diet. Therefore, they may also be considered as essential fatty acids (Rubio-Rodríguez et al., 2010). The food sources high in n-6 fatty acids are vegetable oils, such as soybean oil, corn oil, sunflower oil, safflower oil and cotton seed oils. Linseed oil and canola oil are rich sources for n-3 fatty acids (Russo, 2009) and EPA and DHA are abundantly found in shellfish and fish, such as anchovy, bluefish, herring, mackerel, mullet, sardines, salmon, sturgeon, tuna and trout, which are well known sources of fish oil high in PUFA (Siriwardhana et al., 2012). There is a growing trend in fortifying food products with n-3 and n-6 fatty acids since their clinical advantage has been documented. The health promoting effects of n-3 PUFA extends to improved brain function, reduced CVD, inflammation, aging, rheumatoid arthritis, depression, healthy pregnancy and prevention of certain cancers (Siriwardhana et al., 2012; Rubio-Rodríguez et al., 2010).

The edible oils provide not only nutrition, but also palatability and satiety to the diet. However, lipids are highly susceptible to oxidative deterioration, which is a major cause of quality loss in lipid based food. This includes nutritional loss, rancid flavor, texture, appearance and production of toxic substances, as well as a shorter shelf life. Although unsaturated fatty acids in triglycerides or phospholipids are not responsible for the aroma in food, the off aroma and flavour of their decomposed products are acceptable for only a few food items, like cheese and dried cereals. In order to overcome the oxidation process and protect the functionality of lipid-based foods, different techniques are applied in the food industry. Among them, the use of antioxidants is the most practical and effective method for prevention of lipid oxidation (Chaiyasit et al., 2007; Nawar, 1996; Sun et al., 2011). Natural antioxidants are receiving a great deal of attention since they are safe for

consumption and possess many health promoting activities (Frankel, 1996; Kathirvel and Rupasinghe, 2011).

3.2.2 Mechanism of lipid oxidation

Lipid oxidation occurs in lipid-based foods due to the reaction of unsaturated fatty acyl groups with oxygen via photoxidation, autoxidation or enzyme-catalysed oxidation. The most common pathway of oxidation is autoxidation; unsaturated lipid and oxygen react spontaneously with no involvement of light or any other catalysts (Chaiyasit et al., 2007: Sun et al., 2011). Oxidation usually takes place in three steps; initiation, propagation and termination (Nawar, 1996). In the initiation step, oxidation begins with hemolytic cleavage of the C-H bond on a carbon atom next to a double bond (α-carbon). This process can occur thermally, photochemically or by abstraction of an H atom by an initiator, which is a free radical such as reactive oxygen or nitrogen species (Sun et al., 2011; Porter et al., 1995). During propagation, oxygen reacts with the lipid radical ($L^{\bullet}$) forming lipid peroxyl radical ($LOO^{\bullet}$). These peroxyl radicals can abstract hydrogen from another fatty acid to form a lipid hydroperoxide (LOOH) and another lipid radical ($L^{\bullet}$). Then, the process is terminated by the reaction of two radical species to form a non-radical molecule. Prooxidants, such as transition metal ions, photosensitizers, ultraviolet (UV) light and certain enzymes, accelerate the reaction (Chaiyasit et al., 2007; Wanasundara and Shahidi, 2005).

Below is the simple outline of the reactions in lipid oxidation (Chaiyasit et al., 2007).

Initiation: $R^{\bullet} + LH \longrightarrow L^{\bullet} + RH$

Propagation: $L^{\bullet} + O_2 \longrightarrow LOO^{\bullet}$

$LOO^{\bullet} + LH \longrightarrow LOOH + L^{\bullet}$

Termination: $LOO^{\bullet} + LOO^{\bullet} \longrightarrow LOOL + O_2$

$LOO^{\bullet} + L^{\bullet} \longrightarrow LOOL$

$L^{\bullet} + L^{\bullet} \longrightarrow LL$

The hemolytic cleavage of lipid hydroperoxide yields an alkoxyl radical ($LO^{\bullet}$) and hydroxyl radical ($^{\bullet}OH$). The more energetic alkoxyl radical ($LO^{\bullet}$) can then undergo many reactions, producing secondary oxidation products, which are volatile and responsible for the rancid flavour and odours. Importantly, β scission reaction involving alkoxyl radicals decomposes the unsaturated fatty acids and the formed radicals react with a range of compounds and a variety of secondary oxidation products are yielded, such as aldehydes, ketones, alcohols, furans, hydrocarbons (Chaiyasit et al., 2007). It has been reported in the literature that the physical properties of food have a dramatic effect on lipid oxidation chemistry (Chaiyasit et al., 2007).

3.2.2.1 Lipid oxidation in bulk oil

In general, bulk oil is considered as a homogenous system of triacylglycerols. However, it consists of many minor components, such as mono- and diacylglycerols, free fatty acids, sterols, phospholipids, tocopherols and tocotrienols, trace metals (e.g. iron, copper, manganese, nickel), lipid oxidation products, and water, which also play an important role in oxidation (Chaiyasit et al., 2007). Several suggestions have arisen about the location of the oxidation process in the bulk oil. According to polar paradox theory, it occurs in the oil-air interface, but it has also been suggested that oxidation takes place in the association colloids in bulk oil (Chaiyasit et al., 2007). Association colloids are micelles and lamellar structures, formed by surface active molecules in the presence of a small quantity of water. Therefore, the nano and micro environments within the bulk oil

also play an important role as they can change the physical location of the oxidation substrate, prooxidants and antioxidants. The rate of oxidation of bulk oil increases with the degree of unsaturation of the lipids (McClements and Decker, 2000).

3.2.2.2 Lipid oxidation in colloidal suspensions

Most of the lipid foods are found in colloidal forms which consist of two immiscible liquids, water and oil. It can be oil-in-water emulsion, where oil droplets are dispersed in the water phase (e.g. milk, mayonnaise, salad dressing, dips, sauces, ice-cream) or water-in-oil emulsions, where water droplets are dispersed in the oil phase (e.g. butter and margarine) (McClements and Decker, 2000). These systems are thermodynamically unstable (Dickinson, 1992) and therefore, surface active molecules need to be added to obtain a kinetically stable emulsion by decreasing the surface tension and they are termed emulsifiers. During homogenization, emulsifiers are absorbed into the droplet surface, forming a protective layer so that droplets are not able to come into contact with each other (Chaiyasit et al., 2007).

Lipids in food systems are more commonly found as oil-in-water emulsions (Sun et al., 2011). They consist of three main regions; oil droplet, aqueous phase and the interfacial layer, which is surrounding the oil droplets. The surface active molecules, such as emulsifiers, are found in this narrow region. Therefore, when any compound is added into an emulsion, it moves into one of these three regions, according to its solubility and surface activity (Chaiyasit et al., 2007). The factors which influence the rate of oxidation in oil-in-water emulsions are: fatty acid composition, aqueous phase pH and ionic composition, type and concentration of antioxidant and prooxidants, oxygen

concentration, lipid droplet characteristics such as particle size, concentration and physical state and emulsion droplet interfacial properties, such as thickness, charge, rheology, permeability (Waraho et al., 2011). The surface to volume ratio of oil-in-water emulsion is higher than that of bulk oil. Therefore, the oxidation process is more rapid in oil-in-water emulsions. Further, the prooxidants present in the aqueous phase can react with the oil phase more effectively when there is a high surface area to make interactions (Waraho et al., 2011). However, the advantage is that oxidation control technologies can be incorporated into either the lipid droplet, interfacial or aqueous phase. The difference of oil-in-water emulsions over the bulk oil, in terms of oxidation, is that in emulsions, both antioxidants and prooxidants are present in the aqueous phase. Also, the oil-water interface has an impact on the interaction between the two phases (Waraho et al., 2011). The activity of antioxidants in a colloidal system highly depends on the composition of the system (Schwarz et al., 2000).

3.2.3 Antioxidants in lipid-based food system

Saturated fatty acids are less prone to oxidation than unsaturated fatty acids and therefore, using saturated fatty acids as the major lipid source is one way of minimizing rancidity in emulsions. However, due to the favourable sensory and physical attributes, as well as the clinical benefits of unsaturated fatty acids over the saturated fatty acids, the above strategy is not feasible (McClements and Decker, 2000). Incorporation of antioxidants is the most effective method in inhibiting lipid oxidation in food systems. The safety and health benefits associated with natural antioxidants have attracted both consumer and market attention and there is a progressive use of natural antioxidants in foods, in preference to synthetic antioxidants (Frankel, 1996).

Antioxidants can be categorized into two main groups; primary antioxidants, which are chain breaking antioxidants and secondary antioxidants, which are also known as preventive antioxidants. Primary antioxidants scavenge free radicals and thereby delay or inhibit the initiation step or disturb the propagation of the radical chain reaction of the autoxidation pathway. The formed antioxidant radicals can soon undergo termination by reacting with another free radical molecule. Primary antioxidants in foods are either synthetic or natural antioxidants. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate, and tertiary butylhydroquinone (TBHQ) are synthetic phenolic primary antioxidants. Flavonoids, vitamin E, tocopherols and carotenoids are commonly used natural compounds (Kathrivel and Rupasinghe, 2011). At higher concentrations, phenolic antioxidants can act as prooxidants (Sun et al., 2011; Wanasundara and Shahidi, 2005; Dauqan et al., 2011). The mechanism of primary antioxidants is as follows:

$$LOO\bullet + AH \longrightarrow LOOH + A\bullet$$

$$LO\bullet + AH \longrightarrow LOH + A\bullet$$ (Frankel, 1996)

Secondary antioxidants do not act to stabilize free radicals, but react on other mechanisms, such as chelating metal ions, deactivating singlet oxygen, scavenging oxygen and absorbing UV radiation. Moreover, they can provide H for primary antioxidant radicals and detoxification of ROS enzymatically. Since they enhance the activity of primary antioxidants, they are also known as synergists. Citric acid, ascorbic acid, ascorbyl palmitate, lecithin and tartaric acid are examples of the secondary antioxidants (Sun et al., 2011; Wanasundara and Shahidi, 2005). Quercetin glycosides are a major type of flavonol, abundantly found in apples, acting as antioxidants by stabilizing

n-3 PUFA containing foods (Huber et al., 2009). Polyphenol-rich apple skin extract is an underutilized bio-resource which can be used to prevent fish oil and EPA oxidation (Rupasinghe et al., 2010).

3.2.3.1 Polar paradox theory

The polar paradox theory of antioxidants describes that, polar antioxidants are more effective in the food systems with low surface to volume ratio, such as bulk oil and lipophilic antioxidants are favoured in foods with high surface to volume ratio, such as oil-in-water emulsions. Therefore, polar antioxidants with high hydrophilic-lipophilic balance (HLB), such as propyl gallate, TBHQ, and Trolox are good for applying in bulk oils. Non-polar lipophilic antioxidants of low HLB such as BHA, BHT, and tocopherols can be used in oil-in-water emulsion systems (Porter, 1980). In oil-in-water emulsions, non-polar antioxidants are sufficiently surface active to be oriented in the oil-water interface. When hydrophilic antioxidants partition into the aqueous phase, they get diluted and are not in sufficient concentration to provide enough antioxidant activity. The interfacial phenomenon has a major effect on oxidative stability of the antioxidants (Frankel et al., 1994).

The polar paradox theory may be applicable only over certain concentration ranges of the antioxidants. The solubility and interfacial phenomena dominate one over the other, depending on the concentration of antioxidants (Zhong and Shahidi, 2011). The activity of antioxidants in oil-in-water emulsions cannot be decided considering the polarity of the antioxidant compound as the sole factor (Waraho et al., 2011). Practically, food systems contain many other components and they can interact with antioxidants to alter their functionality (Waraho et al., 2011).

3.3 CELLULAR OXIDATIVE STRESS

3.3.1 Cellular oxidative stress

Molecular oxygen is essential for the aerobic energy production processes in cells. As a by-product of the cellular respiration, partially reduced forms of molecular oxygen, such as superoxide, are generated in the cells and they are termed ROS due to their higher reactivity than molecular oxygen. The mitochondrion is considered as the major intracellular site for producing ROS (Ryter et al., 2007). In addition, activities of several enzymes, such as peroxisomal oxidases, cytochrome P-450 enzymes, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and xanthine oxidase, are responsible for the production of cellular ROS during metabolic reactions as well as during the process of antibacterial defense (Kregal and Zhang, 2007; Harborne and Williams, 2000). Other than the endogenous production, there are exogenous factors, such as xenobiotics, drugs and environmental factors, such as exposure to UV light and ionizing radiation, cigarette smoke, organic and inorganic pollutants, which can result in elevated levels of ROS in cells (Kregal and Zhang, 2007). The oxidative stress in cellular systems can be assessed through measuring its biomarkers, such as production of ROS, antioxidant levels, and products of oxidation and antioxidant/pro-oxidant balance (Powers and Jackson, 2008). Cells themselves contain defense mechanisms for maintaining ROS within safe levels and when this homeostasis cannot be maintained, it is termed oxidative stress (Ryter et al., 2007; Avery, 2011).

3.3.1.1 Reactive oxygen species

Most ROS are chemical species with unpaired electrons, such as superoxide radical ($O_2^{-\bullet}$) and hydroxyl radical ($^{\bullet}OH$). Hydrogen peroxide (H_2O_2) is an example of a non-

radical ROS (Avery, 2011). Superoxide anion and nitric oxide are the primary free radicals generated in the cells and readily react with other molecules to form many other reactive oxygen and nitrogen species (Kregal and Zhang, 2007; McCord, 2000). Superoxide anion is formed in cellular systems as a by-product of the electron transport chain in cellular respiration and as a specific product of enzymatic reactions. It is relatively impermeable for the cellular membranes and has a long half-life which keeps the anion for a considerable time inside the cells, to react with many other molecules. It dismutates spontaneously or in the presence of superoxide dismutase, acts as a biocatalyst, producing hydrogen peroxide in the cells, as below (Powers and Jackson, 2008; Ryter et al., 2007; McCord, 2000).

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \longrightarrow H_2O_2 + O_2$$

Moreover, there are other enzymes that produce H_2O_2 in the cells, such as urate and amino acid oxidases (Powers and Jackson, 2008). Inside the cells, H_2O_2 is relatively membrane permeable, less reactive and a mild oxidant with a long half-life (Ryter et al., 2007). However, the metal catalyzed production of hydroxyl radicals from H_2O_2, via Fenton like reactions, can implicate the toxicity in the cellular environment (Matés et al., 1999).

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH$$

Upon generation, the hydroxyl radicals readily react with the molecules in their vicinity as they are strong oxidizing agents and therefore, considered as the most detrimental ROS in biological systems (Powers and Jackson, 2008).

Singlet oxygen, another type of non-radical ROS, is an excited oxygen molecule with higher oxidizing ability (Powers and Jackson, 2008). Also, nitric oxide is generated in cells through nitric oxide synthases from oxidizing L-arginine amino acid to L-citrulline. It reacts with oxygen forming nitric dioxide and forms peroxynitrite by the reaction with superoxide anion. The generation of peroxinitrite is faster than the reaction producing H_2O_2 from the superoxide anion. Peroxinitrite has an ability to diminish thiol groups, attack DNA molecules and proteins (Ryter et al., 2007; Powers and Jackson, 2008).

3.3.1.2 Effect on cellular environment

Since ROS are generated during normal cellular processes, cells continuously face a risk of ROS damage (Nijveldt et al., 2001). Under normal physiological conditions, homeostasis is maintained through antioxidant defenses. Further, ROS play an important role by being involved in cellular signal transduction (Estany et al., 2007; Avery, 2011). Under oxidative stress conditions, the high levels of cellular ROS are detrimental to biological materials, especially cellular macro molecules; nucleic acids, lipids and proteins, eventually resulting in cellular dysfunction and cell death (Ryter et al., 2007; Pizarro et al., 2009). Various biochemical pathways are involved in causing these effects. Among them, mitogen-activated protein kinase (MAPK) associated pathways and nuclear factor κB (NF-κB) pathway predominate (Powers and Jackson, 2008). Therefore, oxidative stress contributes various pathological conditions, such as cancer, neurological disorders, CVD, lung inflammation, rheumatoid arthritis and aging (Pizarro et al., 2009).

In PUFA, the bis-allylic structures make them more susceptible to the attack by ROS (Kregal and Zhang, 2007). Lipid peroxidation is considered to be a primary result of elevated cellular ROS levels. Oxidation of phospholipids in the plasma membrane and

organelle membranes causes loss of membrane and organelle functions (Ryter et al., 2007). Since cell membranes are selectively permeable by nature, an increase in membrane fluidity, due to membrane lipid oxidation, affects cellular transport ultimately causing a decrease in cell viability (Kregal and Zhang, 2007; Ryter et al., 2007). Lipid peroxidation results in many oxidized products, such as epoxides and aldehydes [e.g. malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), 2-propanol (acrolein), F_2-isoprostane] and they are used as biomarkers of cellular oxidative stress. MDA is mutagenic and causes the formation of DNA adducts. It is reported that some lipid oxidation products activate the stress-response signal transduction pathways. Loss of activities of membrane bound proteins and increased efflux of cytosolic solutes are other detrimental effects of lipid peroxidation (Kregal and Zhang, 2007; Avery, 2011; Deavall et al., 2012).

Oxidative stress can damage both nuclear and mitochondrial DNA and it is one of the major causes for onset of cancer (Waris and Ahsan, 2006; Kregal and Zhang, 2007). The highly reactive hydroxyl radicals abstract H and undergo 'addition' reactions with double bonds in DNA. These reactions lead to the formation of oxidized bases, abasic sites and breaks in the DNA; most of these resulting lesions are mutagenic. The elevated levels of oxidative DNA damage cause pathological conditions, such as acute lymphoblastic leukemia, hematological disorders, Parkinson's disease, Alzheimer's disease and cancer (Cooke et al., 2003). Because of the low oxidation potential, guanine is highly vulnerable to oxidative damage and forms 8-oxo-7,8-dihydroguanine (8-oxoGua) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapGua), which are considered as common

biomarkers of oxidative stress (Deavall et al., 2012). These damages are associated with p53 activated apoptosis (Kregal and Zhang, 2007).

Some proteins, like phosphatases, kinases, transcription factors and metabolic enzymes, are highly sensitive to the oxidative reactions, resulting in dysfunction and harm to cellular homeostasis (Deavall et al., 2012; Avery, 2011). Oxidative damage leads to the aggregation of proteins and it is believed to be the reason for death of neurons in neurodegenerative diseases related to aging. Formation of disulfide bonds at amino acid residues of proteins is a widely studied oxidative modification of proteins (Kregal and Zhang, 2007). Oxidative modification of LDL, the major lipid carrier in plasma, takes place in the artery wall. The PUFA as well as the apolipoprotein B100 (apo B) present in LDL undergo oxidation. The oxidation of PUFA leads to a series of aldehydes and ketones, while apo B is fragmented producing peptides. Oxidized LDL is atherogenic and activates the generation of lesions, associated with the onset of the atherosclerosis. Among them, the fatty streaks including subendothelial lipid-laden foam cells are most commonly observed. In addition, stimulation of monocyte adhesion, enhanced cytotoxicity, alteration of the expression of cytokines and growth factors occur (Yao et al., 2012; Matsuura et al., 2008; Yoshida and Kisugi, 2010).

3.3.1.2.1 Oxidative stress mediated cell death

Oxidative stress can induce cell death via apoptosis and necrosis pathways, but mainly causes necrotic cell death (Pizarro et al., 2009; Nanji and Hiller-Sturmhofel, 1997). Series of cellular biochemical pathways are involved in apoptosis, also described as a programmed cell death. In contrast to that, in necrosis, cells are lysed, due to the acute injuries which occur from the cellular environment (Nanji and Hiller-Sturmhofel, 1997;

Ryter et al., 2007). Therefore, apoptosis has been called cell suicide and necrosis, a biological accident (Nanji and Hiller-Sturmhofel, 1997). These two processes can be identified through the differences in the morphological and biochemical changes taking place in the cells (Ryter et al., 2007).

Apoptosis carries out important functions in the cells by maintaining tissue homeostasis, but is also associated with pathogenesis of diseases (Drakopnagiotakis et al., 2008; Ryter et al., 2007). During the early apoptosis, mitochondria are affected adversely in several ways, such as by impairment of their functions, inhibition of respiratory chain reactions, loss of inner membrane potential and increased permeability of the membrane (Ryter et al., 2007). Also, the outer cell membrane is inversed with the externalization of phosphatidylserine (Fiers et al., 1999). The cardinal characteristic of the later phase of apoptosis is DNA fragmentation into various sizes. During apoptosis, cells shrink and membrane blebbing can be observed (Ryter et al., 2007). Also, cells break down into fragments called apoptotic bodies, which still contain functional cell components due to continuous production of proteins and adenosine triphosphate (ATP) (Nanji and Hiller-Sturmhofel, 1997). Eventually, these apoptotic bodies are taken up by phagocytes (Ryter et al., 2007). There are two apoptotic pathways: mitochondrial dependent intrinsic pathway and receptor dependent extrinsic pathway and both are regulated by Bcl-2 family proteins (Ryter et al., 2007). Many protein responses in the cells are associated with the intrinsic pathway. Further, apoptosis occurs via caspase dependent or caspase–independent pathways (Kregal and Zhang, 2007; Drakopanagiotakis et al., 2008).

In contrast, malfunctioning metabolic processes and loss of cell membrane integrity are the main characteristics of necrotic cell death. Cellular necrosis initiates with the swelling

and loss of functionality of the organelles. Also, bubble like projections called blebs are formed by the cell membrane. After this, the cell membrane ruptures, leaking the cellular components into the nearby cellular environment and this is called cytolysis. This can cause inflammation in surrounding tissues (Nanji and Hiller-Sturmhofel, 1997).

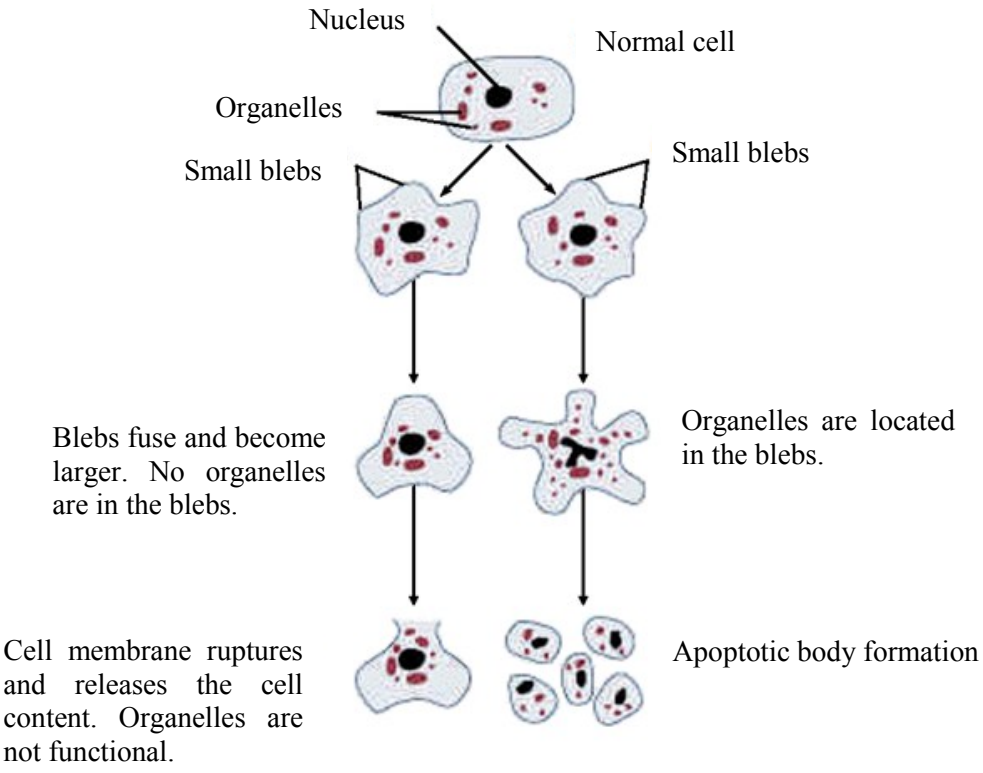


Figure 3.3: Morphological changes in necrosis and apoptosis (Nanji and Hiller-Sturmhofel, 1997)

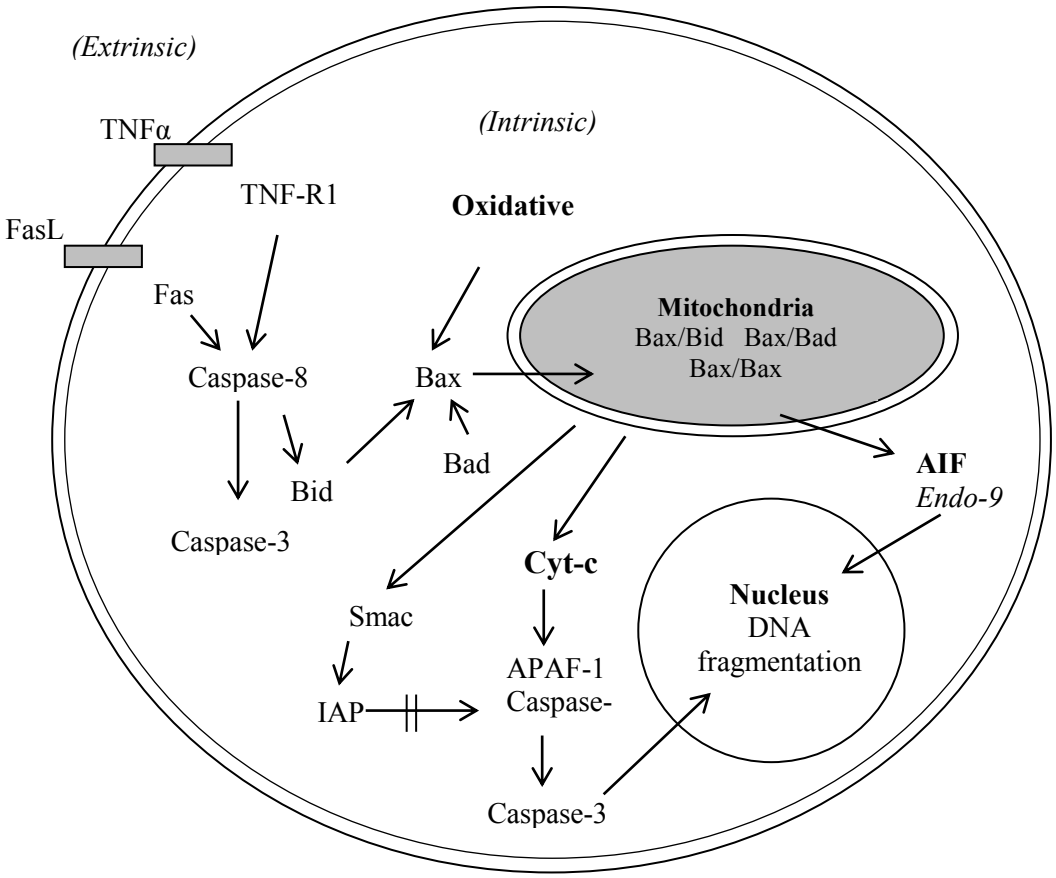


Figure 3.4: Extrinsic (receptor-dependent) and intrinsic apoptosis (mitochondrial) pathway (Ryter et al., 2007). (Bax, Bcl-2-associated X-protein; Bad, Bcl-XL/Bcl-2-associated death promoter; Bid, BH3 interacting domain death agonist; Cyt c, cytochrome c; IAP, inhibitor of apoptosis proteins; TNF α, tumor necrosis factor alpha; TNF-R, tumor-necrosis factor receptor; FasL, Fas ligand: Smac, second mitochondria-derived activator of caspase; APAF-1, apoptotic protease activating factor-1; AIF, apoptosis inducing factor)

3.3.2 Cellular antioxidant defense mechanisms

Living beings themselves have protective mechanisms against damage due to ROS. These antioxidant defense mechanisms mainly include antioxidant enzymes and small molecular weight, non-enzymatic compounds which metabolically eliminate excess ROS from cells and maintain the antioxidant/pro-oxidant balance. Superoxide dismutases (SOD), catalase and different peroxidases, such as glutathione peroxidase, act as primary antioxidant enzymes. SOD are present in mitochondria, cytoplasm, and also extracellularly and convert the superoxide anion to H_2O_2. The catalase enzyme is responsible for the breaking down of H_2O_2 to water and oxygen. Moreover, there are secondary antioxidant enzymes which provide cofactors for the activities of primary antioxidant enzymes. Examples for non-enzymatic antioxidants present are glutathione (GSH), NADPH, thoredoxin, vitamin E and C and trace metals like selenium (Kregal and Zhang, 2007; Ryter et al., 2007; Nijveldt et al., 2001).

3.4 CIGARETTE SMOKE INDUCED INFLAMMATION

3.4.1 Oxidative stress-mediated lung inflammation

Inflammation is a protective biological mechanism which removes harmful agents (e.g. irritants, pathogens etc.) or damaged tissues and supports the repairing of tissues, restoring normal structure and function (Ivanenkov et al., 2008; Rahman and MacNee, 2000). In the inflammation process, the interaction between immune cells and non-immune cells occurs through different kinds of mediators, such as cytokines, growth factors, lipids, and enzymes. Generally, the normal inflammation process can be explained in three phases as acute, subacute and resolution. Vasodilation and capillary permeability take place locally during the acute phase and infiltration of inflammatory

cells to the affected area occurs during the subacute phase causing erythema, pain and edema, which are the cardinal signs of acute inflammation. The healing of the damaged area takes place in the resolution phase (Ballaz and Mulshine, 2003; Moldoveanu et al., 2009). Whereas, if these inflammatory responses occur continuously, they cause excessive tissue/cell damage, leading to chronic inflammation which lasts for several weeks, months or years (Rahman and MacNee, 2000).

It has been shown that lung cells release inflammatory mediators and cytokines/chemokines in response to oxidative/nitrosative stress (Rahman and MacNee, 2000). The cytokines regulate the inflammation process. There are pro-inflammatory cytokines [e.g. Interleukin (IL)-2, Interferon (IFN)-γ, IFN-α, IL-1α,β, TNF-α, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) which induce the inflammatory responses and anti-inflammatory cytokines (IL-4, IL-5, IL-10, and IL-13) which inhibit the inflammatory responses]. In addition, the enzymes phospholipase A2 (PLA2), COX-2 (also known as prostaglandin H synthase), NO synthase (NOS) and 5-LOX are also involved in the inflammatory cascade (Moldoveanu et al., 2009; Ballaz and Mulshine, 2003).

These cytokines/chemokines induce activation of transcription factors, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) to give inflammatory responses through different signalling pathways (Rahman and MacNee, 2000). Under normal physiological conditions, NF-κB is in the cytosol in its inactivated form, but upon activation, it is translocated to the nucleus and increases the release of the inflammatory markers, interleukin-8 and TNF-α (McDonald et al., 2012; Rahman and MacNee, 2000; Rahman et al., 2006). The activation of epithelial cells and resident macrophages are major

inflammatory responses and in addition, other inflammatory cells, such as neutrophils, eosinophils, monocytes and lymphocytes, are also recruited to the site of infection. The activation of the phagocytes generates superoxide anion which causes further oxidative stress in the cellular system. NADPH oxidase, which is found in epithelial cells and phagocytes, is considered as a main enzyme generating ROS. ROS damage cellular membranes by oxidizing the lipids. The formed lipid peroxides are toxic to the cells, but they also help in signal transduction for inflammatory responses (Oh and Sin et al., 2012; Rahman et al, 2006). It has been reported that both the cell-derived ROS and inhaled oxidants, such as environmental pollutants, cause lung inflammation. Moreover, the ROS released by activated phagocytes in the site of inflammation is considered as the main cause for cell damage and apoptosis in chronic inflammation (Ivanenkov et al., 2008; Rahman and MacNee, 2000).

3.4.2 Cigarette smoke related lung inflammation

In the United States, more than 400,000 annual deaths are associated with the exposure to cigarette smoke and, in adults, 13-15 years of life expectancy is lost due to smoking (Lee et al., 2012). Cigarette smoke is a highly complex mixture of more than 4500 components, in both gaseous and particulate phases, including carcinogens (e.g. methylcholanthrene, benzo-α-pyrenes and acrolein), cocarcinogens, mutagens, toxic substances (e.g. carbon monoxide, ammonia, acetone, nicotine and hydroquinone) and oxidants (e.g. superoxide, nitrogen oxides) (Stämpfli and Anderson, 2009). It has been reported that nearly 10^{14} free radicals are in a single cigarette puff (Foronjy and D'Armiento, 2006). Also, studies indicate that most toxic components are present in the particulate phase of the cigarette smoke. Most of the cigarette smoke's constituents are

dissolved in the oral or airway epithelial lining fluid and enter into systemic uptake. Both gaseous and particulate matters first interface with the immune system at the mucosal surfaces lining the oral cavity, sinuses, and airways (Lee et al., 2012; Martey, 2005; Hecht, 1999). The upper respiratory tract is the main target for the oxidants in the gaseous phase of the cigarette smoke, whereas the more stable tar phase oxidants can damage the lungs directly. The oxidative harm for cells results in the damaging of membrane lipids, DNA, proteins and carbohydrates creating pro-inflammatory effects. It is well-established that cigarette smoke constituents are responsible for airway inflammation, asthma and chronic obstructive pulmonary disease (COPD) as the main chronic conditions (Stämpfli and Anderson, 2009).

Isoprostanes are products of membrane phospholipid oxidation and can generate inflammatory responses in the lung. In macrophages, 8-isoprostane induces IL-8 and macrophage inflammatory protein (MIP)-1-alpha. Also, isoprostanes induce the influx of neutrophils to the lung. It has been reviewed by Foronjy and D'Armiento (2006) that an increased amount of serum F2-isoprostane level was reported in active smokers. Moreover, several animal studies have reported elevated levels of 8-OH-dG, an oxidative DNA adduct, upon exposure to cigarette smoke. As well, protein oxidation products were detected in lungs in a study carried out with guinea pigs (Foronjy and D'Armiento, 2006). Free radicals increase the release of mucin from airway epithelial cells through activation of epidermal growth factor receptor and thereby, trigger the MAPK pathway, which is considered to be the most involved pathway in the cigarette smoke induced inflammation process. The MAP kinases, such as extracellular signal-regulated kinases (ERKs), c-jun N-terminal kinases (JNKs) and p38, have a major role in lung inflammatory responses.

MAPK pathways are activated in COPD and asthma patients (Foronjy and D'Armiento, 2006). To protect against exposure to oxidants, the lungs have a well-developed antioxidant system (Rahman and MacNee, 2000).

3.4.3 Chronic inflammation and cancer

Inflammation can persist in an affected tissue, due to the existence of infection, periodic injury in the tissue or lack of anti-inflammatory mechanisms (Rodriguez-Vita and Lawrence, 2010). Although acute inflammation is therapeutic, chronic inflammation leads to many long-term pathological conditions, such as cancer, arthritis, atherosclerosis and autoimmune diseases (Aggarwal and Harikumar, 2009). Among cancer deaths, about 20% are due to inflammation and chronic infections (Greene et al., 2011).

Inflammation has a dual role in the incidence and progression of tumors; it can promote tumorigenesis and on the other hand, it can elevate anti-tumor immunity in the tissue. Inflammatory cells, mostly macrophages infiltrated in the tumor tissues and the cytokines present in the tumor micro-environment, play a critical role in their regulation and cancer progression. T helper-2 (TH2) cytokines, such as IL-4, IL-10 and IL-12, are observed in tumors at elevated levels and promote invasion, angiogenesis and metastasis by activating the macrophages into M2 phenotype. Whereas, T helper-1 (TH1) cytokines, such as IFN-γ and IL-12, promote anti-tumorigenesis. Some cytokines show both pro- and anti-inflammatory effects in the tumor microenvironment. TNF-α blockade is a treatment for some chronic inflammatory conditions, while it worsens others. NF-κB is a transcription factor which acts as a major link between inflammation and cancer; it has a dual nature by promoting and reducing inflammatory cascades. Lipid mediators, such as prostaglandins and leukotrienes which are produced from AA, also possess a very

important role in inducing malignancy in tissues (Moldoveanu et al., 2009; Rodriguez-Vita and Lawrence, 2010; Green et al., 2011).

It has been reported that smoking induces the risk of several cancers such as myeloid leukaemia and those associated with the lungs, bladder, cervix, liver, kidneys and the uterus (Stämpfli and Anderson, 2009). Cigarette smoke is an obvious risk factor for lung cancer due to the mutagenic constituents it contains, but it is reported that only approximately 1-5% of smokers develop lung cancer (Stämpfli and Anderson, 2009). It has been reviewed that patients with COPD have an increased risk for lung cancer. Also, it has been observed that there is a higher risk for lung cancer for smokers with COPD than the norm. Even after discontinuing the smoking, the risk of getting lung cancer can still exist (Brody and Spira, 2006).

3.4.3.1 COX-2 and prostaglandins as mediators of carcinogenesis

The production of eicosanoids is important for the expression of inflammatory responses in the affected tissues and occurs via three major pathways: the COX pathway which forms prostanoids, the lipoxygenase pathway which forms leukotrienes, and the cytochrome p450 pathway (Greene et al., 2011). Phospholipase A2 releases AA from the cellular membrane and then COX enzymes mediate the reaction of adding oxygen molecules to AA to form prostanoids (Hayashi et al., 2011). There are two isoforms of the COX enzyme. COX-1 is the constitutive isoform, which is considered as a house keeping gene. The inducible isoform, COX-2, is usually only expressed when inflammation is stimulated (Willoughby et al., 2000). COX-2 acts on AA in a two-step reaction where AA is first oxidized to form prostaglandin G_2 (PGG_2) using the cyclooxygenase catalytic site and then reduction takes place on PGG_2 to form

prostaglandin H_2 (PGH_2), using the peroxidase catalytic site (Hayashi et al., 2011). PGH_2 is further metabolized to form three main type of prostanoids (eicosanoids): prostaglandin (PGD_2, PGE_2, $PGF_{2\alpha}$), prostacyclin (PGI_2) and thromboxane A2. These prostanoids are responsible for different anti-inflammatory and pro-inflammatory cascades (Greene et al., 2011).

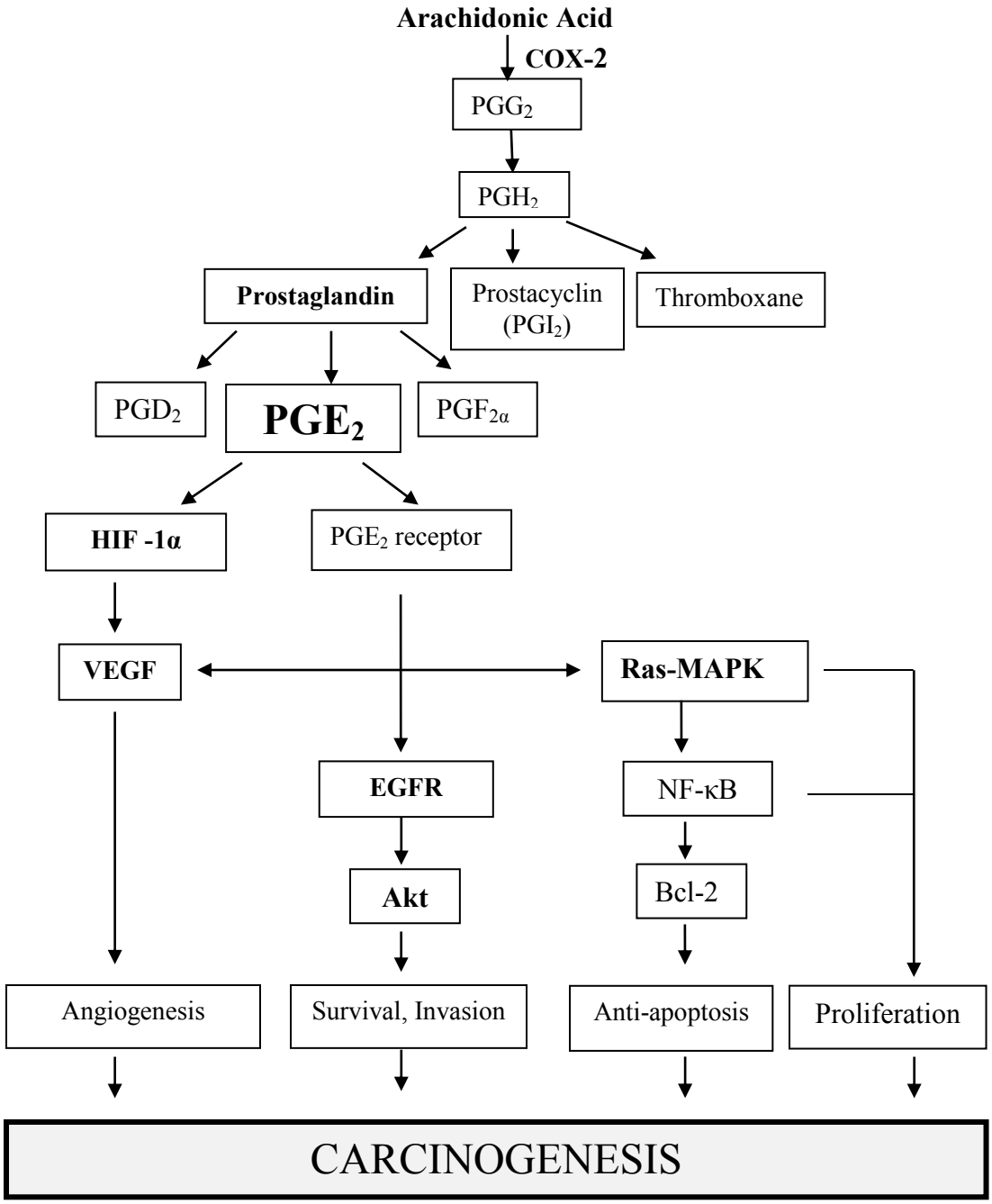


Figure 3.5: Role of PGE_2 in inflammation-induced carcinogenesis (modified from Kundu and Surh, 2008). PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; PGI_2, prostacyclin; PGE_2, prostaglandin E_2; PGD_2, prostaglandin D_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; COX-2, cyclooxygenase-2; HIF-1α, hypoxia inducible factor; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB.

Prostaglandins accelerate carcinogenesis through several mechanisms. One mechanism is associated with lowering the intracellular arachidonic acid levels since depletion of arachidonic acid drains the apoptotic signal, favoring cell survival (Kobayashi and Narumiya, 2002). Prostaglandins modulate cellular processes that govern the cell growth and differentiation. For instance, PGE_2 inhibits apoptosis by inducing the Bcl-2 protooncogene and enhances angiogenesis and adhesion by modulating the integrin expression. Another mechanism involves the effects of malondialdehyde, which is an endogenous mutagen, generated coordinately with the prostaglandin endoperoxides (Fitzpatrick, 2001).

PGE_2 is the most predominant prostaglandin contributing to inflammatory symptoms and tumor progression. Multidrug resistance associated proteins (MRPs) transport PGE_2 into extracellular micro-environments where it binds with specific G-protein coupled receptors in order to exert its functions. Pro-tumorigenic and immunosuppressive effects of PGE_2 are expressed via several mechanisms. PGE_2 induces the production of cytokines in T cells and increases the TH2-type responses while inhibiting the production of TH1 cytokines. Acting on activated macrophages, PGE_2 inhibits its production of certain cytokines; TNF-α, IL-1β, IL-8 and IL-12 (Menter and DuBois, 2012; Kobayashi and Narumiya, 2002).

The suppression of immune responses by inhibiting macrophages, T cells, natural killer cells and other immune cells, PGE_2 acts by inducing the pro-tumorigenic activity in the body. Tumor progression involves tumor inversion, angiogenesis and tumor growth and requires the increase of pro-inflammatory cytokines (Greene et al., 2011). PGE_2-mediated tumor progression includes altering cell morphology and increasing cell motility and

migration (Harris, 2006). PGE_2 is involved with several signalling pathways towards cancer progression, which include hypoxia-inducible factor (HIF-1α), vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR) and peroxisome proliferator-activated receptors. Ras-MAPK pathway and Akt pathway are also believed to be involved in PGE_2 mediated cancer progression (Aggarwal and Harikumar, 2009; Kundu and Surh, 2008).

3.5 ESTERIFICATION OF FLAVONOIDS

Since flavonoids demonstrate a wide range of activities in biological systems, incorporation of flavonoids into foods is an effective approach in formulating nutraceuticals. However, the moderately hydrophilic nature of glycosylated flavonoids results in low solubility and stability in both lipophilic and aqueous media and therefore, their applications are limited (Ardhaoui et al., 2004). Also, their biological activity depends on the degree of lipophilicity which affects their cellular uptake (Katsoura et al., 2007). These properties can be enhanced by modifying the flavonoid structure. The modification of flavonoids, by acylation with hydrophobic groups, improves the solubility in more hydrophobic environments (Ardhaoui et al., 2004). Either esterification or transesterification reactions can be selected to carry out the acylation through chemical, enzymatic or chemoenzymatic methods. However, chemical acylation has been found to be non-regioselective and results in unwanted chemical reactions. Thus, it is necessary to practise many protection or deprotection steps to obtain the desired end product. Therefore, enzymatic approach is the best solution for acylation since these

reactions are regioselective and moreover, mild temperature and pressure conditions give satisfactory results (Lue et al., 2010; Ardhaoui et al., 2004; Chebil et al., 2006; 2007).

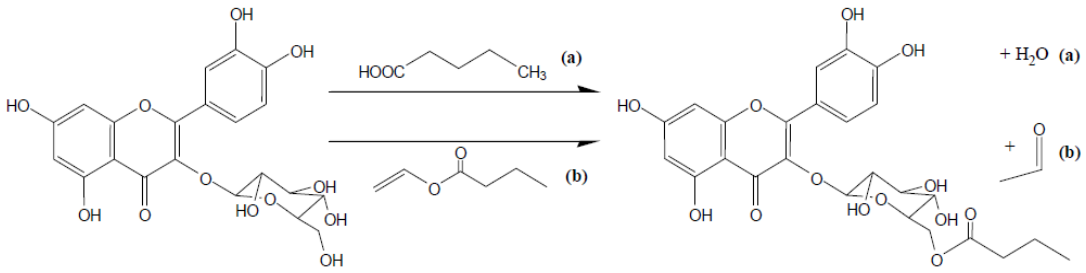


Figure 3.6: Mechanism of flavonoid esterification (a) and transesterification (b) (Chebil et al., 2006)

Several factors affect the enzymatic esterification reaction: nature and concentration of the enzyme, operating conditions (e.g. temperature, by products), nature of solvents used, nature of the substrates and their concentrations (Chebil et al., 2006). Enzymatic acylation can be catalysed by different enzymes, such as proteases, acyl transferases and lipases. Lipases are the most extensively used in various acylation reactions. Most of the flavonoid acylation studies with glycosylated flavonoids to date have used lipases from *Candida antarctica* as a biocatalyst and lipase from *Pseudomonas cepacea* seems to be the best option for aglycon flavonoid esters of fatty acids. An immobilized form of the enzyme is preferred as it forms an easy access for substrate to its catalytic sites and results in easy product recovery (Chebil et al., 2006). *Candida antarctica* lipase B (CAL B) is widely used in both laboratory and commercial scale, mainly due to its ability to accept various substrates, tolerance for non-aqueous medium and resistance for thermal deactivation. CAL B is a member of α/β hydrolase-fold superfamily of enzymes, its

catalytic triad includes Ser105-His224-Asp187, and it follows a ping-pong bi-bi type mechanism as showed in the figure 3.7 (Viskupicova et al., 2009).

Various flavonoids (e.g. quercetin, hesperidin, rutin, esculin, naringin, isoquercetin, isoquercitrin) have been subjected to acylation using *C. antarctica* lipase and acyl donors, such as palmitic acid, cinnamic acid, caffeic acid, coumaric acid, stearic acid, lauric acid, octanoic acids, ceconoic acids, and dodecanoic acids (Ardhaoui et al., 2004, Stevenson et al., 2006, Chebil et al., 2007; Passicos et al., 2004; Ziaullah et al., 2013). Daniel et al. (1997) showed that esculin, which bears a primary hydroxyl group on the glycosyl moiety, is more reactive that rutin, with only secondary hydroxyl groups. It is further explained that the flavonoids with primary hydroxyl groups in the sugar moiety are better substrates for the fatty acids with *C. antarctica* than those with secondary hydroxyl groups (Ardhaoui et al., 2004).

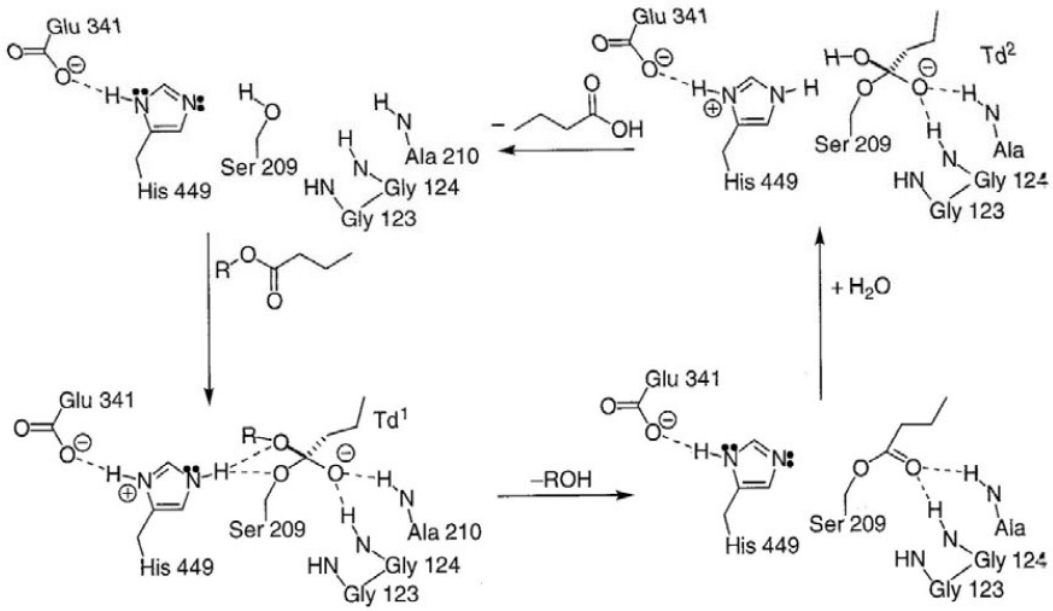


Figure 3.7: Reaction mechanism catalyzed by *Candida antarctica* (Raza et al., 2001)

The effect of the length of the carbon chain on the esterification reaction is still a matter of debate (Viskupicova et al., 2012). Results regarding the influence of carbon chain lengths on the reaction with *C. antarctica* lipase are contradictory. This variability is possibly due to the variations in the reaction media, such as solvent and water content (Ardhaoui et al., 2004). However, according to the studies reported by different authors, acyl acceptor to acyl donor ratio 1:5 is accepted as the best combination (Viskupicova et al., 2009). Solvent used for the reaction has a crucial role in the reaction (Viskupicova et al., 2012), however, the nature of the substrates also contribute to the conversion yield (Chebil et al., 2006). Use of different solvents has been reported, such as 2-methylbutan-2-ol (Ghoul et al., 2006), acetone (Kontogianni et al., 2003), acetonitrile (Nakajima et al., 1999), 2-methylpropan-2-ol (Stevenson et al., 2006). For the enzyme CAL B, acetone and acetonitrile resulted in the best performance (Ardhaoui et al., 2004). Presently, ionic solvents have been widely studied and have been called 'green solvents', since their low toxicity make them potential additives to be used in food, pharmaceutical as well as cosmetic preparations (Viskupicova et al., 2009). Moreover, maintaining a low water content in the reaction media is important. With high water content, thermodynamic equilibrium can be altered, favouring the backward hydrolysis reaction (Viskupicova et al., 2012). Temperature of the reaction mixture is also a key factor which affects the activation as well as the thermal denaturation of the enzyme, viscosity of the medium and the solubility of substrates and products (Chebil et al., 2006). Furthermore, microwave heating is a promising emerging technology for enzyme-catalysed synthesis of long chain acylated analogs of flavonoid glycosides (Ziaullah and Rupasinghe, 2013). The acyl ester derivatives of flavonoids are supposed to bring improved physical, chemical and

biological characteristics to the parent flavonoid compound and, enhancing lipophilicity and ability of penetrating cell membranes are the main expected attributes (Viskupicova et al., 2009).

Quercetin is one of the commonly consumed and widely studied flavonoids and in nature it is mainly available as its glycosides, such as quercetin-3-*O*-galactoside, quercetin-3-*O*-arabiniside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-rutinoside and Q3G (Majewska et al., 2011; Vrhovsek et al., 2004). Q3G is a flavonol in which the sugar group, glucose is attached at hydroxyl group on C-3 position of quercetin molecule (Iwashina, 2000). Among them, Q3G is the most known flavonol that is commonly distributed in plant families and higher amounts quercetin derivatives are found in apple, tea, onions, and broccoli. Also, it has been reviewed that transportation across the intestinal brush border membrane by sodium dependent glucose transporter, make Q3G more effective in biological activities over the other quercetin derivatives. Further, Q3G can be considered as a potent chemoprotectant for cancer due to its high antioxidant activity (Razavi et al., 2009). Also, quercetin-3-*O*-acyl esters with short acyl chains (quercetin-3-*O*-acetate and querctein-3-*O*-propionate) (Saija et al., 2003) and quercetin-3-*O*-palmitate (Sardone et al., 2004) possessed enhanced *in vitro* antioxidant activity and ability to interact with phospholipid membranes. Moreover, quercetin-3-*O*-acyl esters were reported as potential topical prodrugs (Montenegro et al., 2007). Further, fatty acid esters of Q3G from C8-C16 exhibited antiproliferative activity, indicating their potential as anti-tumor drugs (Salem et al., 2010). A similar type of effect was reported with fatty acid esters of rutin, which were able to decrease the VEGF production of K562 human leukemia cells (Mellou et al., 2006).

CHAPTER 4 ANTIOXIDANT PROPERTIES OF LONG CHAIN FATTY ACID ACYLATED DERIVATIVES OF QUERCETIN-3-*O*-GLUCOSIDE

4.1 ABSTRACT

Flavonoids are a diverse group of plant secondary metabolites, known to possess a wide range of health benefits. As a promising source of food antioxidants, their ability to inhibit lipid oxidation in food is highly significant. It was hypothesized that their application in food systems, especially lipophilic systems, can be further enhanced by acylation of flavonoids with fatty acids. Also, fatty acid esters of flavonoids may possess greater bioavailability so that lipid oxidation in biological systems can be inhibited more effectively. Quercetin-3-*O*-glucoside (Q3G) was acylated with six selected long chain fatty acids: stearic acid, oleic acid, linoleic acid, α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), using *Candida antactica* lipase (Novozyme 435®) as the biocatalyst. The antioxidant activities of esterified flavonoids were evaluated using antioxidant capacity assays (FRAP and ORAC) and lipid oxidation model systems of polyunsaturated fatty acid (PUFA)-rich fish oil and human low density lipoprotein (LDL), *in vitro*. The Q3G esters exhibited an antioxidant capacity of 340 – 622 μmol TE/L in FRAP assays and 250 – 500 μmol TE/L in ORAC assays. In the oil-in-water emulsion, Q3G esters exhibited 50% to 100% inhibition in primary oxidation and 30% to 75% inhibition in secondary oxidation. In bulk oil, Q3G demonstrated more than 50% inhibition in primary oxidation and it was significantly ($p \leq 0.05$) higher than the inhibition provided by its esters. Therefore, fatty acid derivatives of Q3G acted as better antioxidants in the oil-in-water emulsion system. EPA, DHA and

ALA derivatives of Q3G showed significantly higher inhibition in human LDL oxidation in comparison to non-acylated Q3G.

Key words: flavonoids, acylation, fatty acids, antioxidant, lipid oxidation, LDL oxidation

4.2 INTRODUCTION

Lipids, which contain both saturated and unsaturated fatty acids, are a main dietary component and n-3 polyunsaturated fatty acids (PUFA) are now widely recognized for their health promoting effects. Lipid based foods are available as bulk oil as well as oil-in-water emulsions, which are the most common. However, foods rich in PUFA are highly susceptible to oxidation, leading to rancidity and nutritional loss (Sun et al., 2011; Nawar, 1996). The oxidation of dietary unsaturated fatty acids results in significant generation of dietary advanced lipid oxidation end-products (ALEs) which are in part cytotoxic and genotoxic. This type of oxidation occurs outside the human body. However, some ALEs are absorbed from the gut and enter the circulation. These ALEs appear to act as injurious chemicals that activate an inflammatory response which in turn can damage organs such as liver, kidney, lung and the gut itself. Therefore, sufficient levels of dietary antioxidants could prevent this form of lipid oxidation (Kanner, 2007).

Lipid oxidation can occur at the cellular level in the body and is associated with many pathological conditions, such as inflammation, autoimmune disease, cardiovascular diseases and aging (Sun et al., 2011). Cellular lipid oxidation can take place in the plasma membrane, as well as in LDL. PUFA within the LDL are susceptible to oxidation by

cellular reactive oxygen species (ROS) and form oxidized LDL or atherogenic LDL. Therefore, LDL oxidation is a causal factor for atherosclerosis (Chalas et al., 2001).

Introducing dietary antioxidants is one of the ways to retard the oxidative stress mediated chronic diseases. Potential safety risks associated with the synthetic antioxidants has created an increasing market demand for natural antioxidants (Kathirvel and Rupasinghe, 2011). Many natural plant polyphenols occur as flavonoids which are a group of phytochemicals abundant mainly in fruit crops. However, lipophilicity of an antioxidant molecule is a critical factor when it is incorporated into different food matrices since the ability of reaching the true site of oxidative damage is controlled by the lipophilic-hydrophilic nature of the antioxidant (Chalas et al., 2001). Therefore, the moderately hydrophilic nature of flavonoids becomes a limiting factor when introducing them into more lipophilic systems (Lue et al., 2010; Montenegro et al., 2007). The structural modification of flavonoid molecules is one of the possible approaches to enhance their incorporation in lipophilic food products such as fish oil. The preparations of lipophilic derivatives of flavonoids, using aliphatic molecules, have been suggested for increasing their solubility in lipophilic systems (Mellou et al., 2005). Enzymatic acylation of natural polyphenolic compounds with long chain fatty acids has been shown to be a promising approach for improving the lipophilic nature of the glycosylated flavonoids (Chebil et al., 2006; Lue et al., 2010; Ardhaoui et al., 2004; Ziaullah et al., 2013). The present study was designed to evaluate the antioxidant activity of long chain fatty acid acylated derivatives of Q3G using their potential to inhibit the oxidation of PUFA in food model systems and the Cu^{2+} and peroxyl radical-induced oxidation of human LDL *in vitro*.

4.3 MATERIAL AND METHODS

4.3.1 Chemicals and supplies

Sodium acetate trihydrate, ferric chloride, phosphate buffer, 2,4,6-Tripyridyl-s-Triazine (TPTZ), spectrophotometric grade dimethyl sulfoxide, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) were obtained from Sigma-Aldrich, St. Louis, MO, USA. Acetic acid, hydrochloric acid, HPLC grade chloroform and HPLC grade methanol were purchased from Fisher Scientific, Ottawa, ON, Canada. Ethanol anhydrous was obtained from Commercial Alcohols, Montreal, QC, Canada. Round bottom 96-well plates were purchased from Corning Incorporated, Edison, NY, USA. Q3G was obtained from Indofine Chemical Company, Hillsborough, NJ, USA. Fatty acids (stearic acid, oleic acid, linoleic acid, α-linolenic acid, EPA and DHA) were purchased from Nu-check Prep, Inc., Elysian, MN, USA. Bulk fish oil was a generous gift from BASF A/S, Malmparken 5, Ballerup, Denmark and was devoid from any antioxidants (See the appendix 2 for the composition of the oil). Trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA) and butylated hydroxytoluene (BHT) were obtained from Sigma-Aldrich (Oakville, ON, Canada). LDL isolated from human plasma (in 150 mM NaCl, 0.01% EDTA, pH 7.4) was purchased from EMD chemicals Inc., Gibbstown, NJ, USA. All other chemicals were purchased from Fisher Scientific, Ottawa, ON, Canada.

4.3.2 Synthesis of fatty acid acylated derivatives of quercetin-3-*O*-glucoside

Synthesis of fatty acid acylated derivatives of Q3G was carried out through enzymatic esterification of Q3G with stearic acid, oleic acid, linoleic acid, ALA, EPA and DHA as acyl donors according to the method described by Ziaullah et al. (2013). Briefly, Q3G (500 mg) and each acyl donor were added into a reaction vessel containing dried 3 Å

molecular sieves in a molar ratio of flavonoid:acyl donor 1:5. Anhydrous acetone was used as the solvent. The acylation was initiated by adding Novozym 435® immobilised lipase from *Candida antarctica* (2 g) as the biocatalyst. Then, the mixture was incubated at 45 ^{o}C while stirring for approximately 48 hrs in a sand bath. Enzymatic conversion of the substrate was qualitatively monitored periodically by thin layer chromatography (TLC) analysis using silica gel plates (TLC Silica gel $60F_{254}$ - aluminium sheets 20 x 20 cm, Merck KGaA, Darmstadt, Germany). Acetone:toluene (50:50) solvent mixture was used as the TLC solvent system, with the addition of few drops of glacial acetic acid and visualized under ultra violet (UV) light and iodide staining. After confirming the completion of the bioconversion, the enzymatic reaction was halted by filtering the immobilized lipase and molecular sieves from the reaction mixture. The acetone was removed by evaporation using vacuum rotary evaporation. To isolate esters, the obtained end product was subjected to silica gel column chromatography using acetone:toluene; 40:60 to 50:50. Preparative TLC was performed under the same conditions as above. The % yield of the esters were calculated according to:

$$\% \text{ Yield} = \frac{\text{Mass of actual yield}}{\text{Mass of theoretical yield}} \times 100\%$$

4.3.3 The ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed according to the method described by Rupasinghe et al. (2008). The working assay reagent (FRAP solution) was prepared daily by mixing 300 mmol l^{-1} acetate buffer of pH 3.6, 10 mmol l^{-1} TPTZ solution and 20 mmol l^{-1} ferric chloride in the ratio of 10:1:1. As the standard material, Trolox solution was prepared by

dissolving 0.025 g of Trolox in 100 ml of 95% ethanol. The appropriate dilutions of Trolox solution were carried out using 95% ethanol to obtain 50, 100, 200, 300, 400, 500, and 1000 μmol l^{-1} concentrations, in order to develop the calibration curve. All the sample compounds were dissolved in 95% ethanol to prepare the desired concentrations. To perform the assay, 20 μl of blank, standard or sample was reacted with 180 μl of FRAP solution in 96-well polystyrene plates after warming to 37 ^{o}C. The absorbance was measured at 595 nm, after 10 min reaction time, including 3 s shaking time, using the FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC, USA). Antioxidant capacity was calculated as μmol l^{-1} Trolox equivalents of 1 mmol l^{-1} of solution.

4.3.4 The oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was carried out using the method described by Rupasinghe et al. (2008). The Trolox solution and AAPH were prepared immediately before the assay. The Trolox standard solution was made using phosphate buffer and suitable dilutions were carried out to develop the calibration curve. The buffer, standard, or sample (35 μl) and 0.957 μmol l^{-1} fluorescein (130 μl) solutions were placed in the 96-well plates and pre-incubated at 37 ^{o}C for 10 min. The fluorescence was measured using FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC, USA) after injection of 35 μl pre-warmed (37 ^{o}C) AAPH to each well, every 60 s. Antioxidant capacity was calculated as μmol l^{-1} Trolox equivalents of 0.001 mmol l^{-1} of solution.

4.3.5 Determination of primary oxidation in bulk fish oil model system

Different concentrations of Q3G and fatty acid acylated derivatives of Q3G (0.5, 1, 5, and 10 mmol l^{-1}), dissolved in dimethyl sulfoxide (1% DMSO), were mixed with bulk fish oil and incubated at 40 ^{o}C for 3 and 5 days. The lipid peroxides which were formed during lipid oxidation were determined as the peroxide value using acetic acid-chloroform method, according to AOCS (2003). Briefly, oxidized fish oil was dissolved in 3:2 acetic acid - chloroform mixture and 0.5 ml of freshly prepared saturated KI solution was added and gently mixed. After 1 min, 30 ml of deionized water was added, followed by addition of 1 ml of starch indicator. The liberated iodine was titrated with 0.1 N of $Na_2S_2O_3$. Percentage inhibition of lipid peroxidation was calculated, based on the peroxide values obtained.

4.3.6 Preparation of aqueous emulsion (oil-in-water) model system

An aqueous emulsion (oil-in-water) of fish oil was prepared following a method described by Rupasinghe et al. (2010). An emulsion of 10 mg of fish oil per 1 ml of buffer (pH 7) containing 0.05 M tris HCl, 0.15 M KCl and 4% Tween 20 as an emulsifier, was prepared by homogenizing the mixture using a polytron homogenizer (model PCU, Drehzahlegler, Switzerland), at 4.5 speed for 30 s.

4.3.7 Determination of primary oxidation in water-in-oil emulsion model system

The procedure for ferric thiocyanate (FTC) test described in Rupasinghe et al. (2010) was followed. Ethanolic solutions (95% ethanol) of Q3G and its esters in 0.5, 1, 5, and 10 mmol l^{-1} concentrations were prepared in disposable 13 × 100 mm borosilicate glass tubes and the solvent was completely evaporated under nitrogen flow. After solubilising the dried compounds with 10 µl ethanol, 80 µl of emulsion was added and vortexed.

Oxidation was induced by adding 10 µl of peroxy radical generator AAPH and incubated at room temperature for 40 min. At the end of the incubation, further oxidation was halted immediately by adding 10 µl of 1000 mg l^{-1} BHT into all the samples. Samples (30 µl) were diluted with 210 µl of 75% ethanol and 30 µl of 3% NH_4SCN was added. After 3 min, 30 µl of 2 mmol l^{-1} ferric chloride in 3.5% HCl was added and absorbance was measured at 495 nm in 96-well microplates, using FLUOstar OPTIMA microplate reader (BMG Labtech, Durham, NC, USA). Blank samples were prepared for all the concentration levels of all the compounds, with no addition of emulsion, in order to eliminate the absorbance by compounds themselves and blank absorbance values were subtracted from the sample absorbance. Percentage inhibition of lipid oxidation was calculated. The hydroperoxides, formed during lipid oxidation, react with ferrous ions to produce ferric ions, which are detected as the ferricthiocyanate red chromogen.

4.3.8 Determining the inhibition of secondary oxidation

Secondary lipid oxidation, in both bulk fish oil and aqueous emulsion (oil-in-water) model systems, were determined by thiobarbituric acid reactive substances (TBARS) assay, as described by Huber et al. (2009) and Rupasinghe et al. (2010), with slight modifications. Desired concentrations of Q3G and fatty acid derivatives of Q3G in 95% ethanol (0.5, 1, 5, and 10 mmol l^{-1}) were placed in disposable 13 × 100 mm borosilicate glass tubes and the solvent was completely evaporated under nitrogen flow. Twenty microliters of ethanol and 80 µl of bulk fish oil or aqueous emulsion were added and vortexed well to ensure complete dispersion of the compounds in the bulk fish oil or aqueous emulsions. Induction of oxidation was achieved by heating the glass tubes,

covered with breathable caps, for 3 hrs at 50 ^{o}C in a shaking incubator (model Apollo HP50, CLP Tools, San Diego, CA, USA).

The TBA reagent (0.375% TBA and 15% TCA in 0.25 M HCl) was added to all the oxidized fish oil or emulsion samples as well as the controls. After vortexing, all the samples were capped and placed in a water bath (Isotemp 205, Fisher scientific, Ubuque IA, USA) at 80 ^{o}C for 15 min. The sample tubes were allowed to cool to room temperature and 2 ml of 1-butanol was added to each sample, followed by vortexing and centrifuging (IEC International Centrifuge, MA, USA) at 40 g for 20 min. After loading the supernatant (1-butanol layer) into 96-well microplates, fluorescence intensity (FI) was measured for excitation at 532 nm and emission at 580, using FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC, USA). The percentage inhibition of the samples was calculated in comparison to controls with no antioxidants, using the below equation.

$$\% \text{ Inhibition} = 100 \, (FI_{control} - FI_{sample}) / FI_{control}$$

4.3.9 LDL oxidation

4.3.9.1 LDL preparation

Prior to dialysis of the LDL, cellulose dialysis tubing (Thermo Fisher Scientific Inc., Ottawa, ON, Canada) was pretreated as follows in order to remove inherent antioxidants. The tubing was cut into the desired length (6-8 cm), soaked in deionized water for 15 min and heated at 80 ^{o}C in a 10 mmol l^{-1} sodium bicarbonate solution for 30 min while stirring. It was transferred into a 10 mmol l^{-1} Na_2EDTA solution and soaked for 30 min. Again, it was transferred to the deionized water at 80 ^{o}C and soaked for 30 min while stirring. The membrane was cooled and submerged in 50% ethanol solution and

refrigerated. To perform the dialysis, the cellulose tubing was washed both inside and outside first with deionised water and then with 10 mmol l^{-1} phosphate buffered saline (PBS) solution containing 0.138 M NaCl and 0.0027 M KCl (pH 7.4, at 25 ^{o}C). One end of the tubing was sealed, LDL was then injected into the open end which was then sealed completely. The sealed tubing was immersed in 10 mmol l^{-1} PBS solution at 4 ^{o}C for 24 hours. Buffer was changed every six hours. Once the dialysis was completed, the dialysed LDL was immediately transferred into an amber coloured vial and stored at -80 ^{o}C for use within two weeks.

4.3.9.2 Determination of protein content in dialysed LDL

Protein content of the dialysed LDL was determined using the Lowry's method (Lowry et al., 1951). Bovine serum albumin (BSA) was used as the standard protein source. Lowry's reagent (1 ml) was added to the blank, standards and dialysed LDL and left for 20 min. Then, Folin Ciocalteu reagent (0.5 ml) was added, followed by immediate thorough mixing and allowed to stand for 30 min. Absorbance was measured at 750 nm. Protein content of the LDL sample was calculated using the calibration curve of BSA (10, 20, 40 and 50 µg/ml). The LDL sample was diluted appropriately with PBS to reach 100 µg/ml protein content.

4.3.9.3 Inhibition of LDL oxidation

Oxidation was induced by two methods: using copper(II) sulphate and peroxy radical generator, 2,2´-azobis(2-methylpropionamidine) dihydrochloride (AAPH). Solutions of Q3G esters and Q3G were prepared in methanol to obtain 1, 10, 100, and 500 µmol l^{-1} concentrations in the reaction mixture. Dialysed LDL (160 µl) was mixed with 20 µl of the flavonoid sample in borosilicate glass tubes and 20 µl of Cu^{2+} (10 µM)/AAPH (5

mM) was added. All the samples were incubated at 37 ^{o}C for 4 hours in a shaking water bath. Solutions of 1 mmol l^{-1} EDTA and BHT were added to terminate oxidation in Cu^{2+} and AAPH- induced systems, respectively. LDL oxidation was determined using the TBARS assay, according to the method described by Xu et al. (2007).

4.3.10 Statistical Analysis

All the analyses were performed in triplicate and *in vitro* antioxidant assays were analysed using one way ANOVA, mean separation was carried out by Tukey's multiple mean separation method. All the other experiments were analysed by two factor factorial design, using general linear model. When the interaction effects were significant, multiple means comparison was carried out by least square means ($P \leq 0.05$) with adjusted Tukey's method. When the main effects were significant, mean separation was done using Tukey's multiple mean separation method (Montgomery, 2012).

4.4 RESULTS

4.4.1 Synthesis of fatty acid derivatives of Q3G

A general reaction for the acylation carried out in the experiment is shown in Figure 4.1, and the structures of six long chain fatty acids used are presented in Table 4.1. The yield for all the six fatty acid derivatives of Q3G was 80% - 97 %. The ^{1}H NMR and ^{13}C NMR data for the synthesised fatty acid derivatives of Q3G are included in appendix 1.

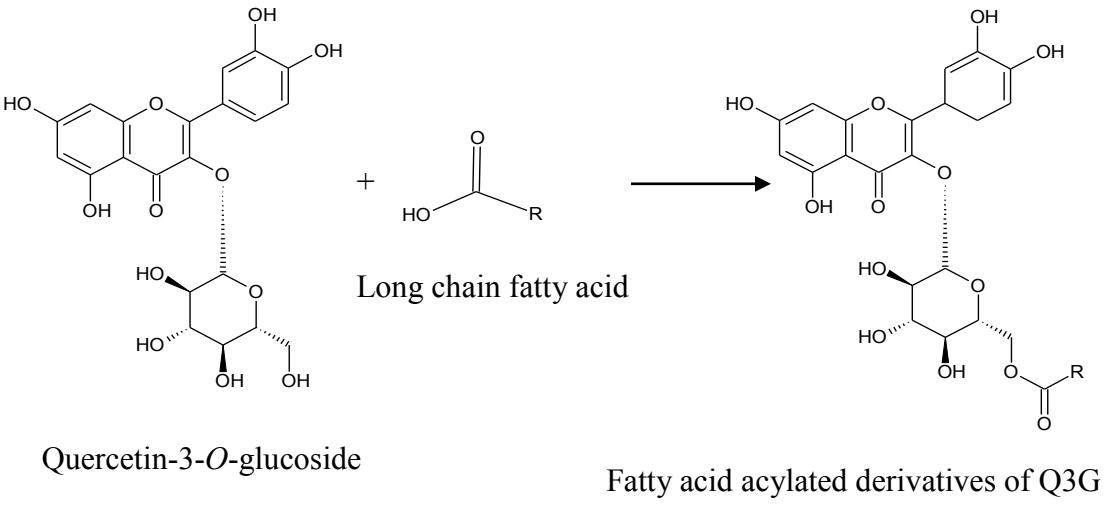


Figure 4.1: The general acylation reaction of Q3G with acyl donor fatty acids

Table 4.1: Six different acyl donor long chain fatty acids used for the esterification

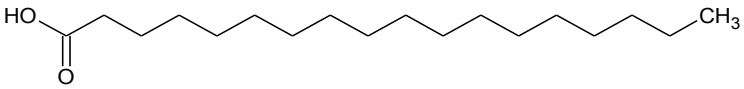
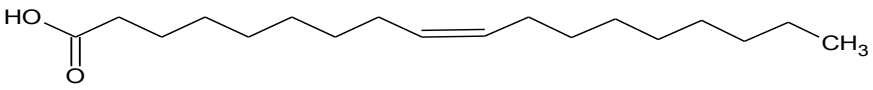
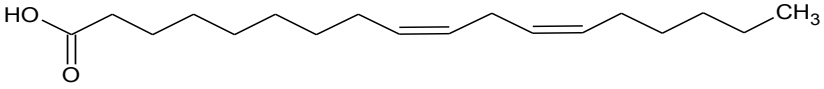
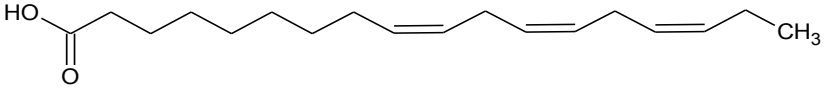
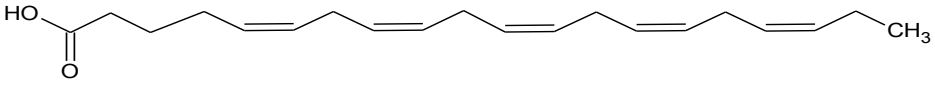
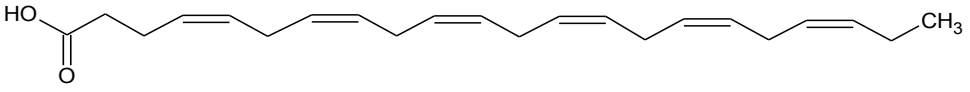
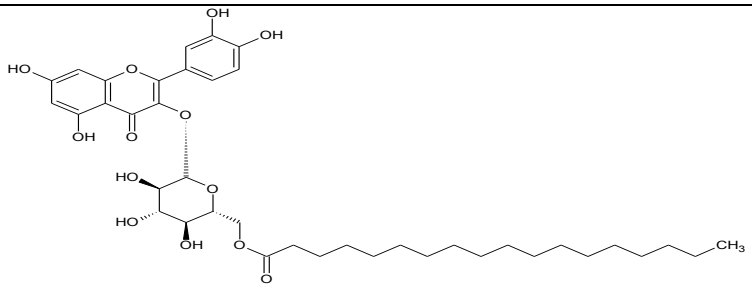
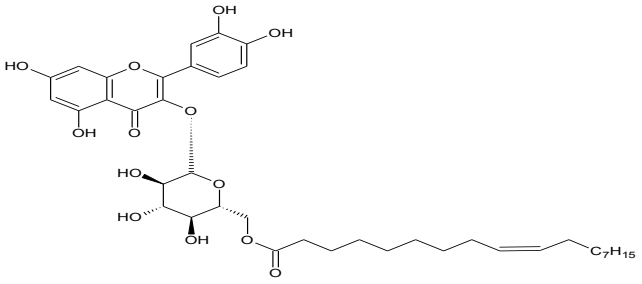
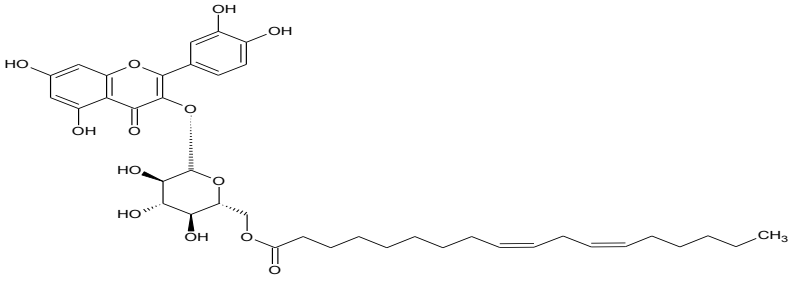
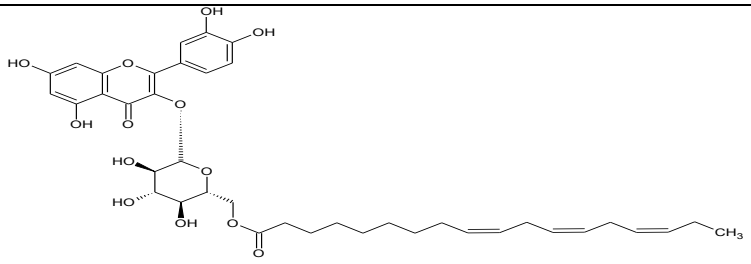
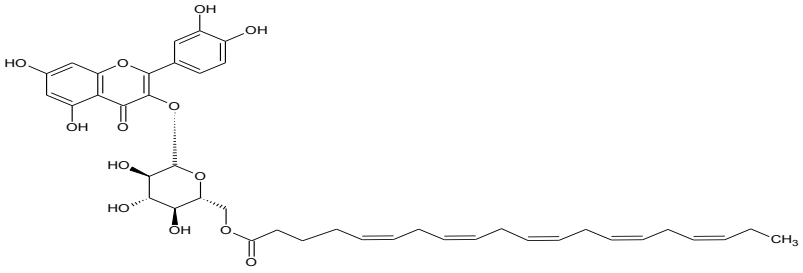
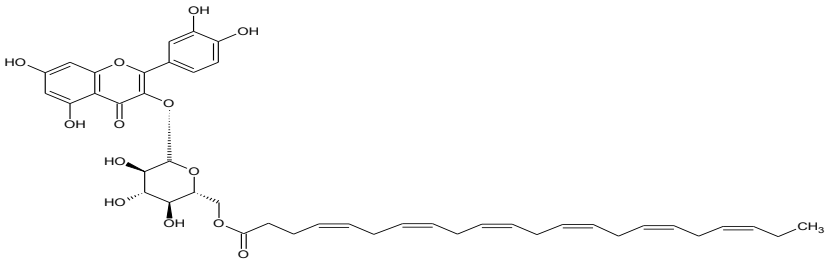
Reaction Number	Structure of acyl donor
Reaction 1	 Octadecanoic acid (Stearic acid; C18:0; $C_{18}H_{36}O_2$; MW = 284.48 g mol^{-1})
Reaction 2	 (9*Z*)-Octadec-9-enoic acid (Oleic acid; C 18:1; $C_{18}H_{34}O_2$; MW =282.4614 g mol^{-1})
Reaction 3	 *cis, cis*-9,12-Octadecadienoic acid (Linoleic acid; C18:2; $C_{18}H_{32}O_2$; MW = 280.45 g mol^{-1})
Reaction 4	 (9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoic acid (α-Linolenic acid; 18:3; $C_{18}H_{30}O_2$; MW = 278.43 g mol^{-1}
Reaction 5	 (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-5,8,11,14,17-icosapentaenoic acid (EPA; 20:5; $C_{20}H_{30}O_2$; MW = 302.451 g mol^{-1})
Reaction 6	 (4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*)-docosa-4,7,10,13,16,19-hexaenoic acid (DHA; 22:6; $C_{22}H_{32}O_2$; MW = 328.488 g mol^{-1})

Table 4.2: Chemical structures and yields of synthesised long chain fatty acid acylated derivatives of Q3G

Reaction Number	Structure and nomenclature of the esters	% yield
Reaction 1	 (6-{[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-chromen-3-yl]oxy}-4,5-dihydroxytetrahydro-2*H*-pyran-2-yl)methyl stearate	97%
Reaction 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 (*Z*)-9-octadecenoate	92%
Reaction 3	 (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 (9*Z*,12*Z*)-9,12-octadecadienoate	94%

Reaction Number	Structure and nomenclature of the esters	% yield
Reaction 4	 (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 (9*Z*,12*Z*,15*Z*)-9,12,15-octadecatrienoate	91%
Reaction 5	 (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-icosapentaenoate	85%
Reaction 6	 (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 (4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*)-4,7,10,13,16,19-docosahexaenoate	81%

4.4.2 *In vitro* antioxidant capacity

The antioxidant capacity was determined using FRAP assays. The fatty acid derivatives of Q3G showed antioxidant capacities ranging from 340 to 613 µmol TE/L while Q3G gave an antioxidant activity of 1500 µmol TE/L (Table 4.3).

However, in the ORAC assay, linoleic acid, ALA and EPA derivatives of Q3G showed significantly ($p \leq 0.05$) higher antioxidant capacity than Q3G (Table 4.3). In this study, no correlation was observed between the saturation number of the fatty acids attached to the esters and their antioxidant capacity. Moreover, the DHA ester also exhibited high antioxidant capacity of 485.9 µmol TE/L.

Table 4.3: Antioxidant capacity of Q3G and its fatty acid derivatives

Compound	FRAP (µmol TE/L^{Z})	ORAC (µmol TE/L^{Y})
Q3G	1500.0 ± 16.7 **a**	475.6 ± 5.2 **b**
Q3G stearate	622.3 ± 27.9 **b**	306.0 ± 10.8 **c**
Q3G oleate	536.0 ± 22.9 **bc**	262.54± 7.1 **d**
Q3G linoleate	340.4 ± 18.9 **d**	491.4 ± 0.8 **a**
Q3G α-linolenate	613.8 ± 62.0 **b**	489.7 ± 1.8 **a**
Q3G eicosapentaenoate	564.4 ± 18.4 **bc**	490.2 ± 4.1 **a**
Q3G docosahexaenoate	485.0 ± 28.1 **c**	485.9 ± 6.1 **ab**

Z= 1 mmol l^{-1} of solution Y= 0.001 mmol l^{-1} of solution

(Means ± Standard Error Mean, followed by the same letter within each column are not significantly different, Tukey's multiple means comparison test, $P \leq 0.05$)

4.4.3 Inhibition of oxidation in bulk fish oil and oil-in-water emulsion

In n-3 PUFA-rich bulk fish oil, the parent compound Q3G showed the highest percentage inhibition of primary oxidation over all the fatty acid derivatives of Q3G at 10 mmol l^{-1}. However, ALA, EPA and DHA derivatives of Q3G demonstrated considerable inhibition of 30% - 40% (Table 4.4). When the secondary oxidation in the bulk fish oil system was measured, all the tested compounds, except for the EPA and DHA derivatives of Q3G, demonstrated a concentration dependent effect where the higher concentrations provided the higher % inhibition. Q3G esterified to linoleate exhibited the highest % inhibition of 70% at the highest concentration of 10 mmol l^{-1} (Table 4.5). In general, the modified esters had no significant ($p \leq 0.05$) effect, compared to their parent flavonoid Q3G, on inhibition of both primary and secondary oxidation in bulk fish oil model system.

In primary lipid oxidation in oil-in-water emulsion (Table 4.6), all the six fatty acid acylated derivatives of Q3G acted as better antioxidants than the parent compound Q3G, showing more than 50% inhibition in the concentration range of 0.5 – 10 mmol l^{-1}. The stearic, oleic and linoleic derivatives of Q3G were effective, with more than 50% inhibition in all the tested concentrations, showing that they are useful as antioxidants, even at low concentration levels such as 0.5 mmol l^{-1}. More than 40% inhibition was observed in the EPA and DHA derivatives in all the tested concentration levels and achieved 100% inhibition at a concentration of 10 mmol l^{-1}. In secondary oxidation of oil-in-water emulsion, all the modified esters had their highest inhibition at 10 mmol l^{-1} level and there was more than 30% inhibition (Table 4.6). Overall, the results show that in the oil-in-water emulsion, the best antioxidant is Q3G stearate, since it reached maximum inhibition at 5 mmol l^{-1} in the primary oxidation. In the case of secondary

oxidation, Q3G stearate is the best antioxidant at 10 mmol l^{-1}. The parent compound Q3G showed lower inhibition than the esters, even at its most effective concentration level of 10 mmol l^{-1}. It can be inferred that in the oil-in-water emulsion system, the fatty acid acylated derivatives of Q3G have a better antioxidant activity than Q3G.

4.4.4 Inhibition of oxidation in human LDL

The ability of the compounds to inhibit the plasma LDL oxidation *in vitro* represents the antioxidant capacity of the compounds to deal with cellular oxidative stress. Over 40% inhibition for Cu^{2+}-induced oxidation has been shown by the EPA derivative of Q3G at 10 µmol l^{-1} and the DHA derivative of Q3G at 500 µmol l^{-1} (Table 4.7). For the AAPH-induced oxidation, the selected concentration range did not demonstrate a significant dose dependent effect, but the EPA and α-linolenic acid esters were more effective than Q3G in protecting the plasma LDL from oxidation. None of the tested compounds showed promise as an excellent inhibitor for preventing oxidation of human LDL. EPA derivative of Q3G, however, showed the highest inhibition in both experiments at concentrations of 500 µmol l^{-1}.

Table 4.4: Inhibition of primary lipid oxidation in bulk fish oil in relation to concentration of the test compounds

Compound	% Inhibition							
	Day 3				Day 5			
	Concentration (mmol l^{-1})				Concentration (mmol l^{-1})			
	0.5	1	5	10	0.5	1	5	10
Q3G	0±0.0**c**	0±0.0**c**	45±7.1**bc**	57±8.7**a**	8±1.5**D**	12±1.4**CD**	34±4.4**BC**	52±6.3**A**
Q3G stearate	0±0.0**c**	0±0.0**c**	24±2.0**bc**	26±4.0**bc**	13±6.0**CD**	22±2.6**CD**	34±2.3**BC**	29±3.2**BC**
Q3G oleate	7±2.8**c**	0±0.00**c**	14±6.5**c**	12±9.3**c**	0±0.0**D**	6±3.8**CD**	26±6.7**CD**	30±15.2**BC**
Q3G linoleate	18±8.4**bc**	16±13.4**bc**	14±0.15**c**	21±2.7**bc**	11±1.0**CD**	22±3.4**CD**	28±3.0**C**	26±5.6**C**
Q3G α-linolenate	14±8.2**c**	5±3.5**c**	12±6.4**c**	17±3.4**bc**	5±3.5**D**	11±4.8**CD**	22±4.1**CD**	40±2.7**AB**
Q3G eicosapentaenoate	18±7.5**bc**	21±4.7**bc**	34±3.8**b**	30±5.0**bc**	16±2.6**CD**	18±0.5**CD**	37±2.1**B**	34±1.6**BC**
Q3G docosahexaenoate	9±7.0**c**	17±1.0**bc**	26±0.8**bc**	21±1.2**bc**	19±2.1**CD**	21±6.2**CD**	30±8.8**BC**	38±1.3**AB**

Means ± Standard deviation, followed by the same letter within day are not significantly different, multiple means comparison by least square means with adjusted Tukey's, $p \leq 0.05$.

Table 4.5: Inhibition of secondary lipid oxidation in bulk fish oil in relation to the concentration of the test compounds

Compound	% Inhibition			
	Concentration (mmol l^{-1})			
	0.5	**1**	**5**	**10**
Q3G	24 ± 5.8**e**	35 ± 2.4**d**	60 ± 2.5**bc**	69 ± 0.1**ab**
Q3G stearate	39 ± 4.38**d**	35 ± 1.2**d**	46 ± 1.2**cd**	52 ± 2.1**bc**
Q3G oleate	40 ± 1.4**cd**	60 ± 0.7**b**	64 ± 1.5**ab**	67 ± 1.7**ab**
Q3G linoleate	34 ± 3.0**d**	49 ± 3.6**c**	63 ± 1.6**ab**	70 ± 0.6**a**
Q3G α-linolenate	38 ± .98**d**	33 ± 4.4**de**	42 ± 0.6**cd**	51 ± 0.9**bc**
Q3G eicosapentaenoate	46 ± 2.6**cd**	40 ± 1.9**cd**	50 ± 4.1**bc**	31 ± 1.1**de**
Q3G docosahexaenoate	50 ± 4.2**cd**	50 ± 3.5**bc**	47 ± 6.4**cd**	33 ± 2.5**de**

Means ± Standard deviation followed by the same letter are not significantly different, multiple means comparison by least square means with adjusted Tukey's, $p \leq 0.05$.

Table 4.6: Inhibition of lipid oxidation in oil-in-water emulsion in relation to the concentration of the test compounds

Compound	% Inhibition							
	Primary oxidation				Secondary oxidation			
	Concentration (mmol l^{-1})				Concentration (mmol l^{-1})			
	0.5	1	5	10	0.5	1	5	10
Q3G	29±6.7**d**	39±9.4**d**	42±9.1**cd**	18±15.3**d**	0±0.0**F**	0± 0.0**F**	9±2.3**EF**	25±1.0**DE**
Q3G stearate	55±6.1**cd**	74±7.1**c**	100±16.9**b**	100±3.6**ab**	16±2.5**E**	20± 5.2**DE**	69±2.3**AB**	78±3.1**A**
Q3G oleate	53±8.5**cd**	56±8.8**cd**	55±4.1**cd**	63±6.0**cd**	11±3.4**EF**	30± 4.1**D**	43±9.7**CD**	49±14.4**C**
Q3G linoleate	47±2.2**cd**	66±0.6**cd**	60±8.1**cd**	73± 4.0**c**	0± 0.0**F**	3±0.96**F**	45±2.0**CD**	67±0.3**AB**
Q3G α-linolenate	13±6.7**d**	15±0.1**d**	34±4.0**d**	64±3.0**cd**	12±1.4**EF**	24±6.5**DE**	51±1.3**BC**	63±0.6**B**
Q3G eicosapentaenoate	42±3.2**cd**	56±5.4**cd**	63±17.3**cd**	65±12.2**cd**	0±0.0**F**	0±0.0**F**	13±1.9**EF**	32± 1.8**D**
Q3G docosahexaenoate	49±5.0**cd**	43±4.0**cd**	54±19.3**cd**	100±8.4**a**	27±9.5**DE**	26±3.8**DE**	53±0.8**BC**	58± 8.0**BC**

Means ± Standard deviation followed by the same letter are not significantly different, multiple means comparison by least square means with adjusted Tukey's, $p \leq 0.05$. Primary and secondary oxidation were analysed separately.

Table 4.7: Inhibition of human LDL oxidation *in vitro* in relation to the concentration of the test compounds

Compound	% Inhibition							
	Cu^{2+} - induction				Peroxy radical - induction			
	Concentration (µmol l^{-1})				Concentration (µmol l^{-1})			
	1	10	100	500	1	10	100	500
Q3G	7±4.5**c**	20±9.2**bc**	20±1.8**bc**	33±8.6**ab**	10±2.6**C**	10± 2.7**C**	16±7.2**C**	29±2.0**C**
Q3G stearate	4±9.4**c**	14±7.5**bc**	21±8.6**bc**	29±4.2**b**	4±5.9**C**	11±5.0**C**	14±7.5**C**	27±8.5**C**
Q3G oleate	8±7.4**c**	18±5.8**bc**	14±5.3**bc**	28±2.8**bc**	12±7.2**C**	9± 2.8**C**	15±3.9**C**	17±13.6**C**
Q3G linoleate	14±3.4**bc**	27±7.8**bc**	29±5.9**b**	34± 6.3**ab**	5± 4.7**C**	15±7.8**C**	23± 5.6**C**	25±5.4**C**
Q3G α-linolenate	40±5.6**ab**	34±5.1**ab**	31±2.9**ab**	31±3.5**ab**	28±9.5**AB**	23±4.3**AB**	23± 2.0**AB**	34±6.3**AB**
Q3G eicosapentaenoate	20±8.8**bc**	43±6.8**ab**	45±3.1**ab**	51±2.6**a**	21±3.8**A**	36±6.7**A**	38±1.7**A**	41± 2.9**A**
Q3G docosahexaenoate	7±5.5**c**	14±7.9**bc**	27±6.7**bc**	43±1.4**ab**	13±3.7**BC**	9±8.1**BC**	30±10.0**BC**	27± 8.1**BC**

Means ± Standard deviation followed by the same letter are not significantly different, multiple means comparison by least square means with adjusted Tukey's and Tukey's multiple means comparison test, $p \leq 0.05$. Cu^{2+}-induction and peroxy radical-induction were analysed separately.

4.5 DISCUSSION

Presently, there is a growing interest in plant polyphenols, which can be applied as natural antioxidants in foods, pharmaceuticals and cosmetics (Viskupicova et al., 2010). Flavonoids are extensively studied for their applicability as antioxidants. Their application can be further enhanced by modifying the flavonoid structure by acylation. In this study, Q3G was successfully acylated with six different long chain fatty acids by lipase-catalysed esterification. In all the reactions, the primary hydroxyl of the glycosyl group of the Q3G molecule was esterified with long chain fatty acids of C18 to C22 having various degrees of unsaturation. The esterification of flavonoid compounds, using *Candida antarctica* lipase as a very effective biocatalyst for various acyl donors, is widely reported in the literature. Naringin (Passicos et al., 2004), chlorogenic acid (Lorentz et al., 2010), rutin (Lue et al., 2010), quercetin (Ardhaoui et al., 2004) , Q3G (Mellou et al., 2006), hesperidin (Chebil et al., 2007) and esculin (Ardhaoui et al., 2004) are some of the extensively used flavonoids in esterification studies, with different acyl donors, such as palmitic acid, oleic acid, stearic acid, lauric acid, myristic acid, γ-linolenic acid and hexadecanedioic acid.

However, the antioxidant ability of the flavonoids is highly dependent on their chemical structure; type, position and number of the functional groups, and they behave differently in different media. According to our results, FRAP and ORAC assays indicate in general that all acylated Q3G esters possess substantial antioxidant capacity. In the literature, lipophilicity has demonstrated its effect on the antioxidant activity in different ways. In some studies, lipophilicity could enhance the activity of parent compound, while in other studies, it resulted in similar or decreased activity compared to the parent compound. The

alkyl esters of caffeic acid displayed enhanced antiradical activity, while dihydrocaffeic acid showed a decreased effect (Silva et al., 2000). Viskupicova et al. (2010) showed that lipophilisation of the rutin molecule using fatty acids had no effect on radical scavenging capacity of rutin. Moreover, Lue et al. (2010) did not observed an improved antioxidant capacity for the synthesised fatty acid esters of rutin. Further, they explained that accessibility of hydroxyl groups to the free radicals is obstructed by the steric hindrance created by long acyl chains of fatty acids and it decreases the radical scavenging ability of the synthesised esters. Moreover, the considerably different reaction conditions used in the antioxidant assays make it difficult to draw unifying conclusions (Sharma and Bhat, 2009).

Oxidation stability is a parameter with greater attention and significance, in terms of evaluating the quality of lipid based foods, which are highly prone to oxidative deterioration. In the present study, the effects of enhanced lipophilicity of these synthesised esters were determined using two lipid oxidation model systems; bulk oil and oil-in-water emulsion based model systems. In oil-in-water emulsion systems, the ester derivatives acted as more effective antioxidants than their parent Q3G. However, Q3G acted as more effective antioxidant for the inhibition of oxidation in bulk oil. The polar paradox theory explains the behaviour of antioxidants in different media; the lipophilic antioxidants are more effective in inhibiting oxidation in less lipophilic media and hydrophilic antioxidants are more effective in inhibiting oxidation in more lipophilic media (Porter, 1980; Frankel, 1996). This is further explained using the interfacial phenomenon; lipophilic antioxidants locate towards the oil-water interface in oil-in-water emulsions, the location where the oxidation is more prevalent and thereby, protect the oil

phase from oxidation. Hydrophilic antioxidants move into the aqueous phase and this dilution make them not sufficiently effective for protecting the oil phase. In bulk oil systems, lipophilic antioxidants move to the oil phase while hydrophilic antioxidants are oriented in oil-air surface protecting the oil form oxidation (Frankel, 1996).

There are a number of previous studies, which have exhibited the effect of the lipophilicity of antioxidants in bulk oil and emulsion model systems. Mateos et al. (2008) explains, using a series of alkyl ester derivatives of tyrosol and hydroxytyrosol, that the lipophilic esters are weaker antioxidants in bulk oil. Esterification with long hydrocarbon chains changed the partitioning behaviour of chlorogenic acid and increased its ability to concentrate at oil-water interface (Sasaki et al., 2010). Further, acylation with two long-chain n-3 PUFA of the epigallocatechin gallate (EGCG) molecule increased its lipophilicity (Shahidi and Zhong, 2011). According to those studies, the acylation improves the lipophilicity of the antioxidants and the lipophilic and hydrophilic antioxidants have different effectiveness in different media. According to the explanations used in the polar paradox theory, it is understood that change in lipophilicity changes the physical location of the antioxidant molecules in different food matrices and this affects the effectiveness of the antioxidants. The observations in the present study indicate that esterification with long chain hydrocarbons improved the lipophilicity of the Q3G which caused the Q3G ester to be orientated with the lipophilic long chain fatty acid embedded in the oil and the polar Q3G in the aqueous phase surrounding the oil droplet. This means that the polar Q3G remains at the oil-water interface which yields protection from the oxidation of the droplet.

It has been explained by Zhong and Shahidi (2012) that the polar paradox theory is applicable only in certain concentration ranges since the effect of solubility starts dominating over the interfacial phenomenon. Therefore, in lipophilic media, more lipophilic antioxidants are effective at the lower concentrations and more hydrophilic antioxidants are effective at their higher concentrations. In the present study, Q3G was not effective in lower concentrations in the bulk oil and it can be due to solubility which is more dominating at the lower concentrations. Since the esters are more lipophilic than Q3G, they are soluble in the lipid matrix playing a dominating role in inhibiting oxidation. Further, Q3G was more effective at its higher concentrations, at which the interfacial phenomenon dominates over the solubility effect. Moreover, long chain fatty acids including n-3 and n-6 PUFA have been used in this study and are known to have numerous health advantages. Therefore, in addition to the protection from oxidation, these novel molecules may provide clinical advantages.

In the present study, EPA ester of Q3G reported the highest % inhibition in both the Cu^{2+}- and peroxyl radical-induced oxidation. Further, α-linolenic ester of Q3G showed significantly higher capacity than Q3G in inhibiting the peroxy radical induced LDL oxidation. These results are supported by the previous study by Chalas et al. (2001) which demonstrated that ethyl esterification of phenolic acids made them better antioxidants in inhibiting LDL oxidation. There are several previous studies which have demonstrated the use of phenolics including flavonoids as the inhibitors of LDL oxidation (Frankel et al., 1993; Safari and Sheikh, 2003; Thilakarathna et al., 2013). Moreover, the beverages prepared from fruits such as red wine and grape juice are rich in flavonoids and have been found to inhibit the human LDL oxidation *in vitro* (Miyagi et

al., 1997; Kerry and Abbey, 1997). Our present study demonstrated that increasing lipophilicity of the Q3G makes them more potent antioxidants in inhibiting the LDL oxidation. Also, it has been postulated that an increase in lipophilicity of the ethyl esters facilitates the incorporation of the antioxidant molecules through the lipid layer of the LDL particles (Chalas et al., 2001). To better understand the mechanisms which make the incorporating of these esters more feasible, further studies are needed.

4.6 CONCLUSION

All the fatty acid acylated derivatives of Q3G showed substantial *in vitro* antioxidant capacity of 340 – 622 μmol TE in the FRAP assay and 262 – 491 μmol TE/L in the ORAC assay. All the derivatives of Q3G showed more than 30% inhibition in secondary oxidation and more than 50% inhibition in primary oxidation in the oil-in-water emulsion. The synthesized compounds were more effective in inhibiting both primary and secondary oxidation in oil-in-water emulsions than Q3G. However, Q3G reported 50-60% inhibition at 10 mM concentration in bulk oil. These modified compounds were not effective in bulk oil system, when compared with Q3G ($p \leq 0.05$). Therefore, these synthesized esters have a potential for use as effective antioxidants in PUFA containing emulsion based foods. The EPA and DHA derivatives of Q3G exhibited more than 50% inhibition in Cu^{2+}-induced LDL oxidation. The EPA and ALA derivatives were more effective in AAPH-induced oxidation than Q3G ($p \leq 0.05$). Therefore, these fatty acid derivatives of Q3G can be introduced as potential drug candidates for pathogenic conditions, such as atherosclerosis and related diseases. Further research is required to understand the biological functions of the modified Q3G.

CHAPTER 5 LONG CHAIN FATTY ACID DERIVATIVES OF Q3G FOR ATTENUATING H_2O_2-INDUCED ACUTE CYTOTOXICITY IN HUMAN LUNG FIBROBLASTS AND PRIMARY HEPATOCYTES

5.1 ABSTRACT

Cellular oxidative stress causes detrimental effects to macromolecules, such as lipids, nucleic acids and proteins, leading to many pathological conditions. Quercetin-3-*O*-glucoside (Q3G), a glycosylated derivative of quercetin (Q), is a natural polyphenolic compound known to possess antioxidant activity. The hydrophilic/lipophilic nature of an antioxidant molecule is considered as an important factor governing the accessibility to the active sites of oxidative damages. Six long chain fatty acid derivatives of Q3G with comparison to Q and Q3G, were evaluated for their cytoprotective activity under H_2O_2-induced oxidative stress using cell viability, lipid peroxidation, DNA fragmentation and fluorescence microscopy studies. Pre-incubation of α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenic acid (DHA) derivatives of Q3G exhibited significantly ($p \leq 0.05$) greater cell viability in both human lung fibroblast (WI-38) and human primary hepatocytes upon exposure to H_2O_2 insult when compared to the control. Cytoprotection due to oleic and linoleic acid derivatives of Q3G was observed only in human primary hepatocytes. All the derivatives, Q3G and Q showed ability to significantly ($p \leq 0.05$) lower production of lipid hydroperoxides compared to the control. However, ALA and DHA derivatives of Q3G resulted in significantly lower lipid hydroperoxidation than Q and Q3G. Based on fluorescence microscopy study, H_2O_2-induced apoptosis was attenuated by the fatty acid derivatives of Q3G. The fatty

acid derivatives of Q3G possess better cytoprotective effect than Q3G against H_2O_2-induced cytotoxicity.

Key words: oxidative stress, H_2O_2, human lung fibroblasts, human primary hepatocytes, lipid hydroperoxidation, apoptosis, cytoprotection

5.2 INTRODUCTION

In biological environments, an excess production of partially reduced forms of molecular oxygen and insufficient antioxidant defense system disturbs the cellular redox balance resulting in oxidative stress (Powers and Jackson, 2008) which is associated with many pathological conditions, such as inflammation, aging, cancer, atherosclerosis, hypertension and diabetes (Neofytou et al., 2012). As a result of anthropogenic activities, a large number of organic and inorganic pollutants are released to the environment causing the generation of elevated levels of reactive oxygen species (ROS) in the human body. In addition to endogenous production of ROS, exposure to exogenous factors causes excess oxidative stress which cannot be counteracted by the antioxidant defence mechanisms in the target organs (Kregal and Zhang, 2007; Wei et al., 2012). Liver is the main target for the toxic effects of xenobiotics resulting in oxidative stress which is involved in initiation and progression of pathogenesis of hepatic damage (Medina and Moreno-Otero, 2005; Gómez-Lechón et al., 2010). The lungs are a highly vascularized organ with a large surface area exposed to air and therefore, it is associated with higher oxygen concentrations than other organs. Consequently, lung is highly subjected to both exogenous and endogenous oxidative stress resulting in the pathogenesis of a number of

chronic obstructive airway diseases such as bronchitis and emphysema (Kang et al., 2005; Boateng and Verghese, 2012; Piao et al., 2008; Zhang et al., 2009).

Therefore, dietary antioxidants are needed to minimize oxidative damage and recently, there is a global trend for using natural phytochemicals as antioxidant supplements (Neofytou et al., 2012, Chen et al., 2010). Antioxidant activity of natural polyphenolic compounds was reported to provide cytoprotective effect under induced oxidative stress *in vitro* and *in vivo* (Chen et al., 2010). The lipophilic/hydrophilic nature of antioxidants is a crucial factor which limits their cellular uptake (Kajiya et al., 2001).

The quercetin molecule is lipophilic in nature, despite a number of the hydroxyl groups present. However, its derivatives possess different degrees of lipophilicity depending on the type of functional group attached to the quercetin molecule and glycosylation is known to increase the hydrophilic character (Huber et al., 2009). In plants, glycosylation is an important modification as it makes the quercetin molecules more cytosol-soluble and also facilitates transport to different plant parts and is stored in vacuoles (Materska, 2008). It was hypothesised that the acylation of the Q3G molecule improves its lipophilicity resulting in enhanced cellular uptake and modified biological activity. This study was designed to evaluate the cytoprotective activity of six long chain fatty acid derivatives of Q3G by determining the % cell viability, release of lactose dehydrogenase (LDH), production of lipid hydroperoxide and apoptotic/necrotic cell death under the H_2O_2-induced oxidative stress in cell culture model systems.

5.3 MATERIALS AND METHODS

5.3.1 Cell cultures

5.3.1.1 Normal human lung fibroblast cell line

Normal diploid human fetal lung fibroblast cell line (WI-38) was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were grown in ATCC formulated eagle's minimum essential medium (EMEM) supplemented with fetal bovine serum (FBS) (ATCC, Manassas, VA, USA) to a final concentration of 10% at 37 ^{o}C in a 5% CO_2 and humidified environment (CO_2 incubator, Model 3074, VWR International, West Chester, PA, USA). Cells were maintained in culture up to 40 population doublings in T-75 flasks (Becton Dickinson Labware, Bedford, USA). Sub-culturing was carried out when the cells reached 80% confluence. The cells were categorized under bio-safety level-1.

Sub-culturing

First, medium was removed followed by rinsing the cell layer with 1 ml of 0.25% trypsin, 0.03% EDTA solution (ATCC, Manassas, VA, USA). Then the solution was removed and additional 1 to 2 ml of trypsin-EDTA solution was added and the flask was allowed to sit at 37 ^{o}C until the cells detached. The fresh complete growth medium was then added to inactivate the trypsin-EDTA solution and dispense the cell suspension into new culture flasks in a sub-culturing ratio of 1:2 to 1:4. Media removal was carried out 1 to 2 times per week. Cells were cryopreserved in complete growth medium with 5% cell culture tested dimethylsulfoxide (DMSO) obtained from ATCC (Manassas, VA, USA).

5.3.1.2 Human normal primary hepatocytes

Fresh human normal primary hepatocytes (h-NHEPS™) were purchased from Lonza (Walkersville, MD, USA). The cells were cultured in hepatocyte basal medium (HBM™) supplemented with the HCM™ [ascorbic acid, bovine serum albumin-fatty acid free (BSA-FAF), hydrocortisone, transferrin, insulin, recombinant human epidermal growth factor (rhEGF), GA-1000 (gentamicin, amphotericin-B)] at 37 ^{o}C in a 5% CO_2 and humidified environment. No sub-culturing was recommended by the manufacturer as cells undergo replicative senescence. The cells were categorized under bio safety level-1.

5.3.2 Preparation of chemicals

H_2O_2 (1 mM) was freshly prepared each time in serum free media using 30% H_2O_2 solution (Sigma Aldriach, ON, Canada). All the test compounds were initially dissolved in DMSO and serial dilutions were carried out using complete growth media to obtain desired concentrations. The DMSO concentration was maintained to be less than 1% (v/v) in the culture plate wells.

5.3.3 Synthesis of fatty acid derivatives of Q3G

Six fatty acids, namely stearic acid, oleic acid, linoleic acid, ALA, EPA, and DHA derivatives of Q3G were enzymatically synthesised according to the method described by Ziaullah et al., (2013). The method is explained in Chapter 4 under section 4.3.2. Briefly, 500 mg of Q3G (Indofine Chemical Company, Hillsborough, NJ, USA) and each acyl donor fatty acids (Nu-Check prep Inc., Elysian, MN, USA) were reacted in a molar ratio of flavonoid:acyl donor 1:5 using anhydrous acetone as the solvent. The acylation was initiated by adding Novozym 435® immobilised lipase from *Candida antarctica* (2 g) as the biocatalyst followed by an incubation at 45 ^{o}C for approximately

48 hours. After the completion of the reaction, product was isolated by silica gel column chromatography using acetone:toluene; 40:60 to 50:50 monitored by preparative thin layer chromatography (TLC).

5.3.4 Instruments

A class II-type A2 biological safety cabinet (Model LR2-452), Esco Technologies Inc. Hartboro, PA, USA; CO_2 incubator (Model 3074), VWR International, West Chester, PA, USA; Inverted microscope (ECLIPSE TS 100/TS 100-F), Nikon Instruments Inc., Melville, NY, USA, supported with a Lumenara Infinity camera (1-2 USB, 2.9 Megapixel) including capture and analyzing software, Infinity Analyze, Lumenara Corporation, Ottawa, ON, Canada; Liquid nitrogen storage vessel, VWR International, West Chester, PA, USA; Haemocytometer (Bright-Line), Hausser Scientific, Horsham, PA, USA; Hand held tally counter, Sigma Aldrich, Oakville, ON, Canada; Stripettor (Stripettor^{TM} Plus), Corning Life Sciences, Lowell, MA, USA; Polystyrene flasks (T-75, canted neck, 0.2 μM vented), Becton Dickinson Labware, Bedford, USA; Bottle top filters (500 ml, 0.22 μm), Corning Life Sciences, Lowell, MA, USA; Sterile disposable pipettes (5 ml, 10 ml, 25 ml), Corning Life Sciences, Lowell, MA, USA; Cryocanes, Thermo Scientific Rochester, NY, USA; Cryotubes, Fisher Scientific, Whitby, Ontario, Canada; Cryogenic vials, Corning Life Sciences, Lowell, MA, USA. FLUOstar OPTIMA plate reader, BMG Labtech, Durham, NC, USA; fluorescence microscope Leica DMBL (20x/.040), Houston, TX, USA with a filter sets for Cyanine-3 (Ex/Em: 550/570 nm) and 7-AAD (7-Aminoactinomycin D, Ex/Em: 546/647 nm) coupled with Nikon Cool Pix 4500 Digital camera, Mississauga, ON, Canada.

5.3.5 Cell viability assay

Cells were cultured in 96-well plates at a density of 1×10^4 cells/100 µl per well and incubated at 37 ^oC for 24 hrs to allow for the attachment of cells. The cells were then treated with 100 µl test compounds (0.01, 0.1, 1, 10, 100 and 200 µM) and incubated at 37 ^oC for 48 hrs. The cells in the control group contained equal volume of DMSO (less than 1%, v/v) without adding any test compounds and were not oxidatively injured. The cells in the model group contained equal volume of DMSO, no addition of test compounds, but oxidative injury by H_2O_2 addition. After incubation, culture medium was removed and cells were washed with phosphate buffered saline (PBS) twice, applying centrifugation at 1000 rpm for 2 min in between. Then, cells were subjected to H_2O_2 insult at 800 µM for lung fibroblasts and 300 µM for normal hepatocytes followed by 3 hr incubation at 37 ^oC. These time-dose combinations for H_2O_2 were selected based on several preliminary experiments, before initiating the cytoprotection experiment, by treating the cells with different H_2O_2 concentrations under several time periods to determine the suitable time-dose combination resulting in 20-30% cell viability. Then, media was removed and cells were rinsed with PBS to remove the oxidant and 100 µl of fresh serum free media was added into the wells. Celltitier 96® aqueous non-radioactive cell proliferation assay (Promega Corporation, Medison, WI, USA) was used to determine cell viability. MTS reagent [10 µl, freshly prepared by adding 2 ml of MTS:100 µl of phenazine methosulfate (PMS)] was added to the each well and incubated at 37 ^oC for 1-4 hrs. MTS is a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-tetrazolium] and PMS is an electron coupling reagent. MTS is reduced by cells into a formazan product that is

soluble in tissue culture medium. The absorbance was then measured at 490 nm using a plate reader (FLUOstar OPTIMA, BMG Labtech, Burham, NC, USA).

For the post-treatment experiments, the cells were initially treated with H_2O_2 for the selected time and concentration at 37 ^oC. Then, the oxidants were removed by rinsing with PBS twice and cells were treated with test compounds (0.01, 0.1, 1, 10, 100, and 200 μM) and incubated in 37 ^oC for 48 hrs. After incubation, cells were washed with PBS and cell viability was determined as below:

$$\% \text{ Cell viability} = \frac{\text{Abs. of the treated wells - Blank}}{\text{Abs. of the control wells - Blank}} \times 100\%$$

(Abs.; Absorbance)

Where the treated wells contained the cells pre-incubated with test compounds followed by oxidative injury, the control wells contained the cells with no incubation of test compounds and no oxidative injury. Blank wells contained culture media only.

5.3.5.1 Cytotoxicity of the fatty acid acylated derivatives of Q3G

WI-38 cells were cultured in 96-well plates at a density of 1×10^4 cells/100 μl per well and incubated at 37 ^oC for 24 hrs. Cells were treated with 100 μl of the test compounds (0.01, 0.1, 1, 10, 100 and 200 μM) and incubated at 37 ^oC for 48 hrs. Then, the culture medium was removed and cells were washed with PBS twice. Cell viability was determined as described under 5.3.4.

5.3.5.2 Cytotoxicity assessment of the free fatty acids used as acyl donors

WI-38 cells were cultured in 96-well plates at a density of 1×10^4 cells/100 μl per well and incubated at 37 ^oC for 24 hrs. Then, cells were treated with 100 μl of the fatty acids

separately: stearic acid, oleic acid, linoleic acid, ALA, EPA, DHA in 0.01, 0.1, 1, 10, 100, and 200 μM concentrations and incubated at 37 ^{o}C for 48 hrs. Cell viability was determined as described under 5.3.4.

5.3.6 LDH release assay

WI-38 cells were cultured in 96-well plates at a density of 1×10^3 cells/100 μl per well and incubated at 37 ^{o}C for 24 hrs. According to the result from the MTS assay, 0.1 and 1 μM concentrations were selected for the test compounds. After treating the cells with 100 μl test compounds, they were incubated at 37 ^{o}C for 48 hrs. The cells in the control group contained equal volume of DMSO (less than 0.1%, v/v) without adding any test compounds and were not oxidatively injured. The cells in the model group contained equal volume of DMSO, no addition of test compounds, but subjected to oxidative injury by H_2O_2 addition. Then the culture medium was removed and cells were washed with PBS twice. Cells were subjected to H_2O_2 insult of 800 μM for 3 hrs. After incubation, the plate was centrifuged at 1000 rpm for 2 min and 50 μl of the supernatant was added into a new 96-well plate. The released LDH was assayed using CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega Corporation, Medison, WI, USA) according to the manufacturer's protocol. Absorbance was measured at 490 nm using plate reader (FLUOstar OPTIMA, BMG Labtech, Burham, NC, USA). After subtracting the absorbance of blank and control wells, from all the model wells and test wells, cytoprotection was calculated as below:

$$\% \text{ Cytoprotection} = \frac{\text{Abs. of the model wells - Abs. of the test wells}}{\text{Abs. of the model wells}} \times 100\%$$

Where, the model wells contained the cells, oxidatively injured without pre-incubation with the test compounds and the test wells contained the cells, pre-incubated with the test compounds followed by oxidative injury. The blank contained only the culture media. The control wells contained the cells with no incubation of test compounds and no oxidative injury.

5.3.7 Lipid peroxidation assay

Lipid peroxidation was measured using a lipid hydroperoxide assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). WI-38 cells were cultured in 24-well plates at a density of 1×10^5 cells/ml per well and incubated in 37 ^oC for 24 hrs. Then, the cells were treated with 1 µM of test compounds for 48 hrs at 37 ^oC. After incubation, the cells were washed with PBS to remove the test compounds and incubated with 800 µM H_2O_2 for 3 hrs at 37 ^oC. At the end of the oxidative injury period, supernatant (200 µl) of each well was taken into glass vials and lipid hydroperoxides were extracted by adding the equal volume of Extract R saturated methanol and 1 ml of cold chloroform followed by centrifuging the pre-vortexed mixture at 1500 × g for five min at 0 ^oC. The bottom chloroform layer (500 µl) was collected to a glass vial and stored on ice. Methanol and chloroform was deoxygenated prior to use by bubbling nitrogen through the solvents for about 30 min. Deoxygenated chloroform-methanol (2:1) mixture (450 µl) was added into each extracted sample of 500 µl. Then freshly prepared standard chromogen (50 µl) was added into the assay vials and vortexed. The assay vials were closed tightly and kept at room temperature for 5 min. A volume of 300 µl from each vial was transferred into a glass 96-well plate and absorbance was read at 490 nm using a plate reader (FLUOstar OPTIMA, BMG Labtech, Burham, NC, USA). Lipid hydroperoxides in the samples

were calculated using the equation obtained from the linear regression of the standard curve prepared using 0, 0.5, 1.0, 2.0, 3.0 and 4.0 nmol of lipid hydroperoxide standards.

5.3.8 Fluorescence microscopy assay

WI-38 cells were cultured in chamber slides (Nunc Lab-Tek II Chamber Slide System, Thermo Fisher Scientific, Ottawa, ON, Canada) at a density of 1×10^5 cells/ml per well and incubated at 37 ^oC for 24 hrs. Cells were treated with 1 μM of test compounds for 48 hrs at 37 ^oC. After incubation, the cells were washed with PBS to remove the test compounds and treated with 800 μM H_2O_2 for 3 hrs at 37 ^oC. The cells were carefully washed with PBS twice to remove the oxidants. The dual detection reagents; apoptosis detection reagent (Annexin V-EnzoGold) and necrosis detection reagent which is similar to the red emitting dye 7-AAD, supplied by GFP-certified™ apoptosis/necrosis detection kit (Enzo Life Sciences International, INC., Plymouth Meeting, PA, USA) were prepared in binding buffer (1×). After careful removal of the supernatant, the prepared detection reagent was dispensed in a volume sufficient for covering the cell monolayer in each slide. The cells were incubated for 15 min at room temperature, protected from the light. Then, the staining solution was flicked onto a paper towel and few drops of binding buffer were added to prevent the cells from drying out. The cells were then observed under fluorescence microscope (DMBL 20x/.040, Leica Microsystems, Houston, TX, USA) with a filter sets for Cyanine-3 (Ex/Em: 550/570 nm) and 7-AAD (Ex/Em: 546/647 nm), coupled with digital camera (Nikon Cool Pix 4500, Nikon Canada Inc., Mississauga, ON, Canada).

5.3.9 DNA fragmentation assay

The ApoTarget™ quick apoptotic DNA ladder detection kit (Invitrogen Corporation, Fraderick, MD, USA) was used to detect the apoptotic DNA fragmentation. WI-38 cells were cultured in 6-well plates at a density of 3×10^5 cells/2 ml per well and incubated at 37 ^oC for 24 hrs. Then the cells were treated with 1 μM of test compounds for 48 hrs at 37 ^oC. After incubation, the cells were washed with PBS to remove the test compounds and treated with 800 μM H_2O_2 for 3 hrs at 37 ^oC. After the oxidative insult, the cells were extracted using trypsin-EDTA and pelleted by centrifuging at 5000 rpm for 5 min. The supernatant was discarded carefully and cells were lysed using Tris-EDTA lysis buffer by careful pipetting up and down several times. Enzyme A solution was added into the crude lysate followed by gentle vortexing and incubating at 37 ^oC in a water bath for 10 min. Then, enzyme B solution was added and incubated at 50 ^oC for 30 min in a water bath. Cold ammonium acetate solution (5 μl) and 100 μl of absolute ethanol (kept at -20 ^oC) were added, vortexed and allowed the DNA to precipitate at -20 ^oC for 10 to 15 min. Samples were centrifuged for 10 min at 14,000 rpm to collect the precipitated DNA and supernatant was carefully discarded. DNA pellet was washed with 70% cold ethanol (0.5 ml). After discarding the supernatant, the DNA pellet was resuspended in DNA suspension buffer and loaded onto 2% agarose gels containing gel red. DNA was visualized by transillumination with UV light.

5.3.10 Cell morphological assessment

WI-38 cells were cultured in 24-well plates at a density of 1×10^5 cells/1 ml per well and incubated at 37 ^oC for 24 hrs. The cells were treated with 1 μM of test compounds for 48 hrs at 37 ^oC. After incubation, the cells were washed with PBS to remove the test

compounds and treated with 800 μM H_2O_2 for 3 hrs at 37 ^oC. Morphology of the cells was examined under inverted microscope (ECLIPSE TS 100/TS 100-F, Nikon Instruments Inc., Melville, NY, USA) using phase contrast optics under 10× magnification. The images were recorded using the Lumenara Infinity camera (1-2 USB, 2.9 Megapixel) including capture and analyzing software (Infinity Analyze, Lumenara Corporation, Ottawa, ON, Canada).

5.3.11 Statistical Analysis

Statistical analyses were carried out using one way ANOVA in Minitab 16 statistical software and two factor factorial design using general linear model in SAS 9.3. Multiple mean comparison was carried out by Tukey's method at $P \leq 0.05$ (Montgomery, 2012).

5.4 RESULTS

5.4.1 Cytoprotective effect of fatty acid derivatives of Q3G

The ability of the fatty acid derivatives of Q3G to overcome the H_2O_2-induced oxidative stress was determined in a dose dependent manner using WI-38 cells (Figure 5.1) and human primary hepatocytes (Figure 5.2). All viability percentages were calculated based on the untreated control with no oxidative injury. In WI-38 cells, 0.01, 0.1, 1, and 10 μM of ALA derivative of Q3G, 10, and 100 μM of EPA derivative of Q3G and 1 and 10 μM DHA derivative of Q3G provided a significant ($p \leq 0.05$) protection against acute oxidative damage, when compared to the model group of H_2O_2 insult without pre-incubation of any test compounds. ALA derivative of Q3G demonstrated a significant cytoprotection over a wide range of concentrations compared to other compounds. It exhibited cytoprotection of 18%, 19%, 26% and 18% at 0.01, 0.1, 1 and 10 μM,

respectively. Further, it is noticeable that ALA showed a significant protection at 0.01 μM which was a 100 times lower concentration than DHA derivative and 1000 times lower than EPA derivative. The concentration of 1 μM was the most effective doses of the ALA derivative while for the EPA derivative, 10 μM and 100 μM concentrations showed 8% and 23% protection respectively and for DHA derivative, 1 μM and 10 μM concentrations showed 7% and 9% protection, respectively. Stearic acid, oleic acid and linoleic acid derivatives of Q3G did not provide significant ($p \leq 0.05$) cytoprotection at any of the tested concentrations. Both 100 and 200 μM concentration of oleic acid, linoleic acid, ALA, and DHA derivatives of Q3G resulted in almost complete cell death, indicating a toxic effect at higher concentrations. Further, EPA also showed the same effect for 200 μM concentration. The parent compound, Q3G showed 7% cytoprotection in 1 μM concentration while it also resulted in complete loss of cell viability at 100 and 200 μM concentrations. The aglycon of Q3G, Q did not provide significantly higher ($p \leq 0.05$) cytoprotection at any of the tested concentrations.

The same experiment was carried out using the human primary hepatocytes (Figure 5.2). The long chain fatty acid derivatives of Q3G: 0.1 and 1 μM of oleic acid, 0.01 and 0.1 μM of linoleic acid, 0.1 μM of ALA, 1 and 10 μM of EPA and 10 μM of DHA exhibited significant ($p \leq 0.05$) protection compared to the model group. However, stearic acid derivative of Q3G did not show a significant ($p \leq 0.05$) cytoprotection at any of the tested concentrations. The oleic acid derivative was able to provide 18%, 32% and 41% cytoprotection in 0.01, 0.1 and 1 μM, respectively. The linoleic acid derivative demonstrated 20% and 33% cytoprotection at 0.01 and 0.1 μM concentrations, respectively. The cytoprotection percentages: for ALA derivative was 15% at 0.1 μM,

for EPA derivative was 19% and 20% at 1 and 10 μM, for DHA derivative was 14% and 32% protection at 1 and 10 μM. All the fatty acid derivatives, except stearic acid, exhibited complete cell death at 100 and 200 μM concentrations. The parent compound, Q3G showed 11% protection at 0.1% μM concentration and 100 and 200 μM concentrations showed very low cell viability of 4-7%. Quercetin did not give a significant ($p \leq 0.05$) cytoprotection at any of the tested concentrations.

5.4.2 Cytotoxic effect of fatty acids derivatives of Q3G

All the fatty acid derivatives of Q3G were tested for their toxicity for WI-38 cells in a dose dependent manner (Figure 5.3). Oleic, linoleic and ALA derivatives of Q3G were not toxic to cells at all of the tested concentrations ranging from 0.01 to 200 μM. Decrease in cell viability was observed for stearic acid (20% decrease at 200 μM), EPA (18% decrease at 100 μM and 30% decrease at 200 μM), DHA (10% decrease at 100 μM and 20% decrease at 200 μM) derivative of Q3G. Quercetin and Q3G did not show cell toxicity at any of the tested concentrations.

5.4.3 Cytotoxic effect of acyl donor free fatty acids

The six long chain fatty acids used for modifying the structure of Q3G were tested for their cytotoxicity against WI-38 cells in a dose dependent manner (Figure 5.4). With the exception of stearic acid, all other fatty acids showed no toxicity for the cells from 0.01 to 100 μM concentration range. Stearic acid reported only 69% viability at 100 μM concentration. However, all six fatty acids exhibited reduced cell viability at the 200 μM level. The cell viabilities at 200 μM were; 50% for stearic acid, 85% for oleic acid, 70% for linoleic acid. ALA, EPA, and DHA showed 100% cell death at 200 μM.

5.4.4 Cytoprotection by fatty acid derivatives of Q3G by LDH release assay

The cytoprotection of fatty acid derivatives of Q3G was also tested using LDH release assay. According to the statistical analysis, the interaction effect of concentration and type of compound was not significant at $P \leq 0.05$. However, 1 μM concentration provided significant protection over the 0.1 μM concentration level. The cytoprotection percentages exhibited by fatty acid derivatives (stearic acid, oleic acid, linoleic acid, ALA, EPA, DHA) of Q3G at 1 μM were in the range of 20% - 31%. At the same concentration, the cytoprotection values for quercetin and Q3G were 16% and 17%, respectively. According to these results, the DHA derivative of Q3G demonstrated the highest protection and all the esters were able to provide higher protection than Q and Q3G (Figure: 5.5).

5.4.5 Lipid peroxidation under H_2O_2-induced cytotoxicity in WI-38 cells

All the fatty acid derivatives of Q3G at 1 μM concentration, showed a significantly ($p \leq 0.05$) lower production of lipid hydroperoxides compared to the H_2O_2 injury control. The decrease in lipid peroxidation by the esters was in a range of 22-27% and ALA and DHA ester showed the highest effectiveness in inhibiting the cellular lipid peroxidation. Q3G and quercetin showed 15% and 18% decrease in the production of lipid hydroperoxides, respectively.

5.4.6 Morphological changes and cell death

The morphological changes of WI-38 cells subjected to H_2O_2-induced oxidative stress with and without addition of test compounds are found in figure 5.7. The morphology of the cells appeared to be altered with the incubation of H_2O_2 when compared to the healthy cells. The cells treated with the test compounds seemed to be less damaged,

shrinked and ruptured compared to the cells which were oxidatively injured without adding the test compounds. The cells underwent pre- and late- apoptosis as presented in figures 5.8 and 5.9, respectively. Staining with fluorescence staining dyes, the early apoptotic cells were colored green and late apoptotic and/or necrotic cells were colored red. The cells pre-treated with test compounds showed less stained area compared to the cells oxidatively damaged, but not treated with test compounds.

5.4.7 DNA Fragmentation

With the oxidative injury with H_2O_2 on WI-38 cells, internecleosomal DNA fragmentation was not observed in the gel electrophoresis.

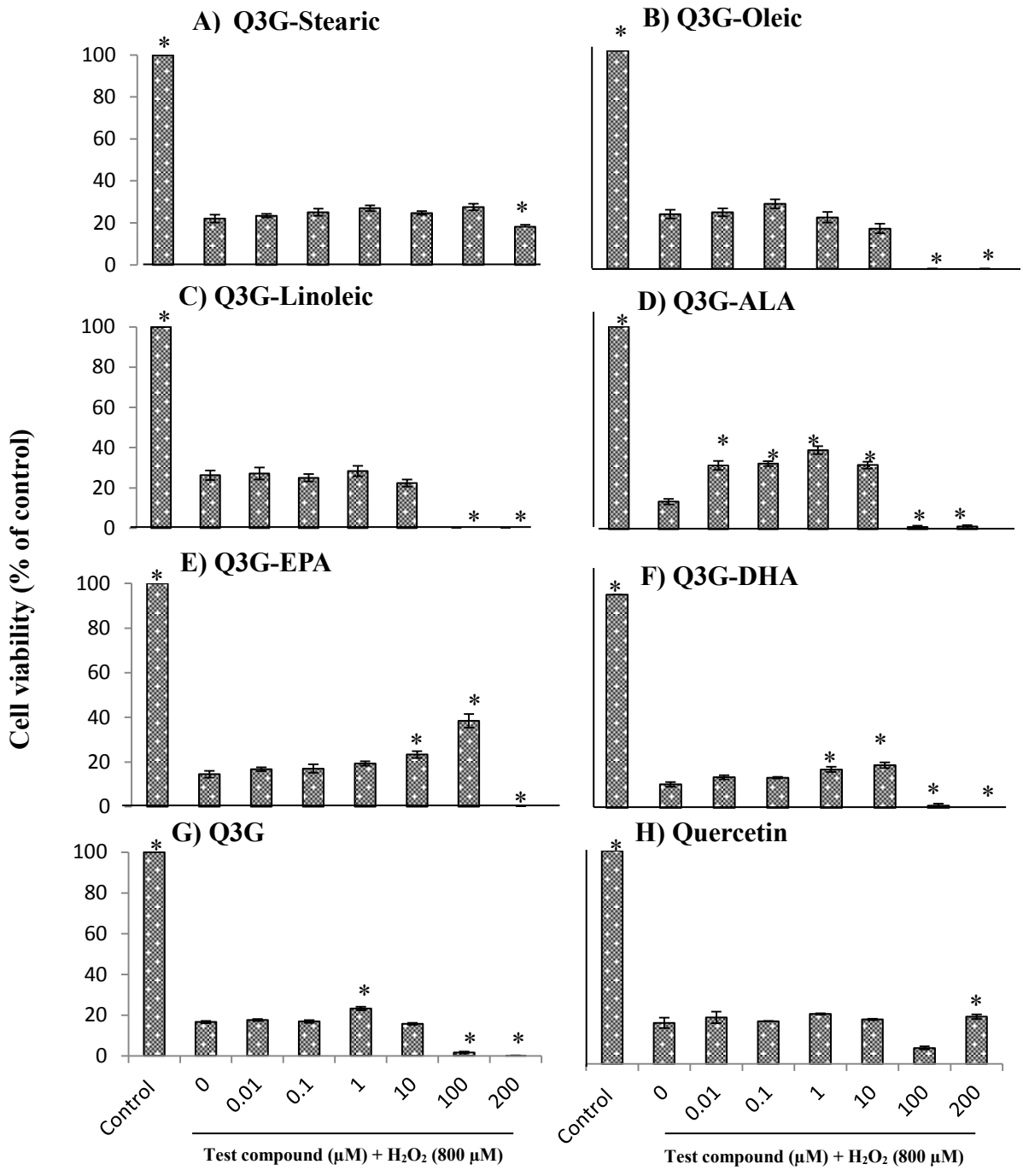


Figure 5.1: Dose dependent cytoprotective effect of test compounds against H_2O_2-induced cytotoxicity in WI-38 cells. A) Stearic acid derivative of Q3G, B) oleic acid derivative of Q3G, C) linoleic acid derivative of Q3G, D) ALA derivative of Q3G, E) EPA derivative of Q3G, F) DHA derivative of Q3G, G) Q3G and H) Q. Cells were pre-incubated for 48 hrs followed by incubation with 800 µM H_2O_2 for 3 hrs. Cell viability is presented as percentage related to the control. Control contains cells with no incubation of test compounds and no oxidative injury. Data are expressed as mean ± SEM (n=6). * $p \leq 0.05$, significantly different from model group. The cells in the model group were subjected to oxidative injury by H_2O_2, but not treated with any test compounds.

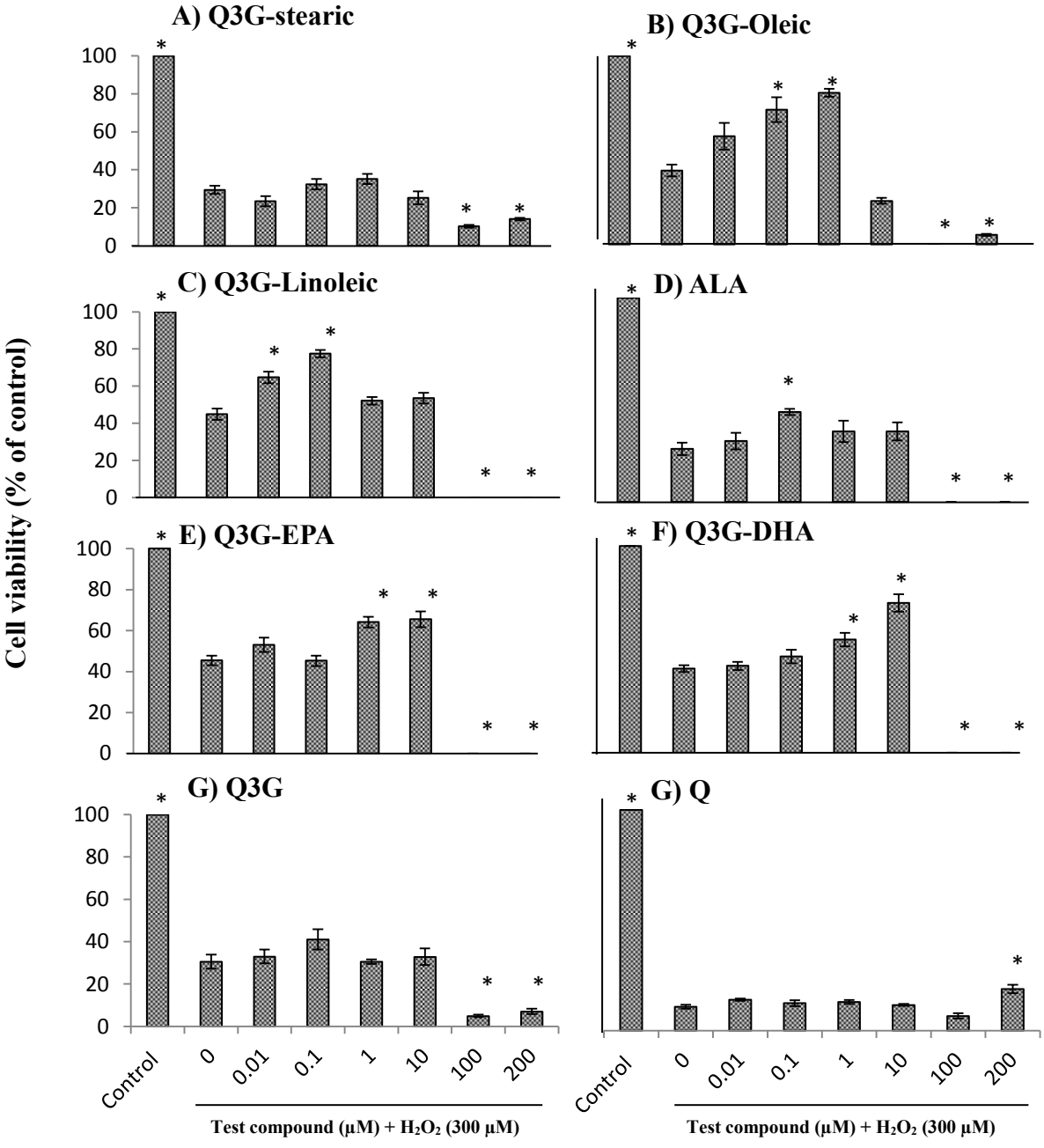


Figure 5.2: Dose dependent cytoprotective effect of test compounds against H_2O_2-induced cytotoxicity in human primary hepatocytes. A) Stearic acid derivative of Q3G, B) oleic acid derivative of Q3G, C) linoleic acid derivative of Q3G, D) ALA derivative of Q3G, E) EPA derivative of Q3G, F) DHA derivative of Q3G, G) Q3G and H) Q. Cells were pre-incubated for 48 hrs followed by incubation with 300 µM H_2O_2 for 3 hrs. Cell viability is presented as percentage related to the control. Control contains cells with no incubation of test compounds and no oxidative injury. Data are expressed as mean ± SEM (n=6). * $p \leq 0.05$, significantly different from model group. The cells in model group were subjected to oxidative injury by H_2O_2, but not treated with any test compounds.

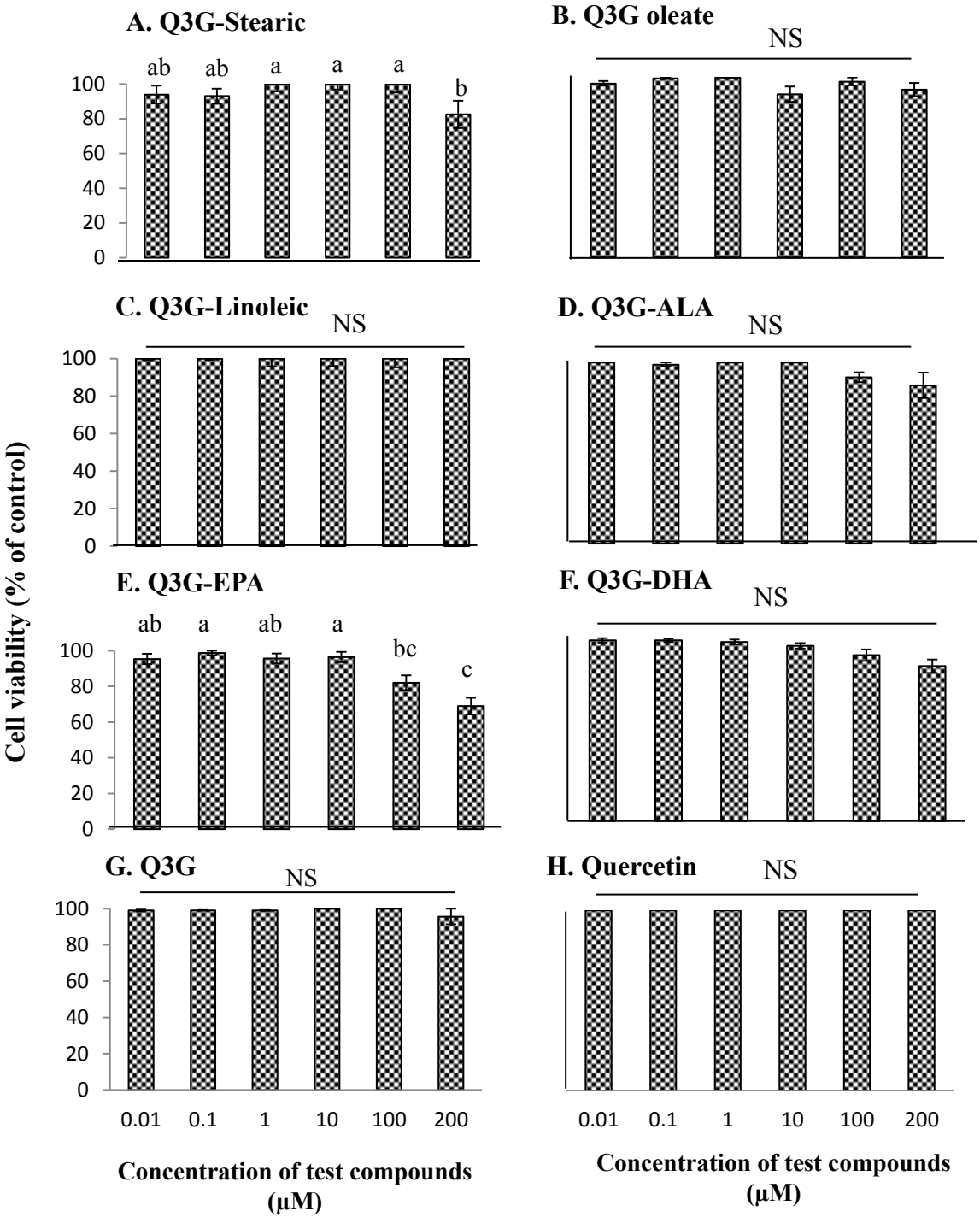


Figure 5.3: Dose dependent cytotoxicity of fatty acid derivatives of Q3G; A) stearic acid derivative of Q3G, B) oleic acid derivative of Q3G, C) linoleic acid derivative of Q3G, D) ALA derivative of Q3G, E) EPA derivative of Q3G, F) DHA derivative of Q3G, G) Q3G and H) Q. Cells were pre-incubated with test compounds for 48 hrs. Cell viability was presented as percentage related to the control. Control contains cells with no incubation of test compounds. Data are expressed as mean ± SEM (n=6), means sharing the same letter within test compound are not significantly different ($p \leq 0.05$).

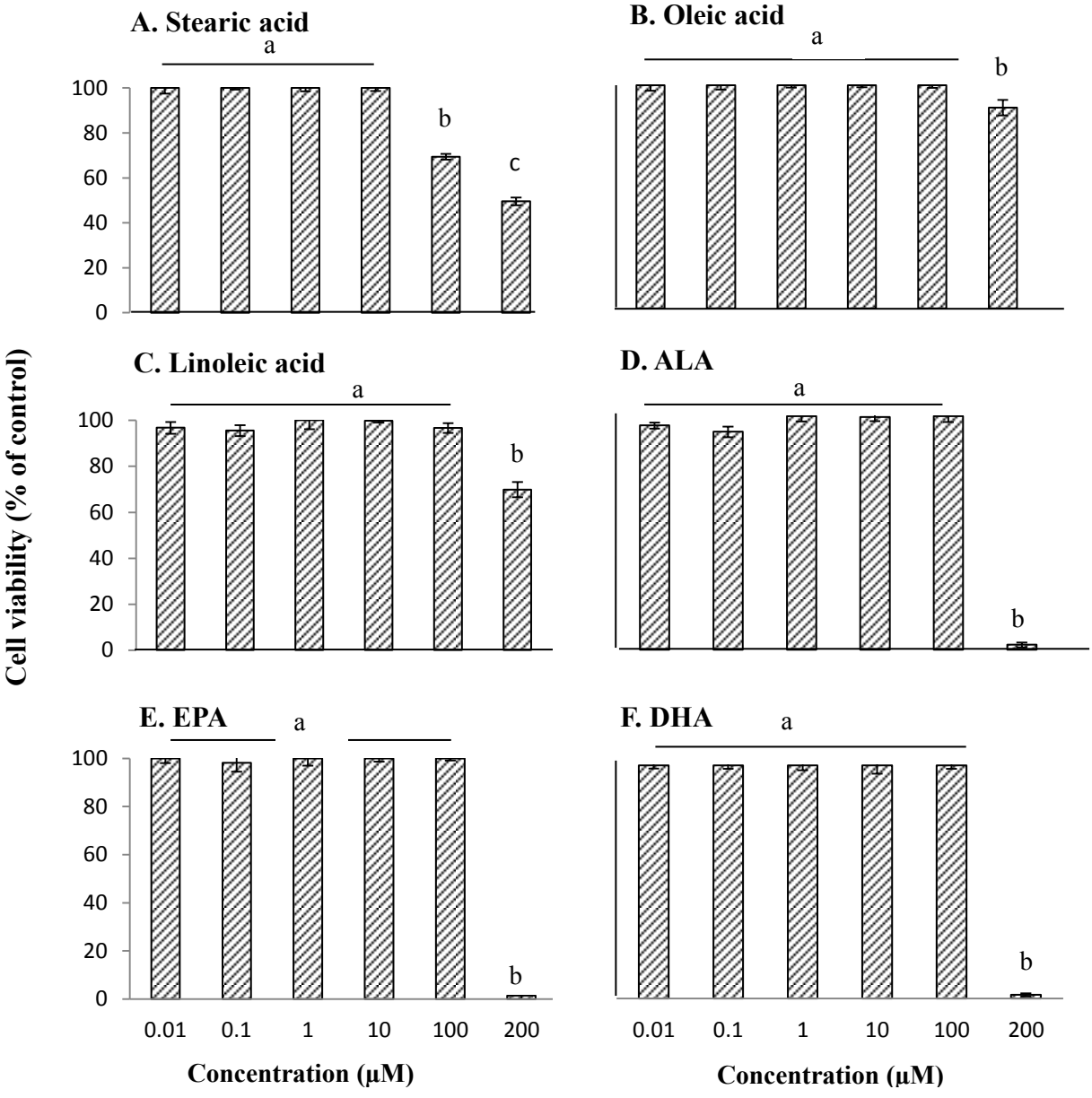


Figure 5.4: Dose dependent cytotoxic effect of free fatty acids on WI-38 cells ; A) stearic acid, B) oleic acid C) linoleic acid D) ALA, E) EPA, F) DHA. Cells were pre-incubated with free fatty acids for 48 hrs. Cell viability was presented as percentage related to the control. Control contains cells with no incubation of free fatty acids. Data are expressed as mean ± SEM (n=6), means sharing the same letter within compound are not significantly different ($p \leq 0.05$).

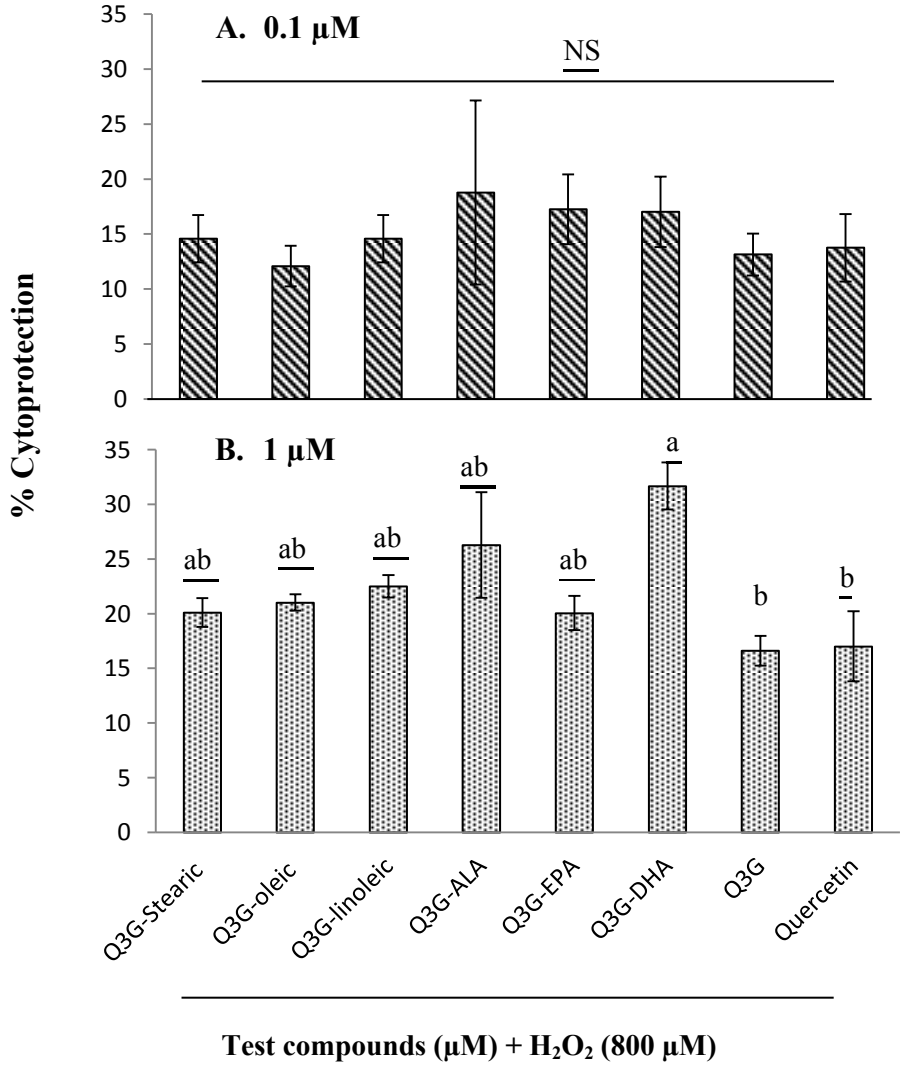


Figure 5.5: Dose dependent cytoprotective effect of six fatty acid derivatives of Q3G, Q3G and Q using release of LDH under H_2O_2-induced oxidative injury on WI-38 cells. Cells were pre-incubated with test compounds at 0.1 µM (A) and 1 µM (B) followed by oxidative injury with H_2O_2 for 3 hrs. Data were expressed as mean ± SEM (n=3), means sharing the same letter within concentration are not significantly different ($p \leq 0.05$).

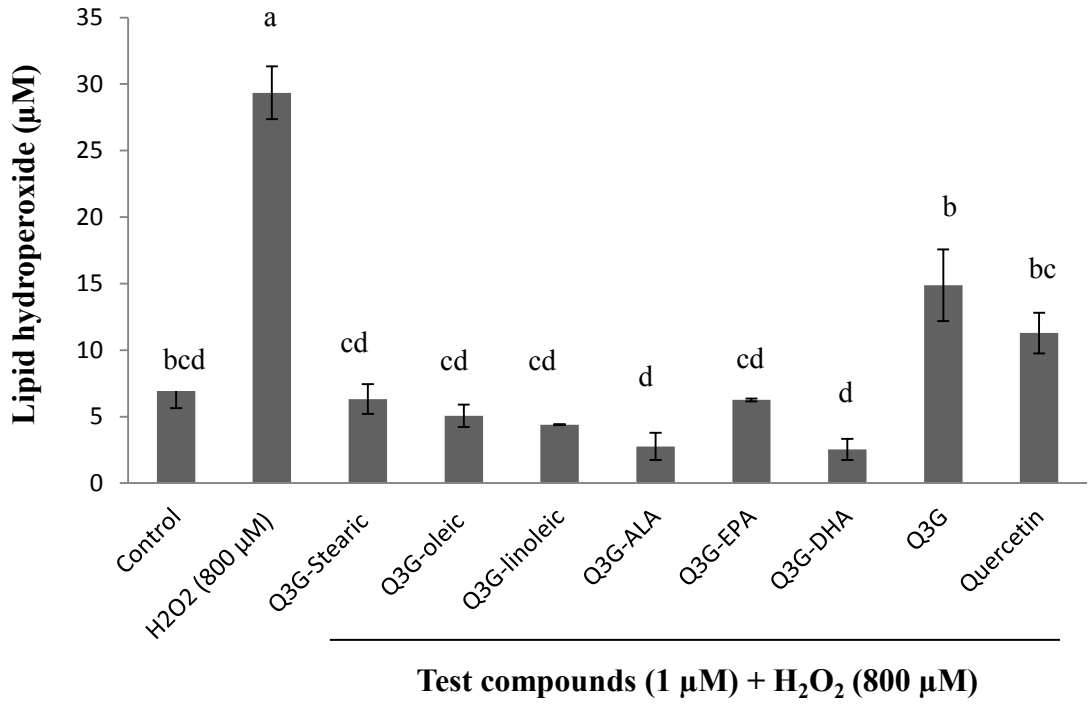


Figure 5.6: Production of lipid hydroperoxides under H_2O_2-induced oxidative injury on WI-38 cells. Cells were pre-incubated with test compounds for 48 hrs followed by 800 µM H_2O_2 for 3 hrs and production of lipid hydroperoxides was determined. Control contains the cells with no incubation of test compounds and no oxidative injury. Data were expressed as mean ± SEM (n=3), means sharing same letter are not significantly different ($p \leq 0.05$).

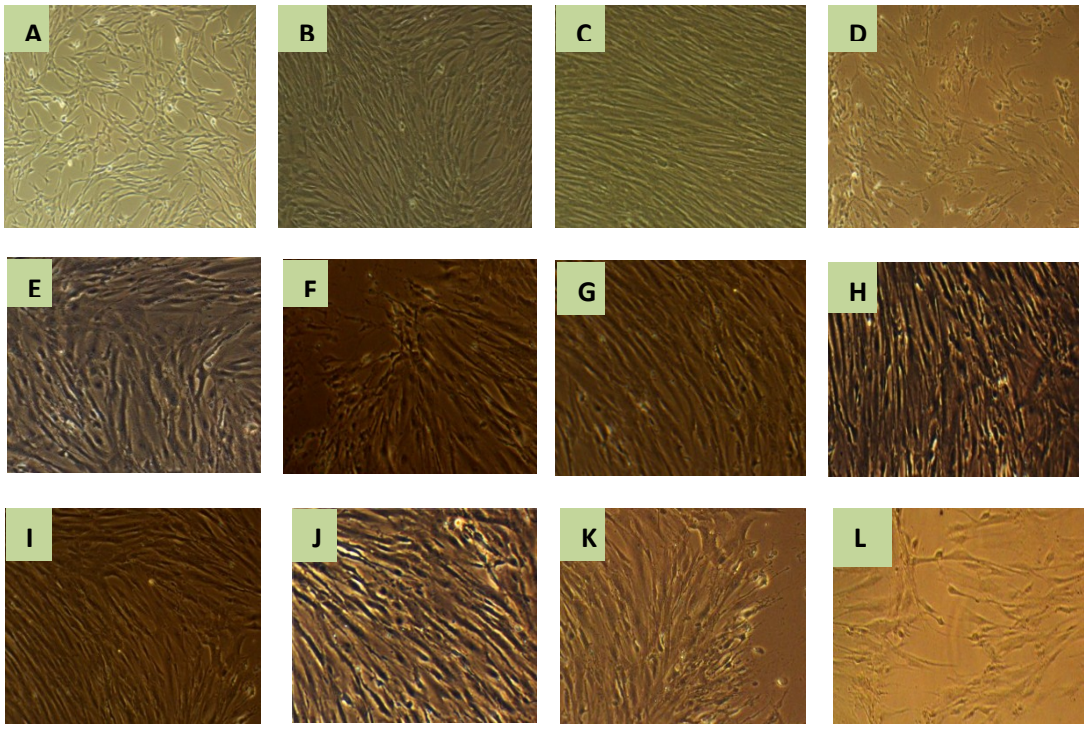


Figure 5.7: Morphological changes of WI-38 cells under an inverted phase contrast microscope (10X). A) to C) WI-38 cells in 70 %, 80%, > 90% confluence respectively. D) Cells incubated only with H_2O_2 (800 µM) for 3 hrs. Images E to C represent the cells pre-incubated with test compounds for 48 hrs followed by H_2O_2-induced oxidative injury: E) stearic acid derivative of Q3G, F) oleic acid derivative of Q3G, G) linoleic acid derivative of Q3G, H) ALA derivative of Q3G, I) EPA derivative of Q3G, J) DHA derivative of Q3G, K) Q3G and L) Q.

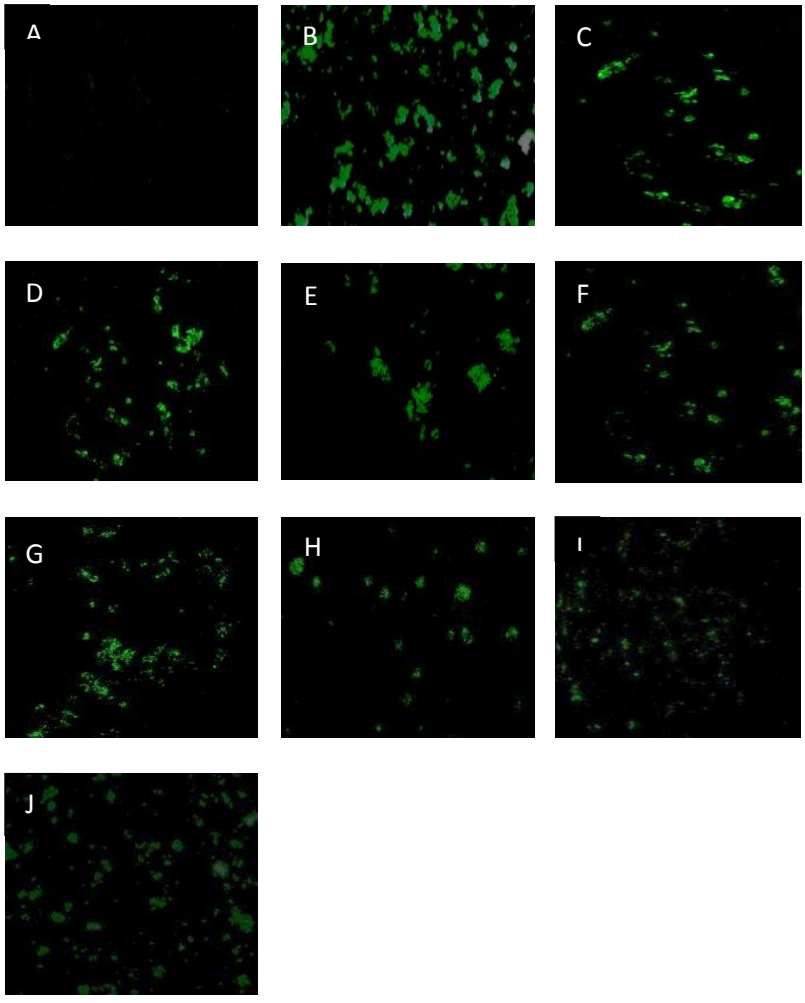


Figure 5.8: Effect of test compounds on H_2O_2 induced apoptotic cell death of WI-38 cells. A) Untreated control; B) Cells treated only with H_2O_2; C) stearic acid derivative of Q3G, D) oleic acid derivative of Q3G, E) linoleic acid derivative of Q3G, F) ALA derivative of Q3G, G) EPA derivative of Q3G, H) DHA derivative of Q3G, I) Q3G, J) Q. Cells were pre-incubated with test compounds for 48 hrs and then subjected to oxidative injury by 3 hr incubation with H_2O_2. Cells were rinsed with PBS, stained with Annexin V and observed under a fluorescence microscope.

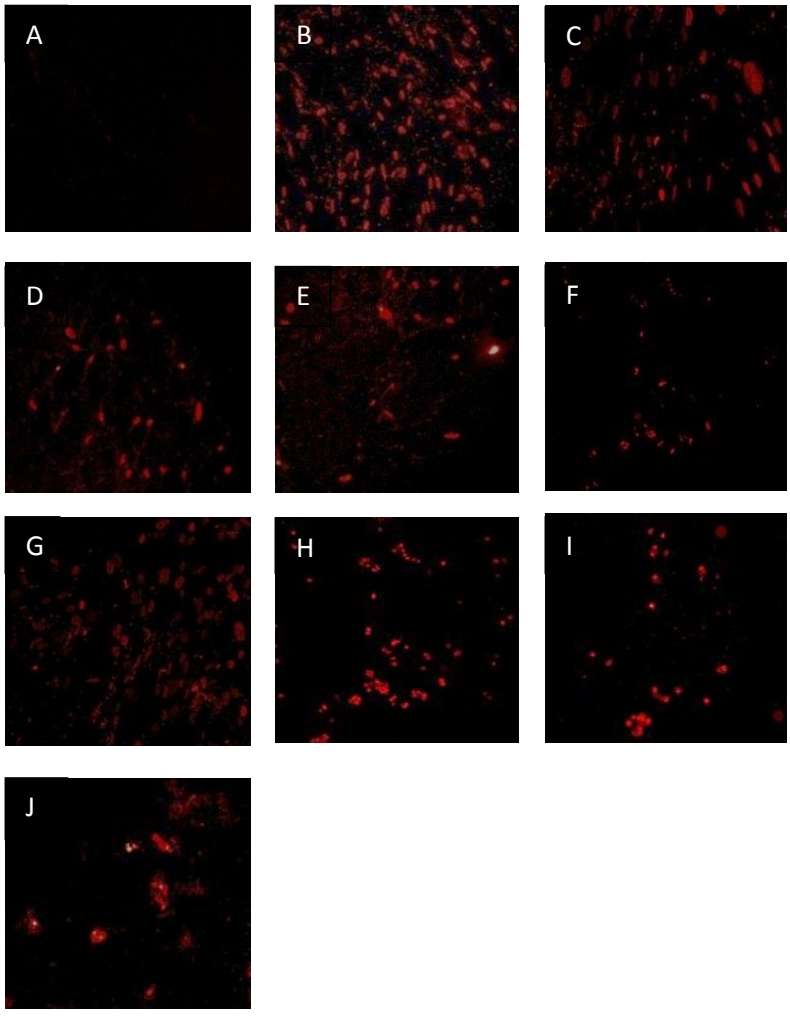


Figure 5.9: Effect of test compounds on H_2O_2 induced late apoptotic/necrotic cell death of WI-38 cells. A) Untreated control; B) cells treated only with H_2O_2; C) stearic acid derivative of Q3G, D) oleic acid derivative of Q3G, E) linoleic acid derivative of Q3G, F) ALA derivative of Q3G, G) EPA derivative of Q3G, H) DHA derivative of Q3G, I) Q3G, J) Q. Cells were pre-incubated with test compounds for 48 hrs and then oxidative injured by 3 hr incubation with H_2O_2. Cells were rinsed with PBS, stained with necrosis detection reagent and observed under a fluorescence microscope.

5.5 DISCUSSION

Plant based polyphenolic antioxidants have been extensively investigated for their potential therapeutic applications against oxidative stress associated pathogenesis of chronic diseases (Laguerre et al., 2011). The focus of the current study was directed on cytoprotection properties of six novel flavonoid derivatives using cell model systems of oxidative stress. The cytoprotective effect of flavonoids and flavonoid-rich extracts under induced oxidative stress conditions in different cell culture model systems are well documented (Nakayama et al., 1997; Kajiya et al., 2001; Piao et al., 2008; Gong et al., 2010). A culture of hepatocytes preserves the metabolic enzymes in the liver *in vivo* providing an *in vitro* model for preliminary screening on hepatoprotective activity of test compounds. Among them, primary hepatocytes are considered to be the closet to the liver *in vivo* (Gómez-Lechón et al., 2011; Zhao and Zhang, 2009). Hesperidin (Chen et al., 2010), a flavonoid extracted from *Artemisia capillaris* (Chu et al., 1999) and an extract of bilberry (Valentova et al., 2007) are reported to have protective effects against *tert*-butyl hydroperoxide-induced oxidative stress in human hepatocytes and rat primary hepatocytes. Moreover, as a highly exposed organ for high oxygen concentration, the lungs are subjected to many airway diseases and the role of human lung fibroblasts is imperative in pulmonary hypertension, pulmonary fibrosis, diffuse alveolar damage by thickening and/or remodeling of airways and parenchyma (Teramoto et al., 1999). Cytoprotective effects of morin (Zhang et al., 2009), heperoside (quercetin-3-*O*-galactoside) (Piao et al., 2008) and modified curcumin (Nakayama et al., 1997) have been reported against H_2O_2-induced cell damage of Chinese hamster lung fibroblasts (Zhang et al., 2009).

The lipophilic/hydrophilic nature of the antioxidants plays a significant role in determining their biological activity as a crucial factor governing the membrane permeability and target accessibility (Garrido et al., 2012). Therefore, conjugation of hydrophobic moiety to the antioxidant molecule is practised widely and it has been shown that hydrophobic groups contribute highly to the cellular uptake and biological activity of phenolic compounds (Locatelli et al., 2013). However, the debate on the role of lipophilicity in enhancing the antioxidant activity still remains unanswered. Recently, it has been reported that lipophilicity acts as a double-edge sword due to the non-linear relationship between lipophilicity and antioxidant activity (Laguerre et al., 2013).

To our knowledge, studies on the effect of increased lipophilicity to mitigate the oxidative stress-induced cytotoxicity are still very limited. Caffeic acid esters with higher lipophilicity exhibited dose-dependent cytoprotection against oxidative damage in rat pheochromocytoma cells (Garrido et al., 2012). Also, stearic acid, EPA and DHA esters of epigallocatechin gallate (EGCG) were more effective than EGCG, in protecting against DNA scission induced by hydroxyl and peroxyl radicals (Zhong and Shahidi, 2012). Antioxidant activity of chlorogenic acid and its methyl, butyl, octyl, dodecyl and hexadecyl esters towards mitochondrial ROS generated in a ROS-overexpressing fibroblast cell line was investigated and long chain esters were found to be more effective in scavenging ROS (Laguerre et al., 2010). Abilities of flavonoids to prevent the formation of DNA damages in cells exposed to H_2O_2 was positive in Jurkat cells and it has been proposed that cytoprotective effect of flavonoids depends on their ability to penetrate through the plasma membrane and to remove loosely bound redox-active iron from specific intracellular locations (Melidou et al., 2005).

H_2O_2 and *tert*-butyl hydroperoxide are commonly used in cell culture studies to mimic oxidative stress *in vitro*. H_2O_2 is a major component of intracellular ROS generated during normal physiological processes and elevated levels are reported under pathological conditions (Zhang et al., 2009; Boots et al., 2007). Due to its less toxic nature and diffusion through biological membranes to reach the target sites, H_2O_2 has been widely used as an inducer of oxidative stress *in vitro* (Wei et al., 2012; Li et al., 2010). Therefore, in the present study, H_2O_2 was used to induce oxidative stress in two cell culture systems. In this study, the fatty acid derivatives of Q3G were better antioxidants than Q3G and Q for attenuating the cytotoxicity induced by H_2O_2 in both primary hepatocytes and lung fibroblasts. However, an aggravated effect was observed at 100 or 200 μM of the test compounds indicating the possible pro-oxidant activity at higher doses under oxidative injury. Q is sparingly soluble in the cell culture medium at higher concentration (Zielinska et al., 2003) and this could be the reason for no cytotoxicity observed at 200 μM for quercetin under induced oxidative stress while it was toxic at 100 μM. There was no cytoprotective effect demonstrated by Q3G against oxidative stress in rat C2 glioma cells while it was not toxic at 100 μM (Zielinska et al., 2003). Our findings in current study are similar. The most probable reason for the cytoprotective effect of Q3G derivatives over Q3G or quercetin is their improved lipophilic nature which provides permeability through cell membranes. However, the toxic effect observed at the higher concentrations is possibly due to several reasons. Firstly, the high intracellular concentrations or interactions with cell membranes could generate pro-oxidative effects. Secondly, the aggregative effect of Q3G and the toxicity of the acyl donor fatty acids at higher doses provide important evidence. The cytotoxicity of fatty acids has been discussed in the literature focusing the effect of structural

diversity in carbon chain. Fatty acid-induced apoptosis and necrosis were exhibited in macrophages while no clear relationship was observed between the toxicity and carbon chain length or the number of double bonds (Lima et al., 2006). However, arachidonic acid, linolenic acid, linoleic acid and oleic acid were reported to be toxic in human lens epithelial cells and it has been further found that saturated fatty acids were less effective than the unsaturated fatty acids (Iwig et al., 2004). However, post treatment with the test compounds was not able to demonstrate any cytoprotection in either cell system (data not shown). The acute cytotoxicity created in the experiment could be too harsh for the cellular system to become regenerated with the activity of test compounds.

Lipid peroxidation is considered as the major mechanism of cell injury caused by H_2O_2 (Zhang et al., 2009; Zhao and Zhang, 2009) and therefore, it is a reliable indicator of the cellular oxidative damage (Yen et al., 2003). Metal ions are capable of catalysing the cytotoxic effect of H_2O_2 and therefore polyphenols effective in chelating the metal ions are protectors against H_2O_2-induced cytotoxicity (Fernandes et al., 2012). Oxidative stress can induce the cell death via either apoptosis or necrosis and the degree of the oxidative insult determines the pathway triggered (Fiers et al., 1999). In a study carried out by Teramoto et al. (1999), H_2O_2 triggered the apoptosis pathway at 10 and 100 μM on human lung fibroblast cells and DNA strand breaks were observed in terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Further, necrotic cell death was observed at H_2O_2 concentrations higher than 1 mM. Digestion of genomic DNA by endonuclease, generating a laddering pattern of small double-stranded fragments is considered as a hallmark of apoptosis. In this fragmentation process, first, DNA is cleaved into high molecular weight (HMW) DNA fragments of 50-300 kb and

secondly these are cleaved into oligonucleosomes (~180 bp) (Higuchi, 2004; Brown et al., 1993). The later fragmentation occurs at internucleosomal level and can be detected as a laddering pattern in conventional agarose gel electrophoresis (Newell et al., 1990). TUNEL assay is efficient for detecting the HMW fragments as every available 3'OH can be tailed by the enzymes (Lecoeur, 2002). There are some cell types that demonstrated apoptosis by only the HMW fragmentation, but not the internucleosomal cleavage and therefore, do not appear as a DNA ladder pattern (Higuchi, 2004; Formichi et al., 2006). Therefore, internucleosomal DNA fragmentation is not necessary to cause apoptotic cell death. The study carried by Formichi et al., (2006), using human fibroblast cells under H_2O_2-induced oxidative stress, observed apoptotic signs under microscopy, but did not observe DNA fragmentation by agarose gel electrophoresis and flow cytometry. However, the cells were positive for TUNEL assay providing a clue for the possibility of HMW fragmentation. They have further concluded that with the induction of oxidative stress, fibroblasts undergo apoptosis with DNA fragmentation via caspase independent pathway and apoptosis inducing factor (AIF); a mitochondrial intermembrane flavoprotein which induced a rapid caspase-independent digestion into ~50 kb fragments, but not oligonucleosomal fragments. Moreover, there are some other studies reported that when apoptosis is induced by different induction techniques in fibroblasts, only the HMW DNA cleavage took place, without signs of typical laddering (Simm et al., 1997; Boularest et al., 2001; Kues et al., 2002; Nagata et al., 2003). The presence of low level of caspase-activated DNase (CAD) and its inhibitor (ICAD) proteins in the fibroblast cells can be the possible reason for low oligonucleosomal DNA fragmentation (Nagata, 2000). The present study showed both apoptosis and late apoptosis cell death in the

fluorescence microscopy study, while no internucleosomal DNA fragmentation was induced by the H_2O_2 injury.

In general, antioxidant activity depends on the number, location and substitution pattern of hydroxyl groups in flavonoids (Bors et al., 1990; Rice-Evans et al., 1996) which scavenge free radicals generating less reactive phenoxyl radicals or chelate transition metal ions suppressing the Fenton reaction (Piao et al., 2008). Chelating property of flavonoids depends mainly on the catechol group on the B ring, 4-carbonyl, 5-hydroxyl group (Piao et al., 2008). Further, the amount of flavonol taken up by the cells and consequently, its cytoprotection effect is governed by the lipophilicity of the structure (Kajiya et al., 2001). Kale extract failed to protect and even aggravated the deleterious effects of H_2O_2-induced acute oxidative stress in Chinese hamster lung fibroblast cells (Fernandes et al., 2012). However, kaemferol-3-*O*-rutinoside, ferulic acid and sinapic acid didn't prevent nor aggravate the toxicity induced by H_2O_2 in V79 cells. It has been postulated that the higher molecular weight and the polar nature of those molecules rather than their aglyones hinder their movements across the cell membrane (Medina and Moreno-Otero, 2005). However, rutin attenuates H_2O_2-induced cytotoxicity and apoptosis in HUVECs (human umbilical vein endothelium cells) via ROS down-regulation, glutathione (GSH) up-regulation and restoration of mitochondrial membrane potential (Gong et al., 2010). In cell culture models, cytoprotective effects of flavonoids are highly linked to their antioxidant activity. Furthermore, lipophilicity of the flavonoid molecule facilitates the passing through the bilayer in the cell membranes. Therefore, lipophilic character together with supportive chemical structure has a significant role in the potency of optimum cytoprotective effects (Zhang et al., 2008).

It is quite clear that the antioxidant activities of the naturally-occurring and preferentially absorbed quercetin derivatives are very different to the quercetin aglycone (Williamson et al., 1996); however, the natural phytochemical form is not necessarily the bioactive form *in vivo* (Spencer et al, 2004). Biotransformation of flavonoid molecules take place in intestine and liver and their absorbed metabolites are crucial for bioavailability (Rice-Evans, 2000). Naturally occurring flavonoids are found mainly in their glycosylated form in which the position and number of glycosylation are responsible for its absorption and bioactivity in human body (He et al., 2006). During the initial phase in flavonoid metabolism, deglycosylation process is known to take place and the resulting aglycones are metabolized into glucuronides, sulphates and *O*-methylated forms (Vitaglione et al., 2012). The effects of these metabolites at the cellular level depend on their interactions with the cell membranes and the uptake into the cytosol which can vary according to the cell type. Furthermore, their potential as mediators in cellular signal transduction is gaining an importance. However, the true bioactive form of the flavonoids is still under debate. This raises the need of better understanding the bioavailability of flavonoids, their availability for absorption (bioaccessibility), absorption, tissue distribution and bioactivity (Stahl et al., 2002), using animal studies.

5.6 CONCLUSION

H_2O_2-induced cytotoxicity was attenuated by ALA, EPA and DHA acid derivatives of Q3G in human primary hepatocytes and WI-38 cells as demonstrated by significantly higher % cell viability ($p \leq 0.05$) under the experimental condition of H_2O_2-induced oxidative injury in cell culture model systems. Oleic acid and linoleic acid derivatives of Q3G exhibited significant ($p \leq 0.05$) cytoprotection only in primary hepatocytes. ALA

and DHA derivatives of Q3G exhibited significantly ($p \leq 0.05$) lower production of lipid hydroperoxides. Low apoptotic cell death was demonstrated with the pre-incubation of cells with the test compounds. Therefore, fatty acid derivatives of Q3G can be considered as cytoprotective agents under oxidative stress.

CHAPTER 6 EFFECT OF LONG CHAIN FATTY ACID DERIVATIVES OF Q3G AGAINST CYTOTOXICITY INDUCED BY SELECTED CIGARETTE SMOKE TOXICANTS ON HUMAN FETAL LUNG FIBROBLASTS

6.1 ABSTRACT

Smoking has become a global health concern due to its association with many disease conditions, such as chronic obstructive pulmonary disease (COPD), cardiovascular diseases (CVD) and cancer. Flavonoids are plant polyphenolic compounds, studied extensively for their antioxidant, anti-inflammatory, and anti-carcinogenic properties. Quercetin-3-*O*-glucoside (Q3G) is a flavonoid which is widely found in plants. Six long chain fatty acid [stearic acid, oleic acid, linoleic acid, α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] derivatives of Q3G were evaluated for their potential in protecting human lung fibroblasts against cytotoxicity induced by selected cigarette smoke toxicants: 4-(methylnitrosoamino)-1-(3-pyridinyl)-1-butanone (NNK), benzo[a]pyrene (BaP), nicotine and chromium(VI). Nicotine and Cr(VI) induced toxicity in fibroblasts and decreased the percentage of viable cells, while BaP and NNK did not affect cell viability. The fatty acid derivatives of Q3G provided protection from nicotine- and Cr(VI)- mediated cell death and membrane lipid peroxidation. Based on the evaluation of inflammatory markers of cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2), the fatty acid derivatives of Q3G were found to be effective in lowering the inflammatory response.

Key words: Cigarette smoke, cytotoxicity, human lung fibroblasts, cell viability, lipid peroxidation, COX-2, PGE_2

6.2 INTRODUCTION

Cigarette smoke is a highly complex mixture of more than 4500 components, in both gaseous and particulate phases, including carcinogens, cocarcinogens, mutagens, toxic substances and oxidants (Stämpfli and Anderson, 2009; Liu et al., 2011; Martey et al., 2005). Nearly 60 carcinogenic chemicals are present in cigarette smoke and they are found in approximate concentrations of 5-20 ng per cigarette (Chen et al., 2006). Smoking is associated with adverse health conditions such as oxidative stress and inflammation, and also is a risk factor for atherosclerosis (Liu et al., 2011). Further, smoking is responsible for tumorigenesis in different organs, causing lung, gastric, pancreatic, bladder cancers and others; therefore, it is considered as one of the major causes of cancer-related deaths, worldwide (Chen et al., 2006). Although smoking is preventable, unfortunately, about 5-6 million deaths per year are reported due to smoking and thus, it has become a global health concern (Liu et al., 2011).

Inflammation has dual roles in promoting tumors and elevating anti-tumor immunity in the tissues (Rodrigues-Vita and Lawrence, 2010). The COX pathway plays a significant function in the production of eicosanoids, which are responsible for expression of inflammatory responses (Greene et al., 2011). Among the two isoforms of COX, COX-1 is a constitutive isoform, considered as a house keeping gene, while the inducible isoform, COX-2, is usually only expressed when inflammation is stimulated. Cigarette smoke is known to be responsible for PGE_2 mediated immunosuppression, which contributes to tumor growth (Willoughby et al., 2000)

Non-steroidal anti-inflammatory drugs (NDAIDs) and selective COX-2 inhibitors (COXIBs) are commonly used in chemotherapy for inflammation mediated

tumorigenesis through blocking PGE_2 synthesis (Hang and Chen, 2011). Moreover, epidemiological studies have revealed that fruit and vegetable consumption is associated with reducing the occurrence of cancer. These plant-based foods are rich in flavonoids which have been found to be antimutagenic and anticarcinogenic (Cui et al., 2008). Q3G can be considered as a potent chemoprotectant for cancer due to its high antioxidant activity (Razavi et al., 2009). Further, fatty acid esters of Q3G from C8–C16 exhibited antiproliferative activity, indicating their potential as anti-tumor drugs (Salem et al., 2010). A similar effect was reported with fatty acid esters of rutin, which decrease the vascular endothelial growth factor (VEGF) production of K562 human leukemia cells (Mellou et al., 2006). Therefore, in this study, it was hypothesised that the fatty acid derivatives of Q3G possess enhanced cytoprotection properties greater than the parent flavonoid, Q3G. This research was designed to evaluate the potential of six long chain fatty acid derivatives of Q3G against the cytotoxicity induced by selected cigarette smoke components, by determining cell viability, membrane lipid peroxidation and production of inflammatory markers in a lung fibroblast cell culture model system.

6.3 MATERIALS AND METHODS

6.3.1 Cell culture

Normal diploid human fetal lung fibroblast cell line (WI-38) was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were grown in ATCC formulated Eagle's Minimum Essential Medium (EMEM), supplemented with 10% fetal bovine serum (FBS) (ATCC, Manassas, VA, USA) at 37 ^{o}C in a 5% CO_2 and in a humidified environment (CO_2 incubator-Model 3074, VWR

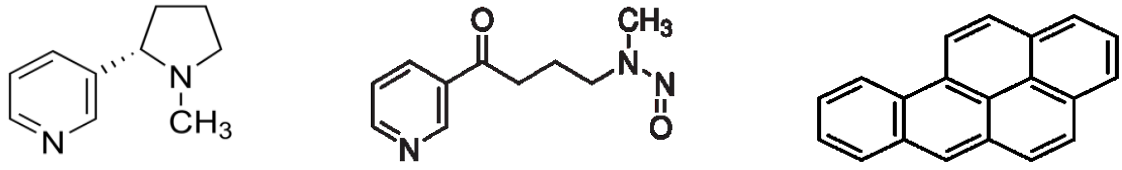
International, West Chester, PA, USA). Sub-culturing and maintaining of the cells were carried out as described in Chapter 5, section 5.3.1.1.

6.3.4 Instruments

A class II-type A2 biological safety cabinet (Model LR2-452), Esco Technologies Inc. Hartboro, PA, USA; CO_2 incubator (Model 3074), VWR International, West Chester, PA, USA; Inverted microscope (ECLIPSE TS 100/TS 100-F), Nikon Instruments Inc., Melville, NY, USA, supported with a Lumenara Infinity camera (1-2 USB, 2.9 Megapixel) including capture and analyzing software, Infinity Analyze, Lumenara Corporation, Ottawa, ON, Canada; Liquid nitrogen storage vessel, VWR International, West Chester, PA, USA; Haemocytometer (Bright-Line), Hausser Scientific, Horsham, PA, USA; Hand-held tally counter, Sigma Aldrich, Oakville, ON, Canada; Stripettor (Stripettor^{TM} Plus), Corning Life Sciences, Lowell, MA, USA; Polystyrene flasks (T-75, canted neck, 0.2 μM vented), Becton Dickinson Labware, Bedford, MA, USA; Bottle top filters (500 ml, 0.22 μm), Corning Life Sciences, Lowell, MA, USA; Sterile disposable pipettes (5 ml, 10 ml, 25 ml), Corning Life Sciences, Lowell, MA, USA; Cryocanes, Thermo Scientific Rochester, NY, USA; Cryotubes, Fisher Scientific, Whitby, ON, Canada; Cryogenic vials, Corning Life Sciences, Lowell, MA, USA. FLUOstar OPTIMA plate reader, BMG Labtech, Durham, NC, USA; fluorescence microscope Leica DMBL (20x/.040), Houston, TX, USA with a filter sets for Cyanine-3 (Ex/Em: 550/570 nm) and 7-AAD (7-Aminoactinomycin D, Ex/Em: 546/647 nm) coupled with Nikon Cool Pix 4500 Digital camera, Mississauga, ON, Canada.

6.3.2 Preparation of chemicals

BaP, NNK, nicotine, Cr(VI) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Canada Co., Oakville, ON, Canada. BaP, NNK and nicotine were initially dissolved in DMSO and made into the desired concentrations, using culture media. DMSO concentration was maintained at less than 0.5% in the culture plate wells. Cr(VI) was prepared in deionized water initially and made into the desired concentrations in culture media.



(-)-Nicotine NNK BaP

Figure 6.1: Chemical structures of nicotine, NNK and BaP

6.3.3 Synthesis of fatty acid derivatives of Q3G

Six fatty acids (stearic acid, oleic acid, linoleic acid, ALA, EPA and DHA) derivatives of Q3G were synthesised according to the method described by Ziaullah et al. (2013). The method is explained in Chapter 4 under section 4.3.2. Briefly, 500 mg of Q3G (Indofine Chemical Company, Hillsborough, NJ, USA) and each acyl donor fatty acid (Nu-Check prep Inc., Elysian, MN, USA) were reacted in a molar ratio of flavonoid:acyl donor 1:5 using anhydrous acetone as the solvent. The acylation was initiated by adding Novozym 435® immobilised lipase from *Candida antarctica* (2 g) as the biocatalyst, followed by an incubation at 45 ^{o}C for approximately 48 hours. After the completion of the reaction,

the product was isolated by silica gel column chromatography, using acetone:toluene; 40:60 to 50:50 monitored by preparative TLC.

6.3.5 Cell viability assay

Cells were cultured in 96 well plates at a density of 1×10^4 cells/100 µl per well and incubated at 37 ^oC for 24 hrs for the attachment of cells. Initial experiments were carried out to determine the suitable time-dose combination required to achieve cytotoxic effect of BaP, NNK, nicotine and Cr(VI). Cells were incubated with different concentrations of the above four components in cigarette smoke at 37 ^oC, for selected time periods. To determine the cytoprotection effect, the cells were pre-incubated with the test compounds: quercetin, Q3G and six fatty acid derivatives of Q3G, in 0.1, 1 and 10 µM at 37 ^oC, for 48 hrs. The cells in the control group contained equal concentration of DMSO (0.1%, v/v) without any test compounds and were not oxidatively injured. The cells in the model group contained equal concentration of DMSO, with no addition of test compounds, but they were treated with cigarette smoke toxicants.

After the pre-incubation, culture medium was removed and cells were washed twice with PBS, and centrifuged at 1000 rpm for 2 min in between. Then, cells were subjected to the treatments of different toxicants: 3 mM Cr(VI), 30 mM nicotine, 100 µM BaP and 3 mM NNK, followed by 6 hr incubation at 37 ^oC. Media were removed and cells were rinsed with PBS to remove the oxidant and 100 µl of fresh serum free media was added into the wells. Celltitier 96® aqueous non-radioactive cell proliferation assay (Promega Corporation, Medison, WI, USA) was used to determine cell viability. MTS reagent (10 µl, freshly prepared by adding 2 ml of MTS: 100 µl of PMS) was added to each well and incubated at 37 ^oC for 1-4 hrs. Absorbance was measured at 490 nm using a plate reader

(FLUOstar OPTIMA, BMG Labtech, Burham, NC, USA). The percentage of viable cells was calculated as follows:

$$\% \text{ Cell viability} = \frac{\text{Abs. of the treated wells - Blank}}{\text{Abs. of the control wells – Blank}} \times 100\%$$

Abs.; Absorbance

Where, the treated wells contained the cells, pre-incubated with test compounds followed by oxidative injury, the control wells contained the cells with no incubation of test compounds and no oxidative injury. Blank wells contained culture media only.

6.3.7 Lipid peroxidation assay

Lipid hydroperoxidation was measured using lipid hydroperoxide assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). WI-38 cells were cultured in 24-well plates at a density of 1×10^5 cells/ml per well and incubated at 37 ^{o}C for 24 hrs. Then, cells were treated with 1 µM of test compounds for 48 hrs at 37 ^{o}C. After incubation, the cells were rinsed with PBS to remove the test compounds and toxicity was induced by addition of cigarette smoke toxicants (3 mM Cr(VI) and 30 mM nicotine) for 6 hrs at 37 ^{o}C.

At the end of the oxidative injury period, the supernatant (200 µl) of each well was pipetted into glass vials and lipid hydroperoxides were extracted by adding an equal volume of Extract R saturated methanol and 1 ml of cold chloroform, followed by centrifuging the pre-vortexed mixture at 1500 × g for five min at 0 ^{o}C. The bottom chloroform layer (500 µl) was collected in a glass vial and stored on ice. Methanol and chloroform were deoxygenated prior to use, by bubbling nitrogen through the solvents for about 30 min. Deoxygenated chloroform-methanol (2:1) mixture (450 µl) was added into each extracted sample of 500 µl. Then, freshly prepared standard chromogen (50 µl)

was added into the assay vials and vortexed. The assay vials were closed tightly and kept at room temperature for 5 min. A volume of 300 µl from each vial was transferred into a glass 96-well plate and absorbance was read at 490 nm, using a plate reader (FLUOstar OPTIMA, BMG Labtech, Burham, NC, USA). Lipid hydroperoxides in the samples were calculated using the equation obtained from the linear regression of the standard curve, prepared using 0, 0.5, 1.0, 2.0, 3.0 and 4.0 nmol of lipid hydroperoxide standards provided with the kit.

6.3.8 Fluorescence microscopy assay

WI-38 cells were cultured in chamber slides (Nunc Lab-Tek II Chamber Slide System, Thermo Fisher Scientific, Ottawa, ON, Canada) at a density of 1×10^5 cells/ml per well and incubated at 37 ^{o}C for 24 hrs. Cells were treated with 1 µM of test compounds for 48 hrs at 37 ^{o}C. After incubation, the cells were washed with PBS to remove the test compounds and treated with cigarette smoke toxicants for 6 hrs at 37 ^{o}C. Cells were carefully rinsed twice with PBS to remove the oxidants. The dual detection reagents, apoptosis detection reagent (Annexin V-EnzoGold) and necrosis detection reagent which is similar to the red emitting dye 7-AAD, supplied by GFP-certified™ apoptosis/necrosis detection kit (Enzo Life Sciences International, INC., Plymouth Meeting, PA, USA), were prepared in binding buffer (1×). After careful removal of the supernatant, the prepared detection reagent was dispensed in a volume sufficient for covering the cell monolayer in each slide. The cells were incubated for 15 min at room temperature, protected from light. Then, the staining solution was flicked onto a paper towel and a few drops of binding buffer were added to prevent the cells from drying out. The cells were observed under a fluorescence microscope (DMBL 20x/.040, Leica Microsystems,

Houston, TX, USA) with filters set for Cyanine-3 (Ex/Em: 550/570 nm) and 7-AAD (Ex/Em: 546/647 nm), coupled with a digital camera (Nikon Cool Pix 4500, Nikon Canada Inc., Mississauga, ON, Canada).

6.3.10 Cell morphological assessment

WI-38 cells were cultured in 24-well plates at a density of 1×10^5 cells/1 ml per well and incubated at 37 ^oC for 24 hrs. Cells were treated with 1 μM of test compounds for 48 hrs at 37 ^oC. After incubation, the cells were rinsed with PBS to remove the test compounds and treated with cigarette smoke toxicants for 6 hrs at 37 ^oC. The morphology of the cells was monitored under an inverted microscope (ECLIPSE TS 100/ TS 100-F, Nikon Instruments Inc., Melville, NY, USA), using phase contrast optics under 10X magnification. The images were recorded using the Lumenara Infinity camera (1-2 USB, 2.9 Megapixel), including capture and analyzing software (Infinity Analyze, Lumenara Corporation, Ottawa, ON, Canada).

6.3.11 PGE_2 assay

The PGE_2 Express EIA kit, supplied by Cayman Chemical Company (Ann Arbor, MI, USA), was used to determine the PGE_2 concentration in the cell culture supernatant. WI-38 cells were cultured in 24-well plates at a density of 1×10^5 cells/1 ml per well and incubated at 37 ^oC for 24 hrs. Cells were treated with 1 μM of test compounds for 48 hrs at 37 ^oC. After the incubation, the cells were washed with PBS to remove the test compounds. Then, cells were treated with BaP (1 μM), nicotine (600 μM), NNK (50 μM) and Cr(VI) (150 μM) and incubated at 37 ^oC for 6 hrs. After incubation, culture media supernatant (200 μl) was collected. Enzyme immunoassay buffer, standards, samples, tracer and antibody were added into appropriate wells in recommended

volumes. The covered plates were then incubated at room temperature on an orbital shaker for 1 hr. The wells were emptied and rinsed with wash buffer five times. The tracer was added to the total activity wells and the chromogen, Ellman's reagent (200 µl), was added to all remaining wells. The plate was covered and incubated for 60-90 min at room temperature, with gentle shaking. The absorbance was read at 410 nm using a plate reader (FLUOstar OPTIMA, BMG Labtech, Burham, NC, USA). The concentration of PGE_2 in each sample was expressed in pg/ml.

6.3.12 COX-2 assay

The human COX-2 ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA) was used to determine the production of COX-2 in cell culture lysates. WI-38 cells were cultured in 24-well plates at a density of 1×10^5 cells/1 ml per well and incubated at 37 ^oC for 24 hrs. Cells were treated with 1 µM of test compounds for 48 hrs at 37 ^oC. After incubation, the cells were washed with PBS to remove the test compounds. The cells were treated with BaP (1 µM), nicotine (600 µM), NNK (50 µM) and Cr(VI) (150 µM) and incubated at 37 ^oC for 6 hrs. Cell culture lysates were prepared in a buffer solution containing (10 mM Tris, pH 8.0, 0.15 M NaCl, 1 mM EDTA and 1 % Triton x-100). Standards, samples and the assay buffer, which was provided with the kit, were added into appropriate wells in 100 µl aliquots and the covered plate was incubated at 37 ^oC for 1 hr. The wells were decanted and washed 7 times with 200 µl of wash solution. The labeled antibody (100 µl) was pipetted into each well, except the blank. The covered plate was incubated at 4 ^oC for 30 min, after which the contents were decanted and rinsed 9 times with 200 µl of wash buffer. Then, 3,3',5,5'-tetramethylbenzidine (TMB) substrate (100 µl) was added to each well and incubated for 30 min at room temperature in the

dark. Stop solution (100 µl) was added to each well and absorbance was measured at 450 nm. The COX-2 concentration in each sample was expressed in ng/ml.

6.3.13 Statistical analysis

Statistical analysis was carried out using one way ANOVA in Minitab 16 statistical software and a two factor factorial design, using general linear model in SAS 9.3. Multiple mean comparison was carried out by Tukey's method at $P \leq 0.05$ (Montgomery, 2012).

6.4 RESULTS

6.4.1 Cytoprotective effect of fatty acid derivatives of Q3G

The ability of fatty acid derivatives of Q3G to attenuate the cytotoxicity, induced by cigarette smoke constituents, was determined in a dose dependent manner, using WI-38 cells. All the viability percentages were calculated based on the control, with no addition of test compounds and with no oxidative injury. When considering cytotoxicity induced by Cr(VI) (Figure 6.2), only DHA derivative of Q3G showed significantly higher % cell viability than the model at 0.1 µM ($p \leq 0.05$). The concentration of 10 µM showed the toxic effect of the compounds. Stearic acid, oleic acid, ALA, EPA, and DHA derivatives were effective at a very low concentration of 0.1 µM and linoleic acid derivative of Q3G, Q3G, and Q showed the highest protection at 1 µM, which is ten times higher than the effective concentration level of the other test compounds. DHA derivative of Q3G showed significantly ($p \leq 0.05$) higher cytoprotection than the model group at 0.1 µM.

The cytotoxicity induced by nicotine was attenuated by oleic acid, linoleic acid, ALA, EPA and DHA derivatives of Q3G and Q (Figure 6.3). The significantly higher cytoprotection was demonstrated by linoleic acid derivative of Q3G at 10 μM and DHA derivative of Q3G at both 1 and 10μM ($p \leq 0.05$).

The highest percentage protection was demonstrated by linoleic acid derivative of Q3G (10 μM, 16%), EPA derivative of Q3G (10 μM, 10%) and DHA derivative of Q3G (1 μM, 18%). Moreover, linoleic acid, and DHA derivatives of Q3G demonstrated significantly ($p \leq 0.05$) higher cytoprotection when compared with the respective model group. BaP and NNK did not induce cytotoxicity at the tested concentrations (data not presented).

6.4.2 Lipid peroxidation under induced cytotoxicity

All the fatty acid derivatives of Q3G at 1 μM concentration demonstrated significantly ($p \leq 0.05$) lower production of lipid hydroperoxides, under both Cr (VI)- and nicotine-induced cytotoxicity models, compared to the respective model groups treated only with either Cr(VI) or nicotine (Figure 6.4 and 6.5). For Cr (VI)-induced cytotoxicity, the decrease in lipid peroxidation by fatty acid derivatives of Q3G, in a range of 60-90 % and ALA, EPA and DHA derivatives of Q3G showed the highest effectiveness in inhibiting membrane lipid peroxidation. For nicotine induced cytotoxicity, the decrease in lipid peroxidation by the fatty acid derivatives of Q3G was in a range of 76-90% and the DHA derivative demonstrated the highest protection from membrane lipid peroxidation.

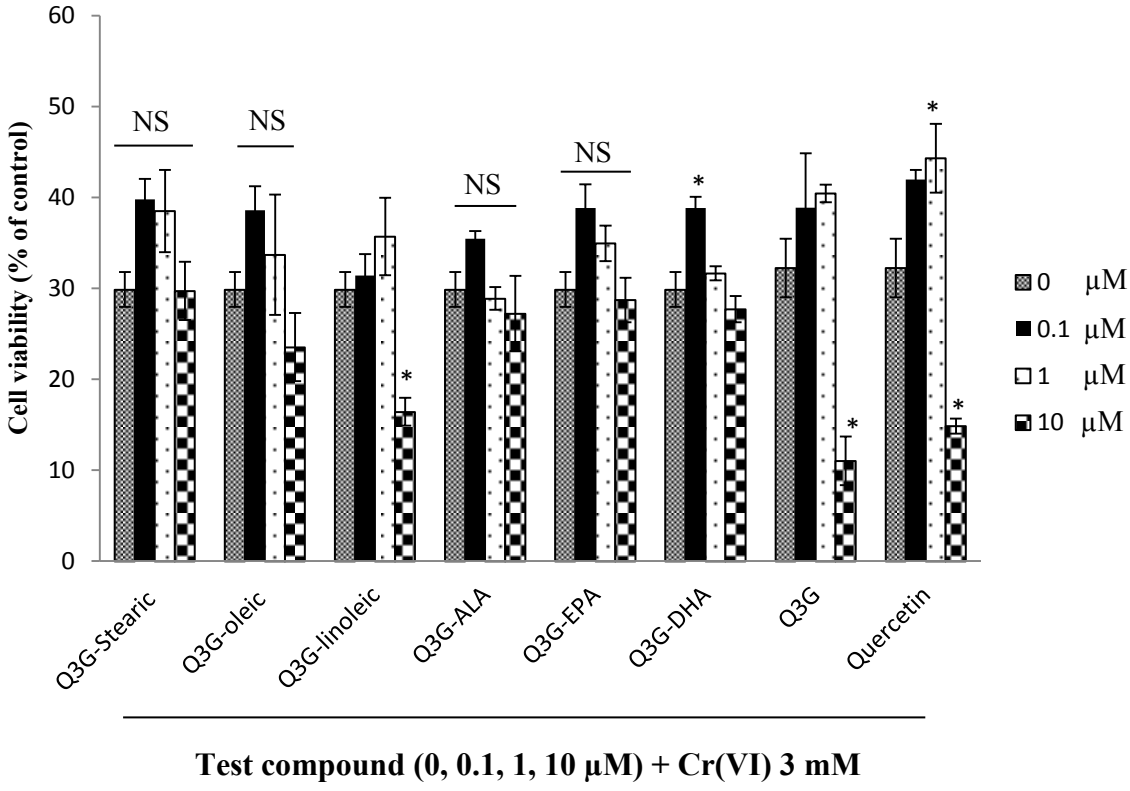


Figure 6.2: Dose-dependent effect of test compounds on cell viability against Cr(VI)-induced cytotoxicity in WI-38 cells. Cells were pre-incubated with 0, 0.1, 1 and 10 µM of test compounds (stearic acid derivative of Q3G, oleic acid derivative of Q3G, linoleic acid derivative of Q3G, ALA derivative of Q3G, EPA derivative of Q3G, DHA derivative Q3G, Q3G and Q) for 48 hrs, followed by 6 hr incubation with 3 mM Cr(VI). Cell viability is presented as percentage related to the control. Control contains the cells with no incubation of test compounds and no induced toxicity. Data are presented as mean ± SEM (n=3). P ≤ 0.05, significantly different from the model group. The cells in the model group were induced toxicity with Cr(VI), but not treated with test compounds.

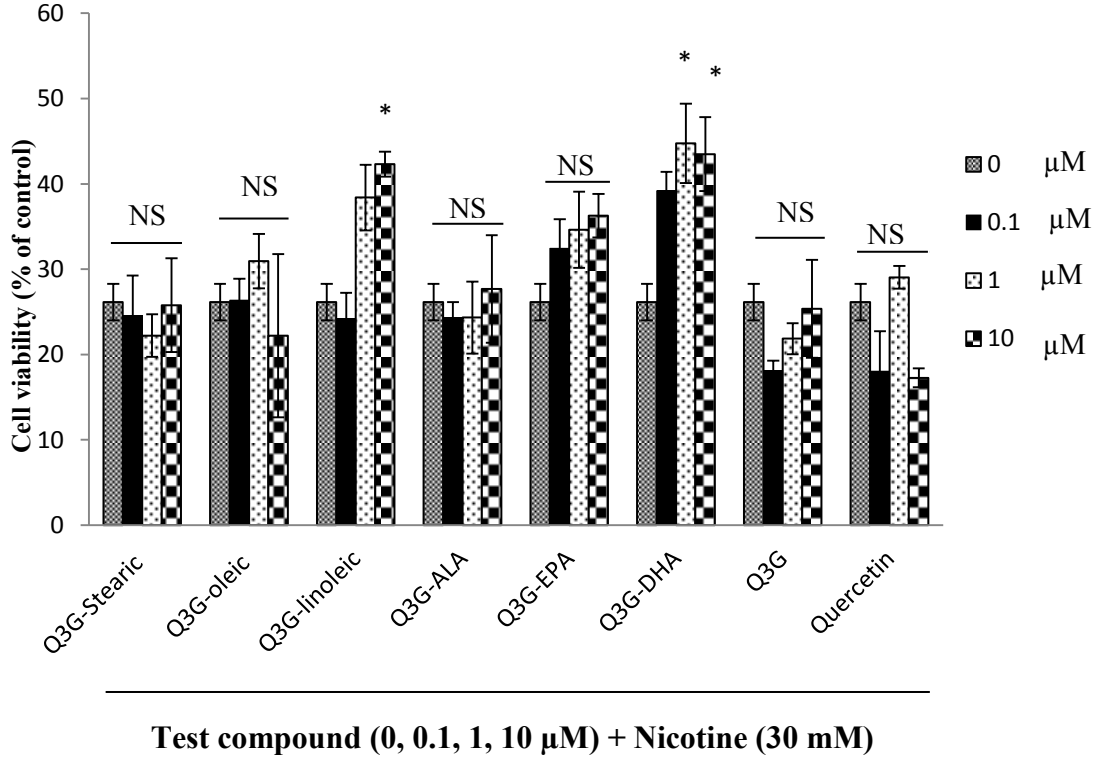


Figure 6.3: Dose-dependent effect on cell viability against nicotine-induced cytotoxicity in WI-38 cells. Cells were pre-incubated with 0, 0.1, 1 and 10 µM of test compounds (stearic acid derivative of Q3G, oleic acid derivative of Q3G, linoleic acid derivative of Q3G, ALA derivative of Q3G, EPA derivative of Q3G, DHA derivative Q3G, Q3G and Q) for 48 hrs, followed by 6 hr incubation with 30 mM nicotine. Cell viability is presented as percentage related to the control. Control contains the cells with no incubation of test compounds and no induced toxicity. Data are presented as mean ± SEM (n=3). * P ≤ 0.05, significantly different from the model group. The cells in the model group were induced toxicity with nicotine, but not treated with test compounds.

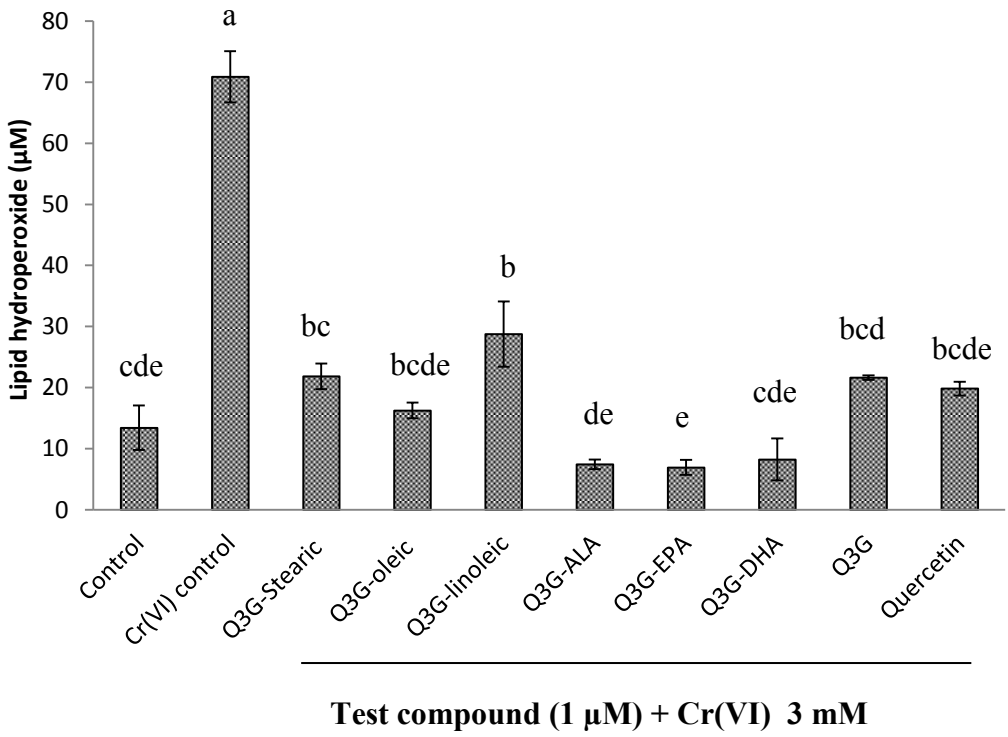


Figure 6.4: Production of lipid peroxides under Cr(VI)-induced cytotoxicity in WI-38 cells. Cells were pre-incubated with 1 µM of test compounds (stearic acid derivative of Q3G, oleic acid derivative of Q3G, linoleic acid derivative of Q3G, ALA derivative of Q3G, EPA derivative of Q3G, DHA derivative Q3G, Q3G and Q) for 48 hrs, rinsed with PBS, followed by 6 hr incubation with Cr(VI). Control contains the cells with no incubation of test compounds and no induced toxicity. Data are presented as mean ± SEM (n=3), means sharing the same letter are not significantly different.

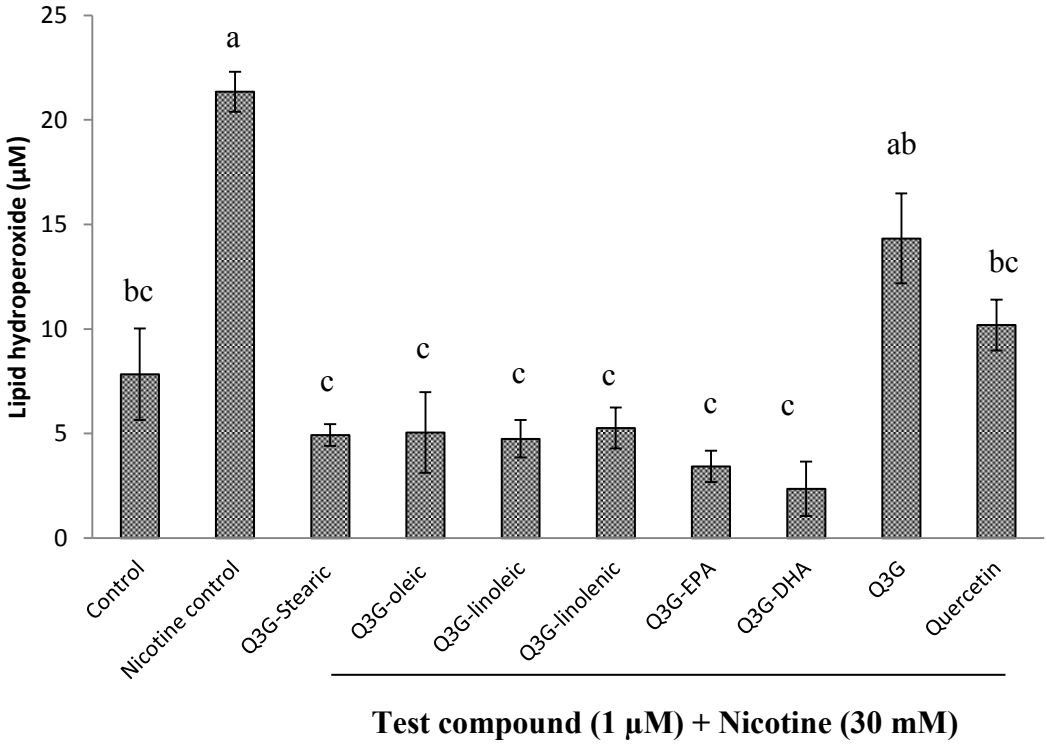


Figure 6.5: Production of lipid peroxides under nicotine-induced cytotoxicity in WI-38 cells. Cells were pre-incubated with 1 µM of test compounds (stearic acid derivative of Q3G, oleic acid derivative of Q3G, linoleic acid derivative of Q3G, ALA derivative of Q3G, EPA derivative of Q3G, DHA derivative Q3G, Q3G and Q) for 48 hrs, rinsed with PBS, followed by 6 hr incubation with nicotine. Control contains the cells with no incubation of test compounds and no induced toxicity. Data are presented as mean ± SEM (n=3), means sharing the same letter are not significantly different.

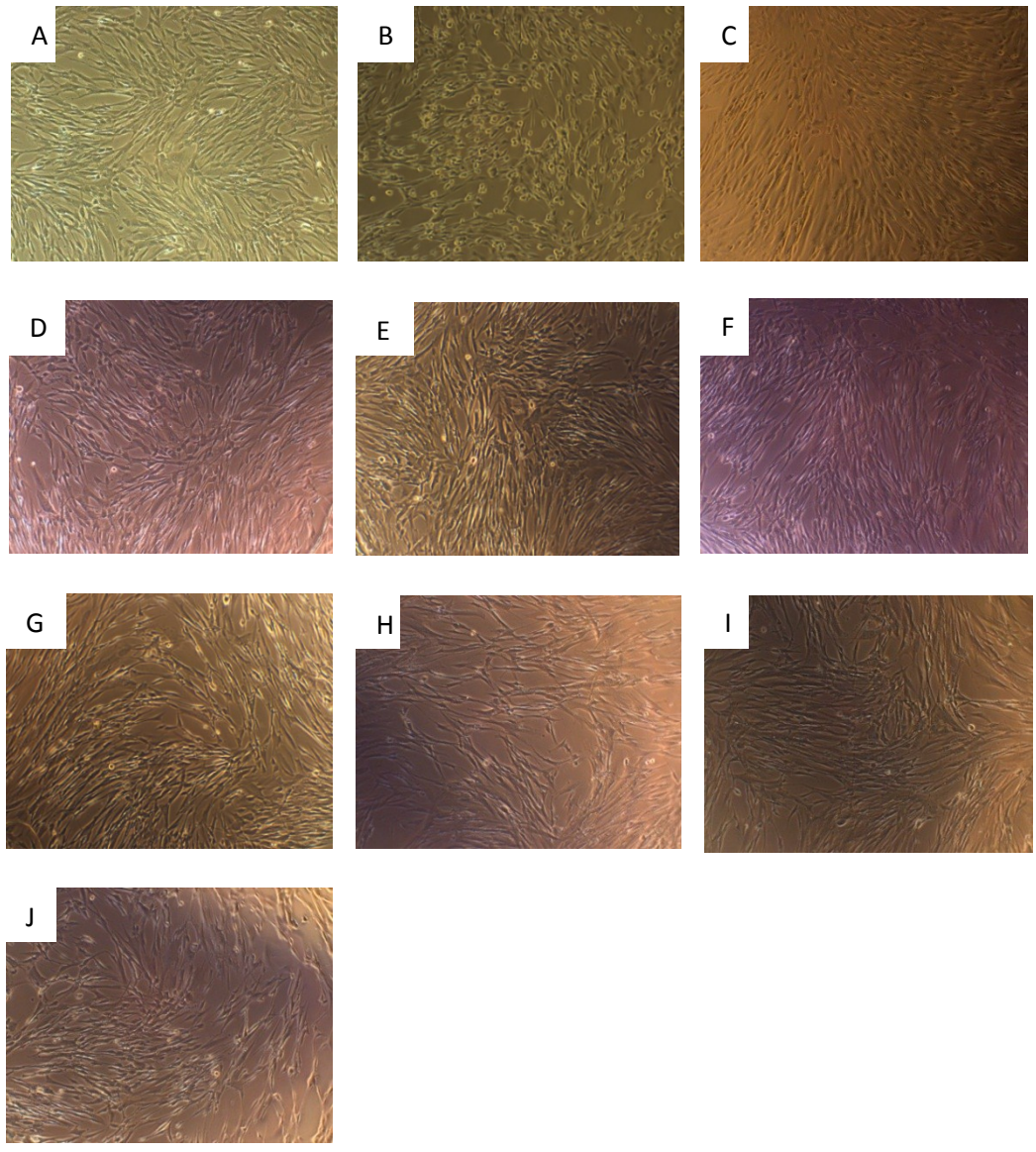


Figure 6.6: Morphological changes of WI-38 cells under Cr(VI)-induced cytotoxicity using inverted phase contrast microscope(10X). A) Untreated control, B) cells treated only with Cr(VI), C) stearic acid derivative of Q3G, D) oleic acid derivative of Q3G, E) linoleic acid derivative of Q3G, F) ALA derivative of Q3G, G) EPA acid derivative of Q3G, H) DHA derivative of Q3G, I) Q3G and J) Q. Cells were pre-treated with test compounds for 48 hrs and then toxicity induced for 6 hrs by Cr(VI).

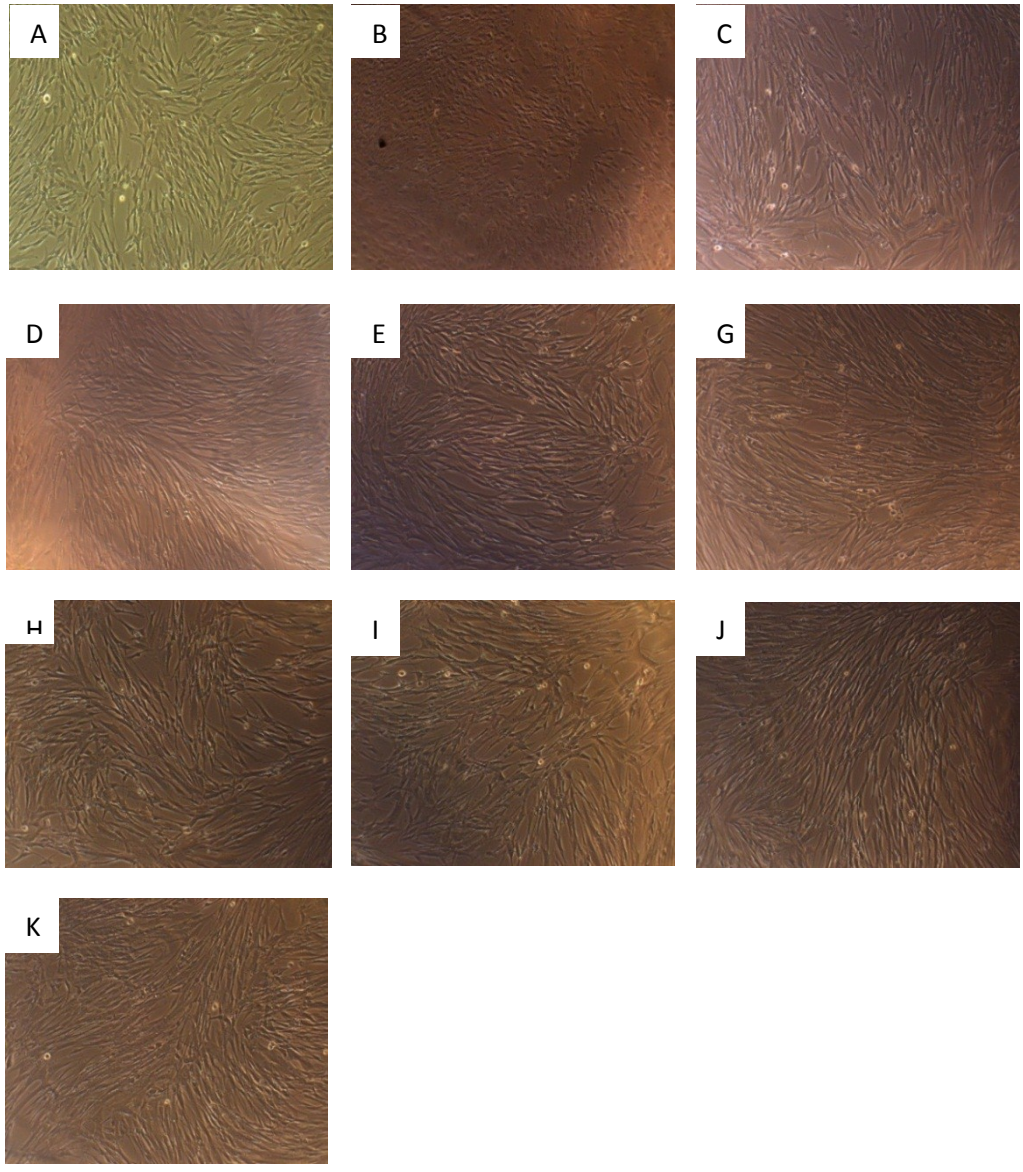


Figure 6.7: Morphological changes of WI-38 cells under nicotine-induced cytotoxicity using inverted phase contrast microscope (10X). A) Untreated control, B) cells treated only with Cr(VI), C) stearic acid derivative of Q3G, D) oleic acid derivative of Q3G, E) linoleic acid derivative of Q3G, F) ALA derivative of Q3G, G) EPA acid derivative of Q3G, H) DHA derivative of Q3G, I) Q3G and J) Q. Cells were pre-treated with test compounds for 48 hrs and then toxicity induced for 6 hrs by nicotine.

6.4.3 Morphological changes and cell death

The morphological changes of WI-38 cells subjected to nicotine and Cr(VI), with and without addition of test compounds, are found in Figures 6.6 and 6.7. The morphology has been clearly altered by the addition of these toxicants, showing cell loss, cell rounding and shrinking. The cells treated with fatty acid derivatives of Q3G were less damaged, compared to the cells without addition of the test compounds. The cells that underwent pre- and late-apoptosis after the addition of Cr(VI) are presented in Figures 6.8 and 6.9. Early apoptotic cells were colored in green and late apoptotic and/or necrotic cells were colored in red. More cells in the only Cr(VI) treated group underwent apoptotic cell death than the cells treated with test compounds. However, it is apparent that nicotine treatment caused more necrotic type cell death or turned the cells into late apoptotic phase sooner (Figure 6.10 and 6.11).

6.4.4 Induction of inflammatory markers

The tested cigarette smoke toxicants induced an increase in COX-2 and PGE_2 levels in the cells within 6 hr incubation, while the potential of fatty acid derivatives of Q3G to inhibit the COX-2 pathway was determined. BaP-induced COX-2 level was lowered to the range of 1 to 4 ng/ml with the treatment of fatty acid derivatives of Q3G (Figure 6.12). Moreover, ALA, EPA, DHA derivatives of Q3G lowered the production of COX-2 when induced with BaP. Nicotine-induced COX-2 was lowered by ALA and EPA derivatives of fatty acids into less than 2 ng/ml. DHA derivative of Q3G showed the lowerest production of COX-2 with NNK insult, while, oleic, ALA and DHA derivatives of Q3G lowered Cr(VI)-induced COX-2 into less than 4 ng/ml. When considering the PGE_2 levels, induced by different treatments of cigarette smoke toxicants, EPA and

DHA derivatives of Q3G lowered the BaP-induced PGE_2 concentrations into less than 20 ng/ml, while linoleic, ALA, EPA and DHA derivative of Q3G significantly lowered the nicotine-induced PGE_2 into less than 40 ng/ml. ALA, EPA and DHA derivative of Q3G were effective in inhibiting the NNK-induced PGE_2 production, while only ALA derivative was effective for Cr(VI)-induced inflammation. In general, Q and Q3G were not effective, compared to the fatty acid derivatives of Q3G, in lowering the cigarette smoke toxicant induced production of COX-2 and PGE_2.

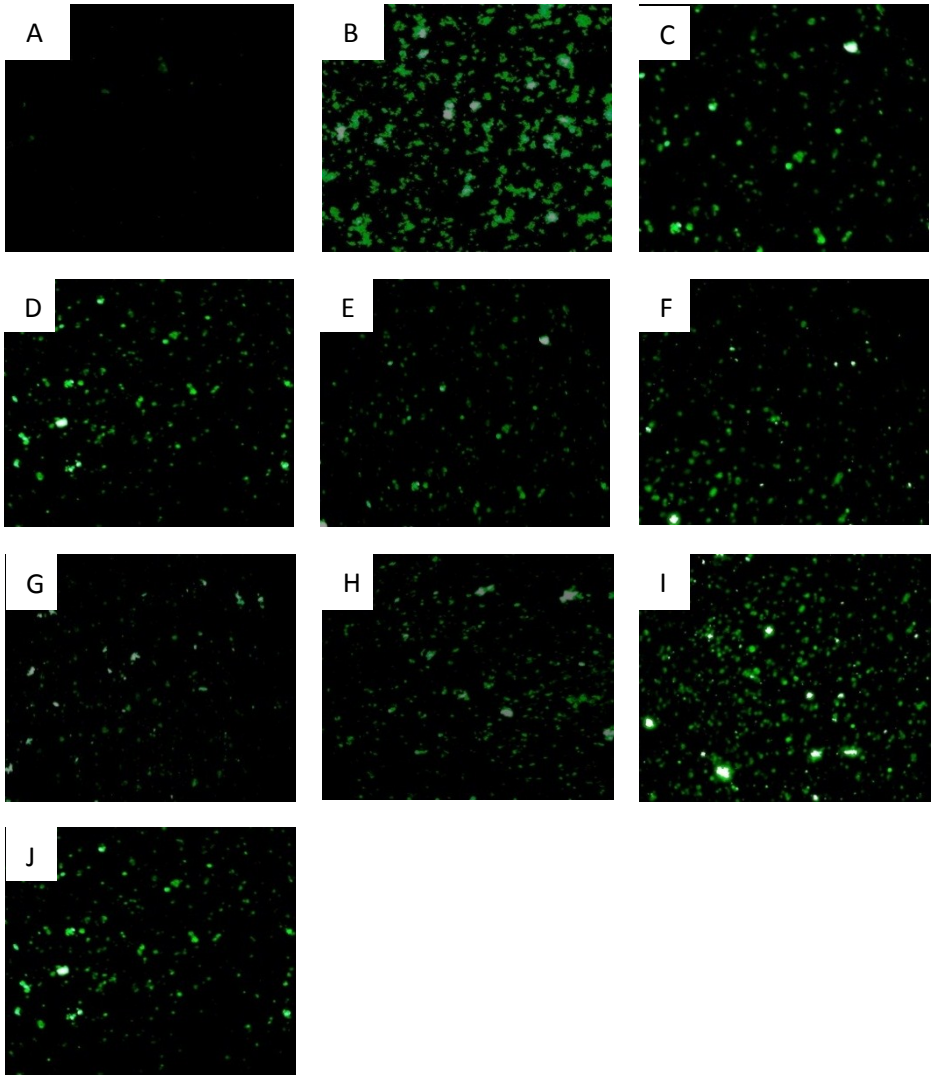


Figure 6.8: Effect of test compounds on Cr(VI)-induced apoptotic cell death of WI-38 cells. A) Untreated control, B) cells treated only with Cr(VI), C) stearic acid derivative of Q3G, D) oleic acid derivative of Q3G, E) linoleic acid derivative of Q3G, F) ALA derivative of Q3G, G) EPA acid derivative of Q3G, H) DHA derivative of Q3G, I) Q3G and J) Q. Cells were pre-treated with test compounds for 48 hrs and then toxicity induced for 6 hrs by Cr(VI). The cells were washed with PBS, stained with Annexin V and observed under a fluorescence microscope.

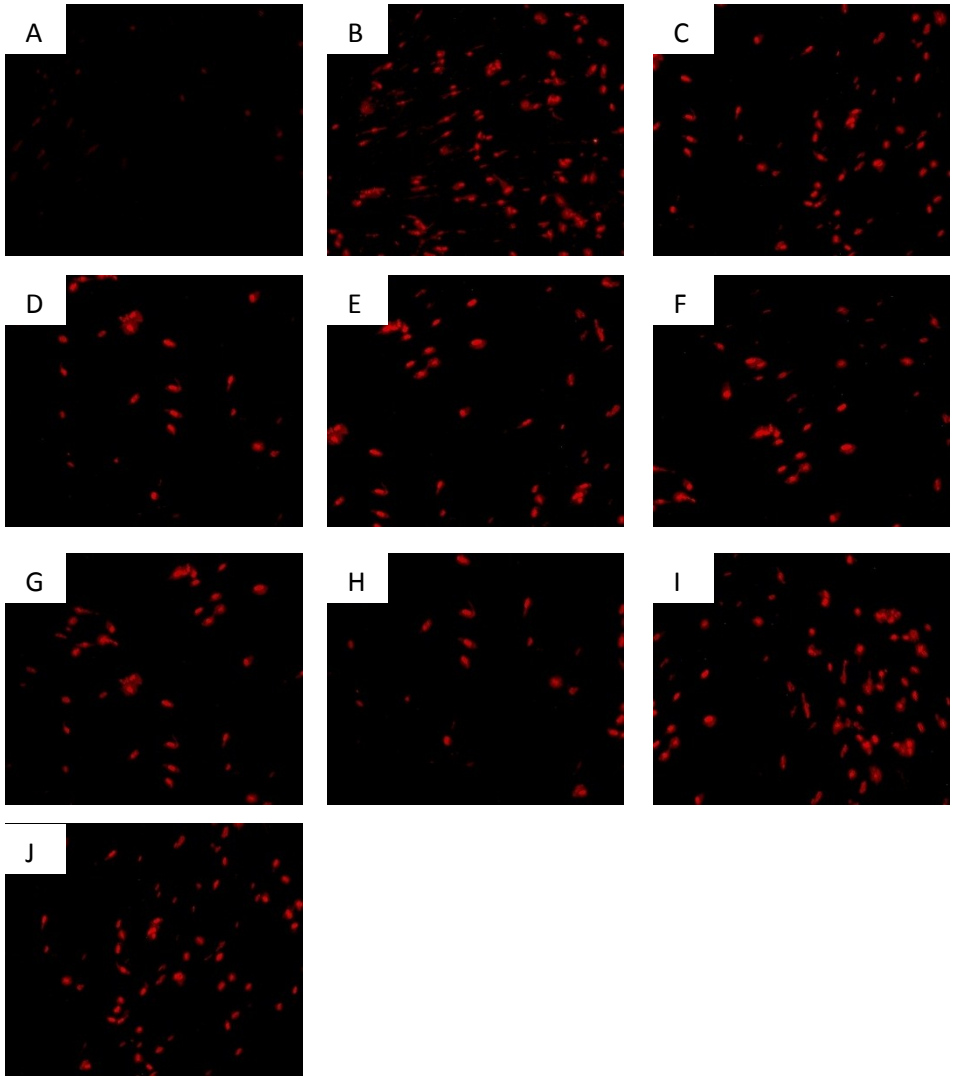


Figure 6.9: Effect of test compounds on Cr(VI)-induced late-apoptotic/necrotic cell death of WI-38 cells. A) Untreated control, B) cells treated only with Cr(VI), C) stearic acid derivative of Q3G, D) oleic acid derivative of Q3G, E) linoleic acid derivative of Q3G, F) ALA derivative of Q3G, G) EPA acid derivative of Q3G, H) DHA derivative of Q3G, I) Q3G and J) Q. Cells were pre-treated with test compounds for 48 hrs and then toxicity induced for 6 hrs by Cr(VI). The cells were washed with PBS, stained with necrotic detection reagent and observed under a fluorescence microscope.

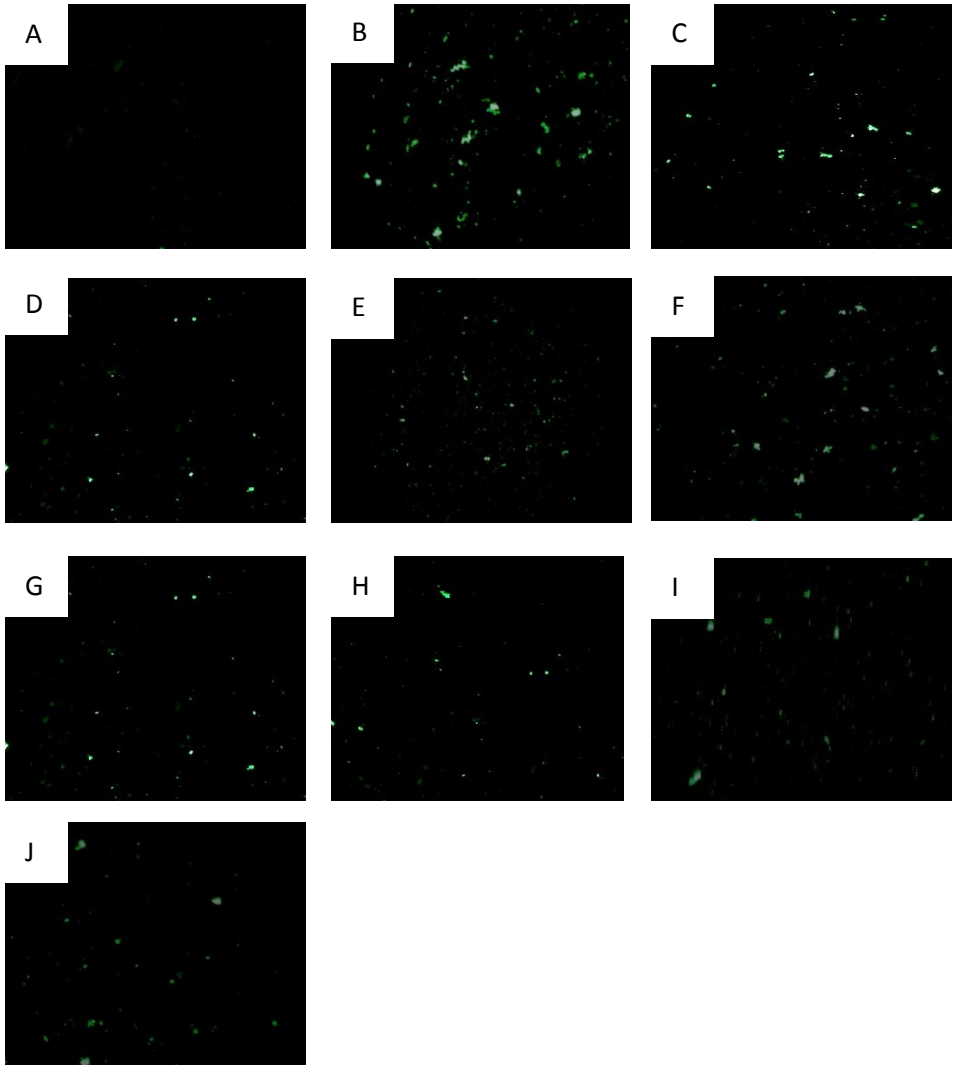


Figure 6.10: Effect of test compounds on nicotine-induced apoptotic cell death of WI-38 cells. A) Untreated control, B) cells treated only with nicotine, C) stearic acid derivative of Q3G, D) oleic acid derivative of Q3G, E) linoleic acid derivative of Q3G, F) ALA derivative of Q3G, G) EPA acid derivative of Q3G, H) DHA derivative of Q3G, I) Q3G and J) Q. Cells were pre-treated with test compounds for 48 hrs and then toxicity induced for 6 hrs by nicotine. The cells were washed with PBS, stained with Annexin V and observed under a fluorescence microscope.

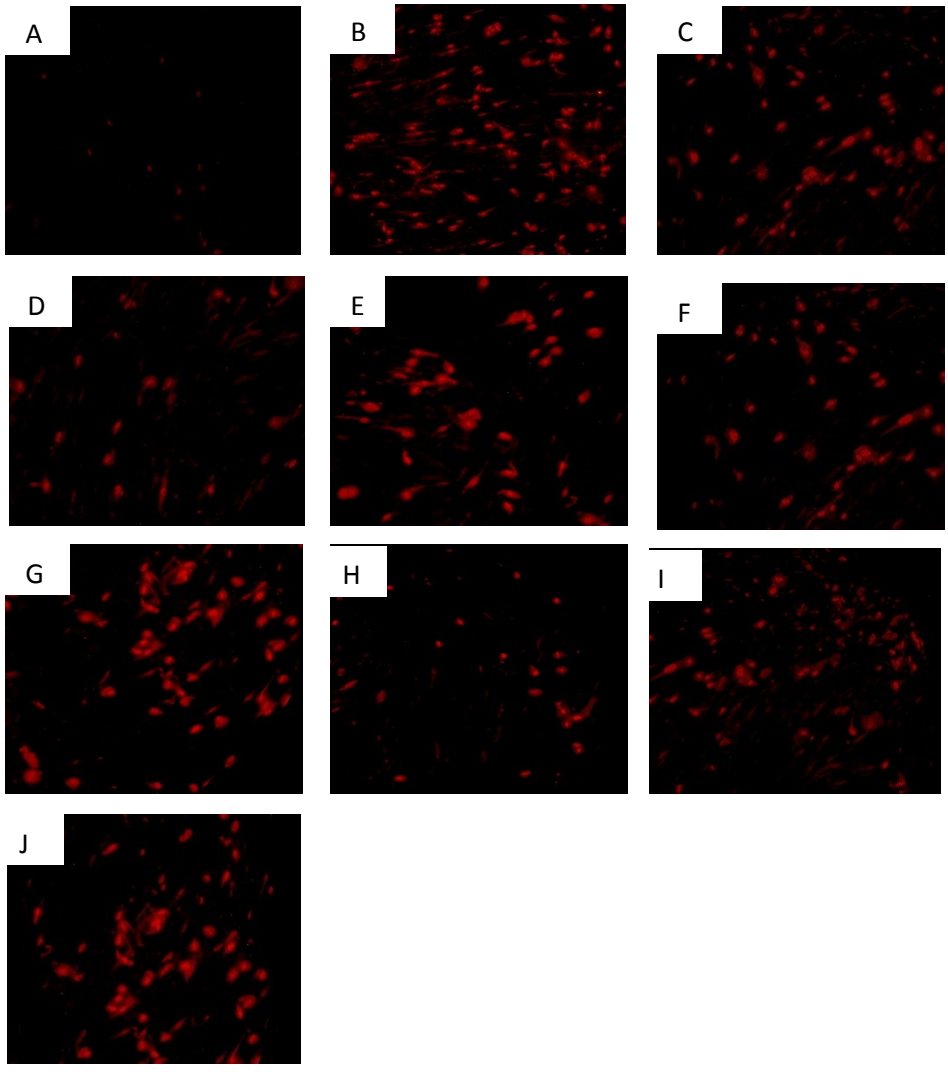


Figure 6.11: Effect of test compounds on nicotine-induced late-apoptotic/necrotic cell death of WI-38 cells. A) Untreated control, B) cells treated only with nicotine, C) stearic acid derivative of Q3G, D) oleic acid derivative of Q3G, E) linoleic acid derivative of Q3G, F) ALA derivative of Q3G, G) EPA acid derivative of Q3G, H) DHA derivative of Q3G, I) Q3G and J) Q. Cells were pre-treated with test compounds for 48 hrs and then toxicity induced for 6 hrs by Cr(VI). The cells were washed with PBS, stained with necrotic detection reagent and observed under a fluorescence microscope.

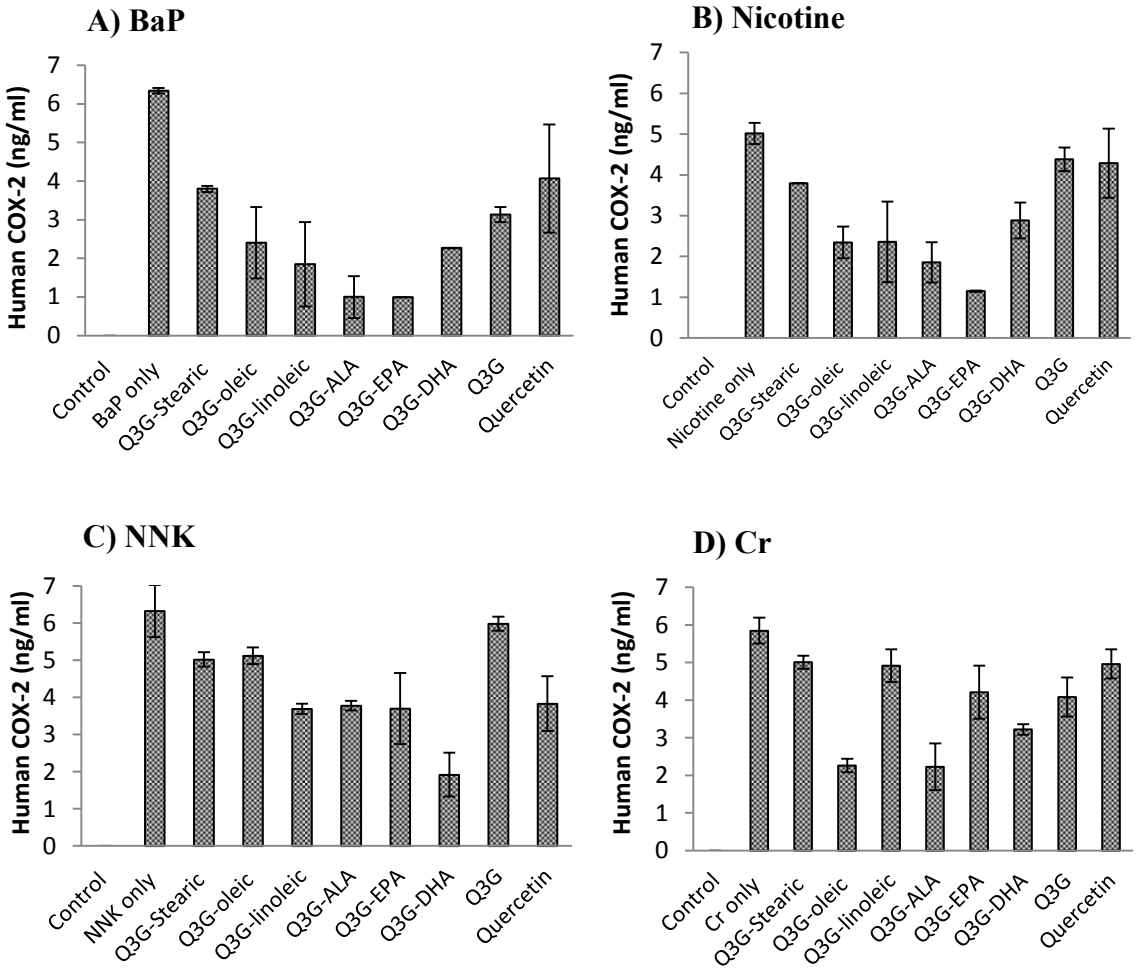


Figure 6.12: Production of human COX-2 under nicotine-induced cytotoxicity in WI-38 cells. Cells were pre-incubated with 1 µM of test compounds (stearic acid derivative of Q3G, oleic acid derivative of Q3G, linoleic acid derivative of Q3G, ALA derivative of Q3G, EPA derivative of Q3G, DHA derivative Q3G, Q3G and Q) for 48 hrs, followed by 6 hr incubation with cigarette smoke toxicants. Control contains the cells with no incubation of test compounds and no induced toxicity. COX-2 protein was quantified using ELISA. The cells in the model group were induced toxicity, but not treated with test compounds.

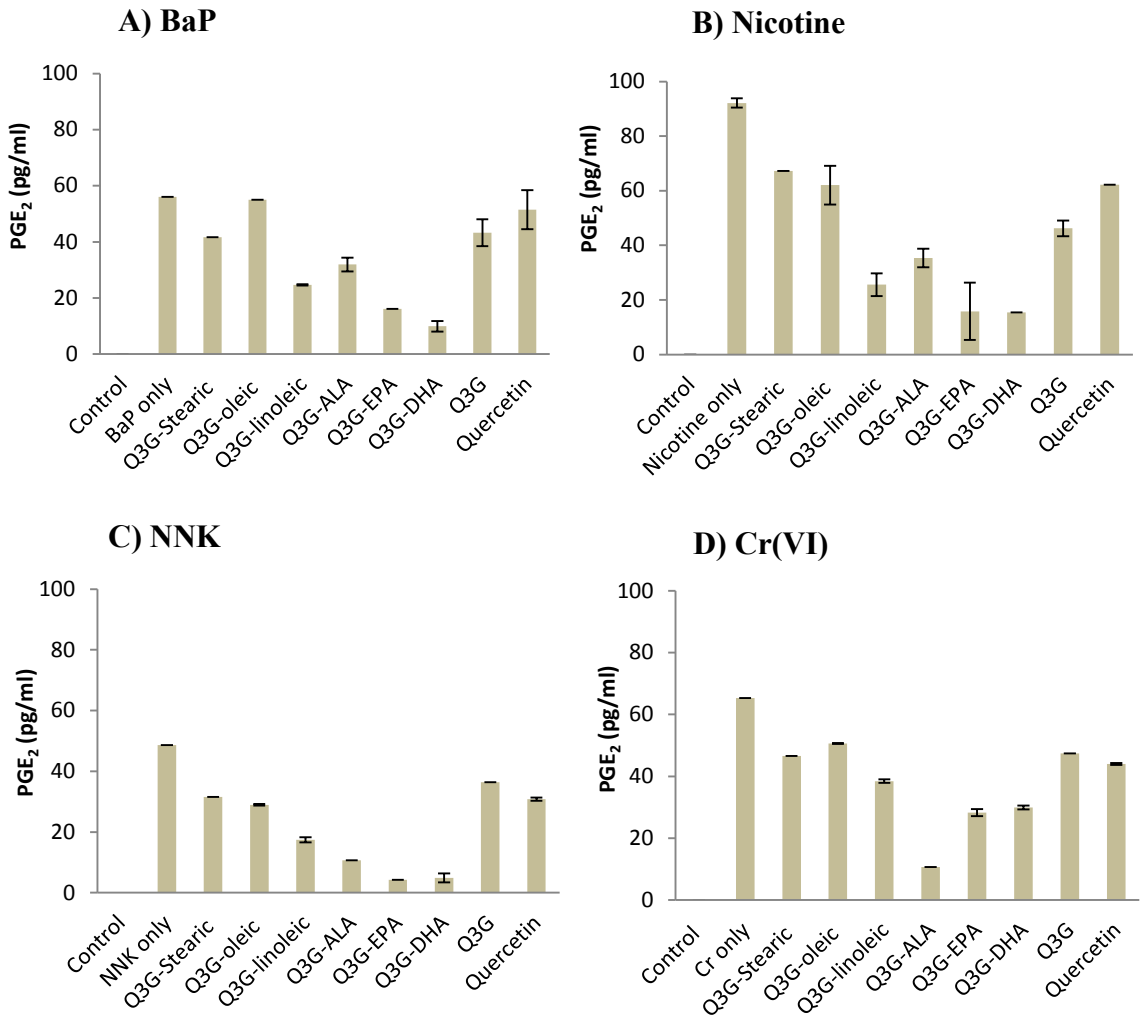


Figure 6.13: Production of PGE_2 under nicotine-induced cytotoxicity in WI-38 cells. Cells were pre-incubated with 1 µM of test compounds (stearic acid derivative of Q3G, oleic acid derivative of Q3G, linoleic acid derivative of Q3G, ALA derivative of Q3G, EPA derivative of Q3G, DHA derivative Q3G, Q3G and Q) for 48 hrs, followed by 6 hr incubation with cigarette smoke toxicants. Control contains the cells with no incubation of test compounds and no induced toxicity. PGE_2 was quantified using ELISA.

6.5 DISCUSSION

Exposure to cigarette smoke is associated with many disease conditions, including tissue damage, cell death and airway inflammation (Toorn et al., 2007). Lung fibroblasts provide the structural support to the alveolar compartments and further, being a major target for cigarette smoke, have been reported to play an important role in the response against adverse effects of many inhaled toxicants (Dreij et al., 2010; Baglole et al., 2008; Matsuzuka et al., 2009). The adverse effects of cigarette smoke, such as tissue damage and cell death via apoptosis and necrosis and inhibition of cell proliferation, have been reported through studies of lung fibroblasts *in vitro* (Nakamura et al., 1995). Moreover, it has been shown that lower concentrations or shorter application periods of cigarette smoke induced apoptosis, while higher concentrations or longer application periods induced necrosis in human lung fibroblasts (Ishii et al., 2001; Toorn et al., 2007; Carnevali et al., 2003).

Also, it has been reported that the oxidative stress condition initiated upon exposure to cigarette smoke is involved with inflammatory cascades and cell death in disease conditions such as COPD (Toorn et al., 2007). Smoking is associated with down-regulation of cell proliferation and up-regulation of the inflammation process (Lu et al., 2007). It has been shown that exposure to cigarette smoke extract results in up-regulation of COX-2 and PGE_2 in normal human lung fibroblasts (Martey et al., 2004). In humans, fibroblasts are a major type of cells which express COX-2, leading to the production of prostaglandins (Baglole et al., 2008). Fibroblasts are activated by lung irritants and thereby stimulate lung inflammation and further, they are thought to be the targets of water-soluble components of cigarette smoke (Martey et al., 2005). COX-2, not COX-1,

is the responsible enzyme for the PGE_2 generation after exposure to cigarette smoke extract (Martey et al., 2004). COX-2 has a role in activating inflammation and cancer, since COX-2 and prostaglandin PGE_2 are found to stimulate cell proliferation, suppression of apoptosis, induction of tumor angiogenesis and promotion of invasiveness (Kundu and Surh, 2008).

To mimic the exposure of cigarette smoke, this study used four major carcinogenic and toxic substances which are present in cigarette smoke. BaP and NNK represent the two major classes of lung carcinogens (Martey et al., 2004). The role of NNK, a tobacco specific N-nitrosamine, as a cancer causing agent, has been extensively studied (Chen et al., 2006) and it is reported to induce tumorigenesis (Hecht, 1998) and lung adenocarcinoma, independent of the route of administration (Rosa et al., 1998), in rats, mice and hamsters. Further, DNA damage is reported in mice with oral administration, as well as single intraperitoneal injection of NNK (Rosa et al., 1998). The cytochrome P450 pathway, as well as the cyclooxygenase pathway via COX-1 and COX-2 in the presence or absence of microsomal proteins, is involved in the metabolic activation of NNK, leading to its carcinogenic effects (Rioux and Castonguay, 1998). NNK-induced lung tumorigenesis and DNA damage in mice was attenuated by flavonoid epigallocatechin gallate (Xu et al., 1992). Black tea polyphenols with antiproliferative activity have also been found to inhibit tumorigenesis induced by NNK (Rosa et al., 1998). The tumorigenesis induced by NNK in the lung can be attenuated by selective COX-2 inhibitors (Matsuzuka et al., 2009). In the present study, loss of cell viability was not observed due to exposure to NNK, which is in accordance with an earlier study that NNK activated the extracellular signal-regulated kinase (ERK1/2) pathway, stimulating cell

proliferation in human mammary epithelial cells (Chen et al., 2006). The induction of inflammatory markers was inhibited with the application of fatty acid derivatives of Q3G, which are supposed to possess enhanced cellular uptake. Therefore, the tested compounds have the ability to act as inhibitors of cyclooxygenases, in turn being potential anti-inflammatory agents. *In vitro* studies have demonstrated that the gas phase of cigarette smoke causes lipid peroxidation in blood plasma (Hecht, 1999) and NNK- induced COX-2 expression in rat lung cells (El-Bayoumy et al., 1999), while resulting in tumorigenesis in mice (Prokopczyk et al., 2000).

BaP is a polyaromatic hydrocarbon (PAH) present in cigarette smoke and is considered to be a chemical responsible for cancer. It is listed as a group 2A carcinogen, which is carcinogenic to animals and is possibly carcinogenic to humans, according to the International Agency for Research on Cancer (IARC) (Sang et al., 2012). Among PAHs, BaP is the most studied compound (Hecht, 1999; Lin et al., 2004). BaP must be metabolically activated to express its carcinogenic effects (Gower and Wills, 1984) and cytochrome P450s and cyclooxygenase enzymes are associated with metabolically activating BaP into its carcinogenic metabolites. It has been reported that COX-1 mediates the metabolism of BaP to BaP-7,8-diol and to BaP-7,8-diol-9,10-oxide, which forms DNA adducts (Marnett, 1990). The activation of BaP-7,8-diol induces COX-2 expression. Therefore, BaP and its metabolites are inducers, as well as substrates for COX (Wiese et al., 2011). The oxidation of B[*a*]P-7,8-dihydrodiol to B[*a*]P-diolepoxide, a highly mutagenic carcinogen, is catalysed by COX-2. This cyclooxygenase activity is important in extra-hepatic tissues where P450 activity is low (Angus et al., 1999, Sohn et al., 2008). Activation of the carcinogens by these extra-hepatic enzymes plays a

significant role in normal tissue or tumors such as colon, lung, breast and skin (Wiese et al., 2011). Also, COX-2, induced by procarcinogens, such as BaP, in turn, is responsible for conversion of BaP to BaP-7,8-diol-9,10-epoxide. This can amplify the tumorigenic effect of BaP (Sonaware et al., 2011). However, some authors have demonstrated that BaP shows mutagenicity and DNA adduct formation, with or without metabolic activation (Krishnamurthi et al., 2003).

It has been reported that BaP induces COX-2 expression in fibroblasts (Martey et al., 2004), epithelial cells (Yan et al., 2000), lymphocytes (Anto et al., 2002) and oral epithelial cells (Kelley et al., 1997). The up-regulation of COX-2 and microsomal PGE synthase in lung cancers has been reviewed by Marty et al. (2004). It has been found that cigarette smoke activates the aryl hydrocarbon receptor (AhR) in normal human lung fibroblasts and thereby, induces the expression of COX-2 and PGE_2. Also, the nuclear factor κB (NF-κB) pathway regulates COX-2 expression (Marty et al., 2004, 2005). In the present study, loss of cell viability was not observed in the tested concentrations for BaP and this is in accordance with previous literature which showed increased cell viability with treatment of 10 μM of BaP in WI-38 cells (Sohn and Kim, 2008). Further, in studies carried out to determine DNA adduct formation in human diploid lung fibroblasts after exposure to 1 μM BaP, the DNA adducts were formed via induction of P53 and P21WAF1 proteins, showing that cells activate the parent BaP to their reactive metabolites. Further, cell cycle G1 arrest was not observed, whereas there was a delay in the S phase, which is called 'S phase checkpoint'. However, cell viability was not affected and it has been explained that activation of P53 and P21WAF1 genes may prevent DNA replication without inducing apoptosis, allowing DNA repair

(Binkova et al., 2000). Similarly, in another study with MRC-5, human fetal lung fibroblast cells, treated with 8 to 128 μM of BaP, showed no significant changes in cell viability at 4 and 12 h incubation (Yang et al., 2011).

Nicotine, a major alkaloid present in tobacco, itself is not carcinogenic. However, addiction to nicotine is considered as the reason for people to continue smoking (Jin et al., 2003). Tar-containing nicotine, distilled from burning cigarettes, is deposited in the small airways and alveoli, followed by rapid absorption. Absorbed nicotine exposes the whole body to the effects of nicotine (Upadhyaya et al., 1999; Jin et al., 2003). During the curing process of tobacco, nicotine can be converted to NNK, which is carcinogenic (Upadhyaya et al., 1999). Nicotine is a primary constituent in cigarette smoke, inducing the production of COX-2 and PGE_2 in dendritic cells (Vassallo et al., 2008), microglia (De Simone et al., 2005), fibroblasts (Ho and Chang, 2006), monocytes (Payne et al., 1996) and whole blood (Saareks et al., 1998). The mechanism by which nicotine induces the COX-2 inflammatory pathway results in the production of PGE_2 and it is thought to happen via nicotine acetylcholine receptors (nAchRs) (Arredono et al., 2003) or NF-κB activation (Nakao et al., 2009). The elevated ROS production by nicotine activates mitogen-activated protein kinases (MAPKs) and NF-κB and the activated NF-κB induced COX-2 expression and further increases PGE_2 release (Lin et al., 2010). A traditional Korean medicinal extract, composed of Oriental herbs (Sejin-Eum), showed inhibitory effect towards nicotine-induced cytotoxicity in human embryonic lung fibroblasts (Jin et al., 2003). Similarly, the present study reports the effectiveness of fatty acid derivatives of Q3G in providing cytoprotection against nicotine-induced toxicity. However, treatment with higher concentrations of nicotine induced cytotoxicity, while

lower concentrations increased cell viability (data not shown). This study's findings are supported by research which showed increased cell viability with 10 μM of nicotine on WI-38 cells and it has been further explained that nicotine may contribute to lung cancer by inducing cellular growth (Sohn et al., 2008). Moreover, nicotine is known to activate growth promoting pathways promoting lung cancer (Zhang et al., 2006).

Cr(VI) is considered to be a human respiratory tract irritant and carcinogen, which causes lung and nasal cancer. Insoluble forms of Cr(VI) are believed to be more carcinogenic, due their persistence in the tissues, while soluble forms are reduced into non-toxic forms in the body. However, K and Na chromates are known to induce toxicity in cell culture systems (De Flora et al., 1997). As an established lung carcinogen, tumors and neoplastic transformation were reported by Cr(VI) in toxicological studies (Bao et al., 2012). Cr(VI)-mediated cell death is thought to be due to apoptosis (Blankenship et al, 1994).

The inverse relationship between the consumption of fruits and vegetables and cancers has been identified by epidemiological studies (Cui et al., 2008). Among tobacco smokers, consumption of epicatechin, catechin, quercetin, and kaempferol has been demonstrated to have an inverse association with lung cancer (Cui et al., 2008). Quercetin exerts a protective effect on aqueous cigarette tar extract-induced impairment of erythrocyte deformability (Begum and Terao, 2002). Nobiletin, a polymethoxy flavonoid, has been found to interfere with the production of PGE_2 via the selective down-regulation of the COX-2 gene in human synovial fibroblasts (Lin et al., 2003). Further, silymarin, a natural flavonoid derivative present in milk thistle, has given a protective effect against BaP induced oxidative stress and immunotoxicity in human

peripheral blood mononuclear cells (Vijayaraman et al., 2012) and erythrocytes (Kiruthiga et al., 2007). In general, the fatty acid derivatives of Q3G showed inhibitory activity towards the toxicity induced by cigarette smoke toxicants. COX-2 pathway, leading to the inflammatory cascades, was affected with the application of some of these compounds. However, future research is needed, using animal models and human clinical studies, in order to confirm the activity of these novel compounds.

6.6 CONCLUSION

The cigarette smoke toxicants, nicotine and Cr(VI) induced cytotoxicity in WI-38 cells and decreased the percentage cell viability while increasing the production of lipid hydroperoxides. Pre-incubation of the cells with the fatty acid derivatives of Q3G provided protection against nicotine- and Cr(VI)-induced toxicity. However, the fatty acid derivatives of Q3G provided an inhibitory effect by decreasing COX-2 and PGE_2 production. Therefore, it can be generally concluded that fatty acid derivatives of Q3G are more effective than their parent Q3G in inhibiting cigarette smoke-induced cytotoxicity. Further, the biological activity of Q3G molecules is enhanced by the acylation with fatty acids.

CHAPTER 7 GENERAL DISCUSSION

As one of the most ubiquitously found flavonoid in plants, quercetin-3-*O*-glucoside (Q3G) has been extensively studied for its potential biological activities. However, lipophilic/hydrophilic nature of flavonoid glycosides is an important factor governing the ability to cross biological membranes and the biological activity in human body. Therefore, conjugation of a hydrophobic moiety to the flavonoid molecule is investigated for improving cellular uptake and target accessibility of flavonoids. This phenomenon was examined in this study by acylation of Q3G with six different long chain fatty acids. The overall objective of the study was to determine the effect of increased lipophilic nature of the Q3G, in a food model system and biological systems *in vitro* on antioxidant activity and cytoprotection, respectively. Finally, it was shown that these derivatives are potent antioxidants in experimental model systems containing an interfacial membrane (emulsion) or a biological membrane. Lipid oxidation in oil-in-water emulsions was inhibited effectively by the acylated forms of Q3G when compared to its antioxidant effects in the bulk oil system. However, their behaviour has to be further investigated in order to apply them for bulk food systems. Further, inhibition of LDL oxidation and, attenuation of induced acute oxidative stress and cigarette smoke constituents-induced toxicity in cell culture model systems demonstrated the potential of the fatty acid derivatives of Q3G in providing advantage as natural cytoprotective agents. It is apparent that the hydrophobic moiety seems to have an effect on permeability to cell membranes and interactions with cellular signaling pathways. Therefore, this study raises the need of better understanding of the cellular mechanisms which are involved in the cytoprotection

and the bioavailability of the fatty acid derivatives of Q3G using animal models and clinical trials to reach further conclusions.

Oxidation of lipids in natural and processed food is one of the major types of their quality deterioration. Due to different physicochemical characteristics in these foods, the physical location that lipid oxidation takes place can vary and therefore, it has been one of the major factors that has to be considered when selecting suitable antioxidants for each of the food systems. Antioxidants can act via different mechanisms such as scavenging free radicals, chelating metal ions, deactivating singlet oxygen, scavenging oxygen and absorbing UV radiation. However, the extent of lipophilicity is a governing parameter of their suitability for bulk oils or emulsions, which are in oil-in-water or water-in-oil forms. Moreover, the safety and health benefits associated with natural antioxidants have attracted both consumer and market attention. Therefore, the fatty acid derivatives of Q3G would have potential as antioxidants; however the sensory properties, shelf-life, storage conditions and safety should be further studied before incorporating them into bulk foods.

Although reactive oxygen species (ROS) are important in cellular signal transduction, the excessive levels of cellular ROS under oxidative stress are detrimental to biological materials such as macro molecules; nucleic acids, lipids and proteins, eventually resulting in cellular dysfunction and cell death. The evaluation of the biochemical pathways such as mitogen-activated protein kinase (MAPK) associated pathways and nuclear factor κB (NF-κB) would be helpful to understand the role of fatty acid esters of Q3G in preventing oxidative stress mediated pathological conditions, such as cancer, neurological disorders, cardiovascular disease (CVD), lung inflammation, rheumatoid

arthritis and aging. Cigarette smoke is an obvious risk factor for inflammation mediated lung cancer due to the mutagenic constituents it contains. Furthermore, MAP kinases, such as extracellular signal-regulated kinases (ERKs), c-jun N-terminal kinases (JNKs) and p38, have a major role in lung inflammatory responses. MAPK pathways are activated in chronic obstructive pulmonary disease (COPD) and asthma patients (Foronjy and D'Armiento, 2006).

Biotransformation of flavonoid molecules take place in intestine and liver and those metabolites are important in cellular absorption and the biological activity. Since the naturally-occurring form of flavonoids is not necessarily their bio-active form, it is interesting to further study flavonoid metabolism and the effects of these metabolites at the cellular level, including their potential as mediators in cellular signal transduction which depends on their interactions with the cell membranes and the uptake into the cytosol. Further, *in vitro* experimental conditions that were used to measure the antioxidant activity of lipophilised molecules are not always satisfactory to reach conclusions about their behaviour in the real *in vivo* situations. Therefore, the effect of the hydrophobic nature of the compounds may substantially vary *in vivo* conditions. Further studies are required to evaluate the effect of the fatty acid derivatives of Q3G using appropriate animal systems especially to understand their stability in the human digestive system and then to recognize clinical applications.

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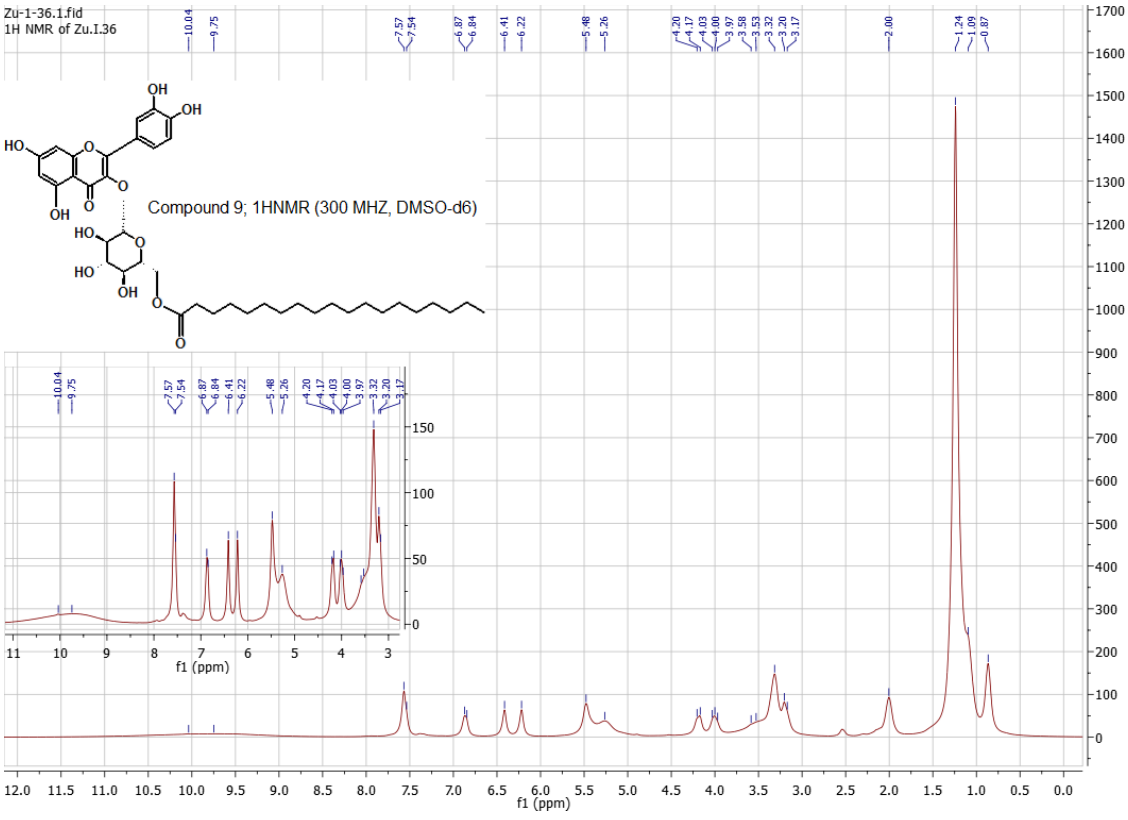
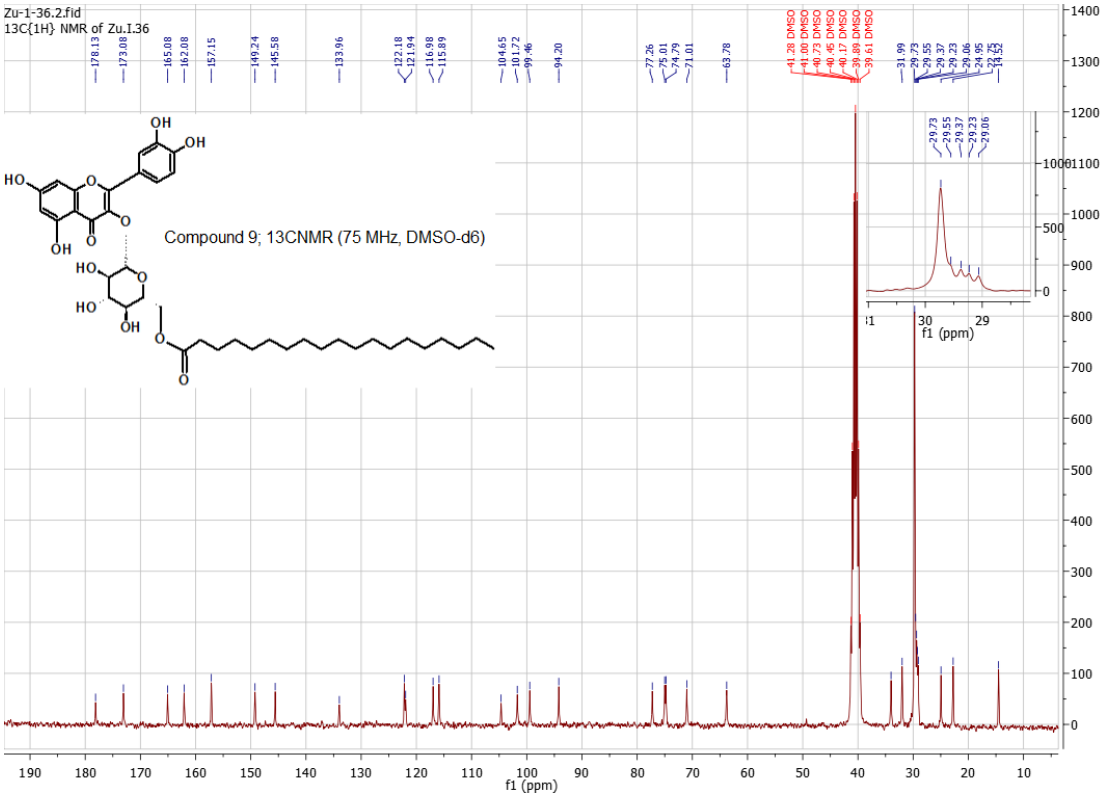
APPENDIX 1: ^{1}H NMR AND ^{13}C NMR DATA FOR THE SYNTHESISED FATTY ACID DERIVATIVES OF Q3G

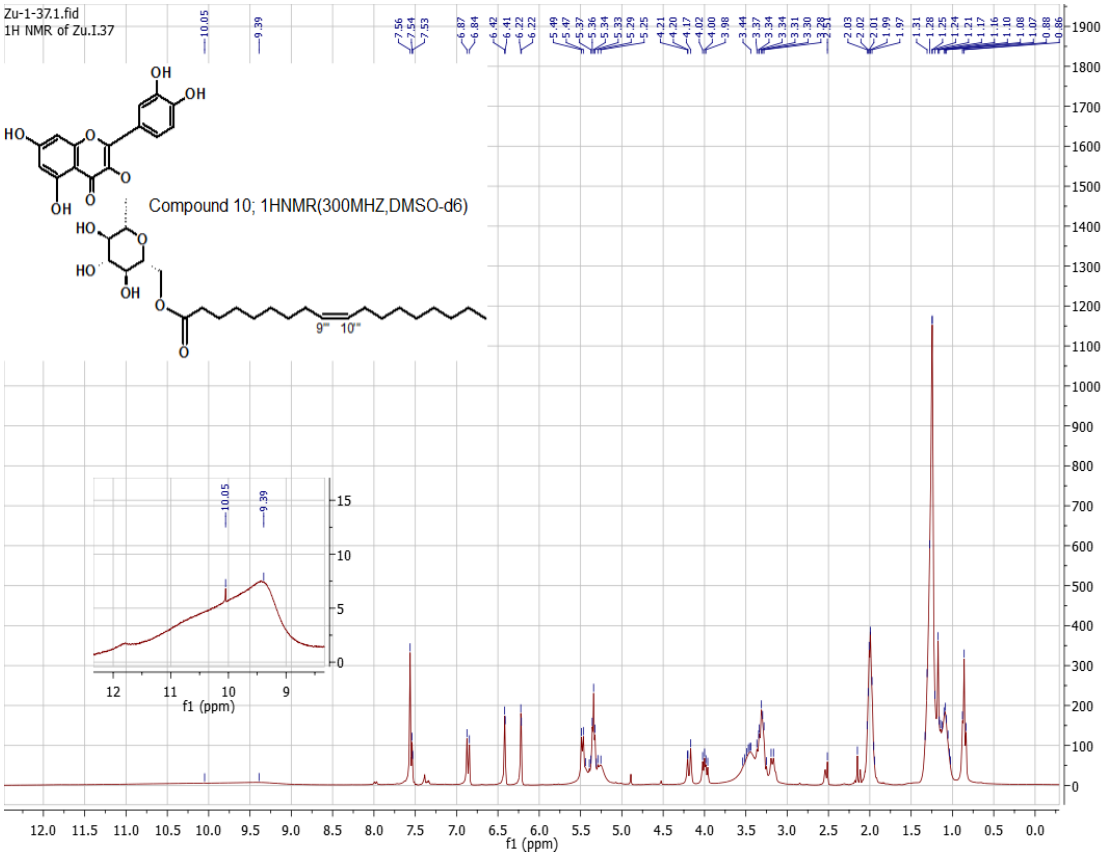
^{1}H NMR for stearic acid derivative of Q3G:



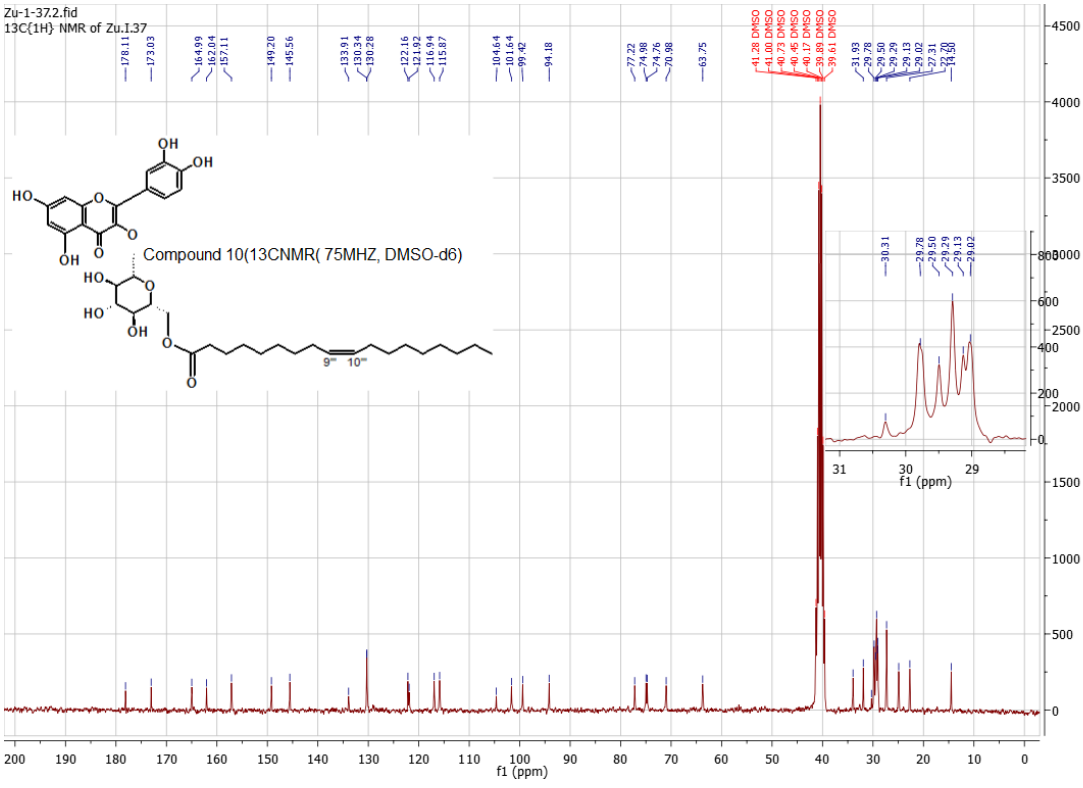
^{13}C NMR for stearic acid derivative of Q3G:



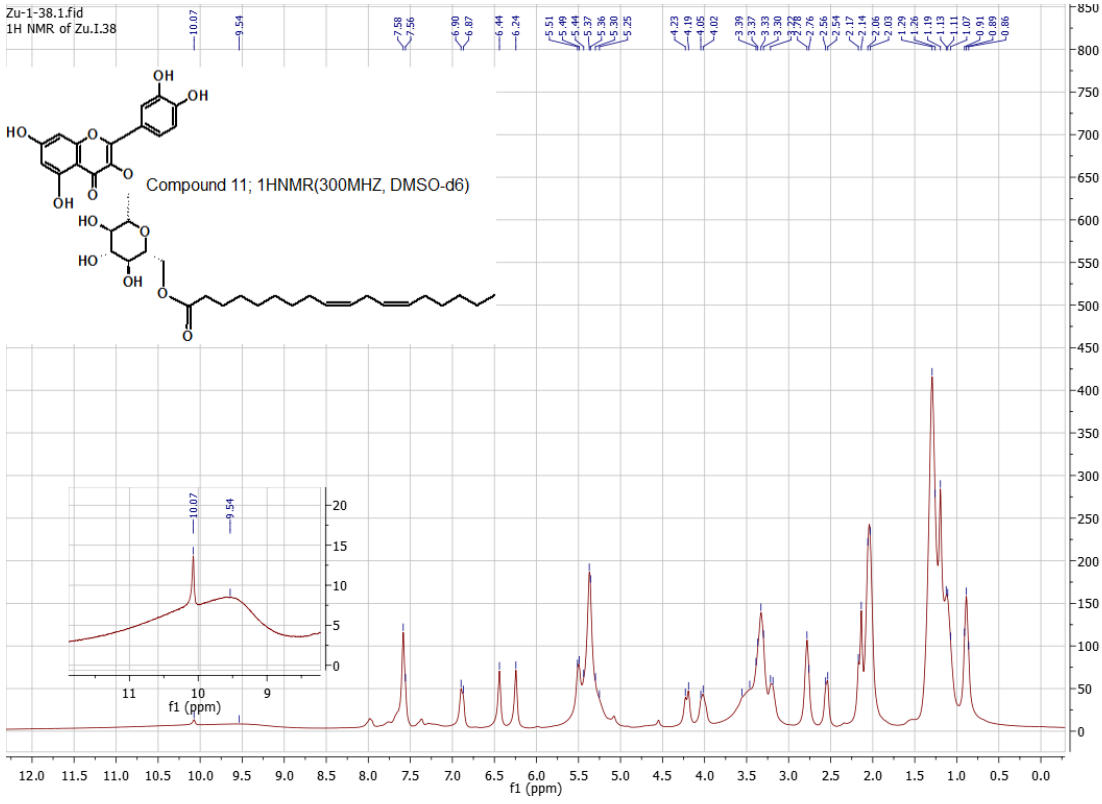
^{1}H NMR for oleic acid derivative of Q3G:



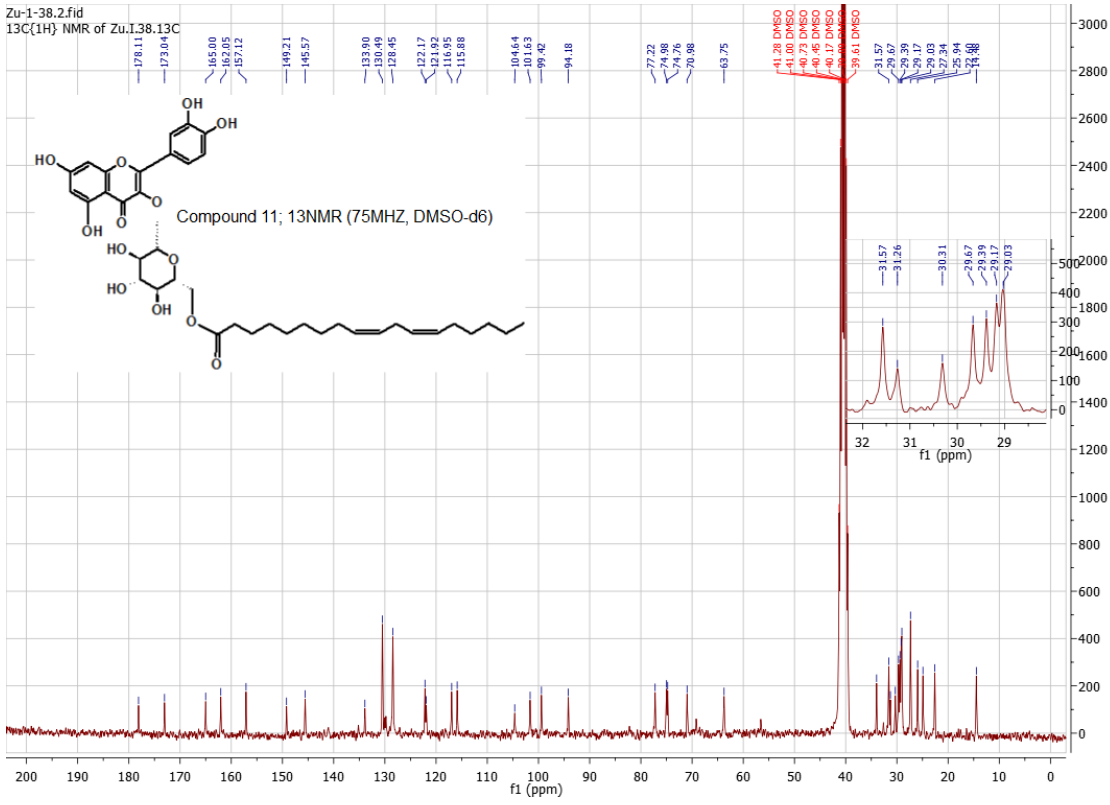
^{13}C NMR for oleic acid derivative of Q3G:



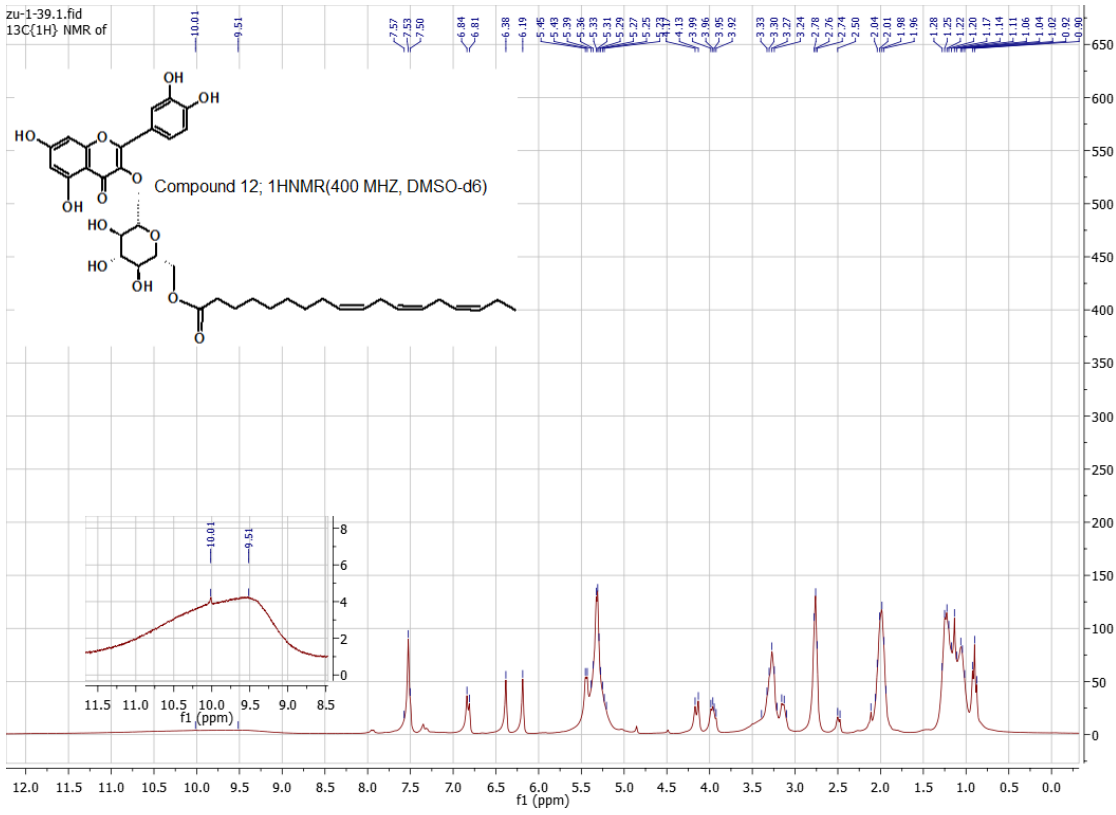
^{1}H NMR for linoleic acid derivative of Q3G:



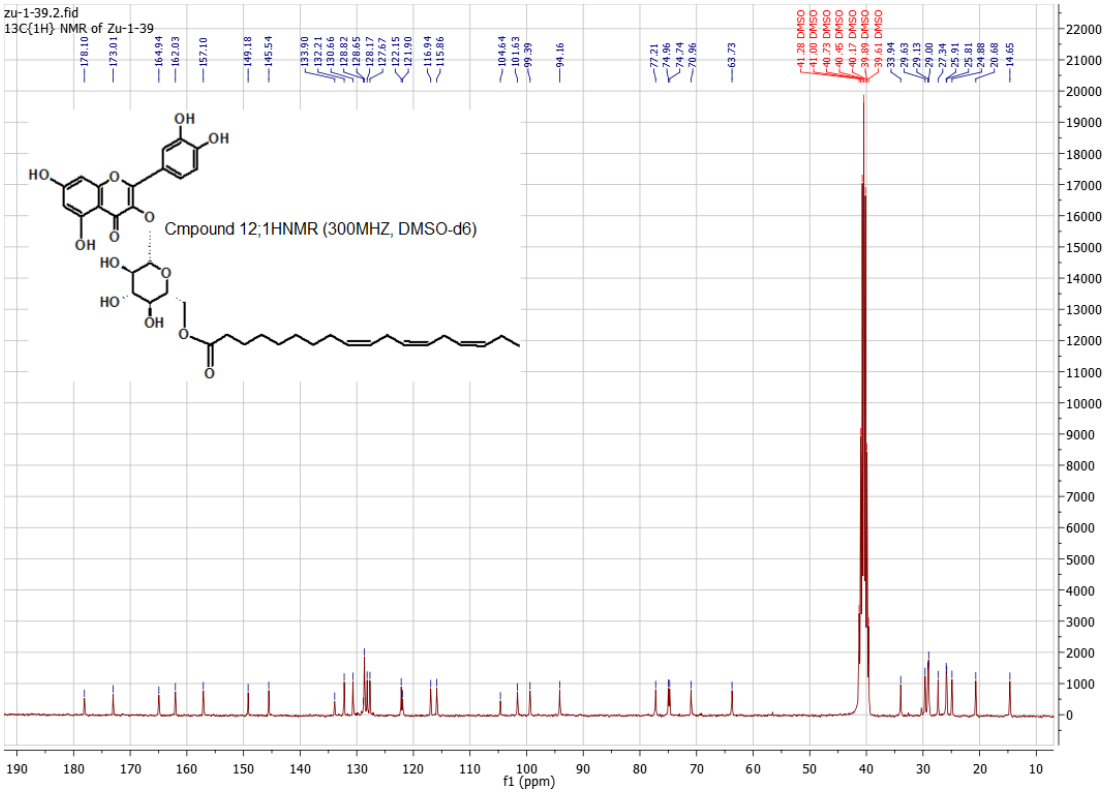
^{13}C NMR for linoleic acid derivative of Q3G:



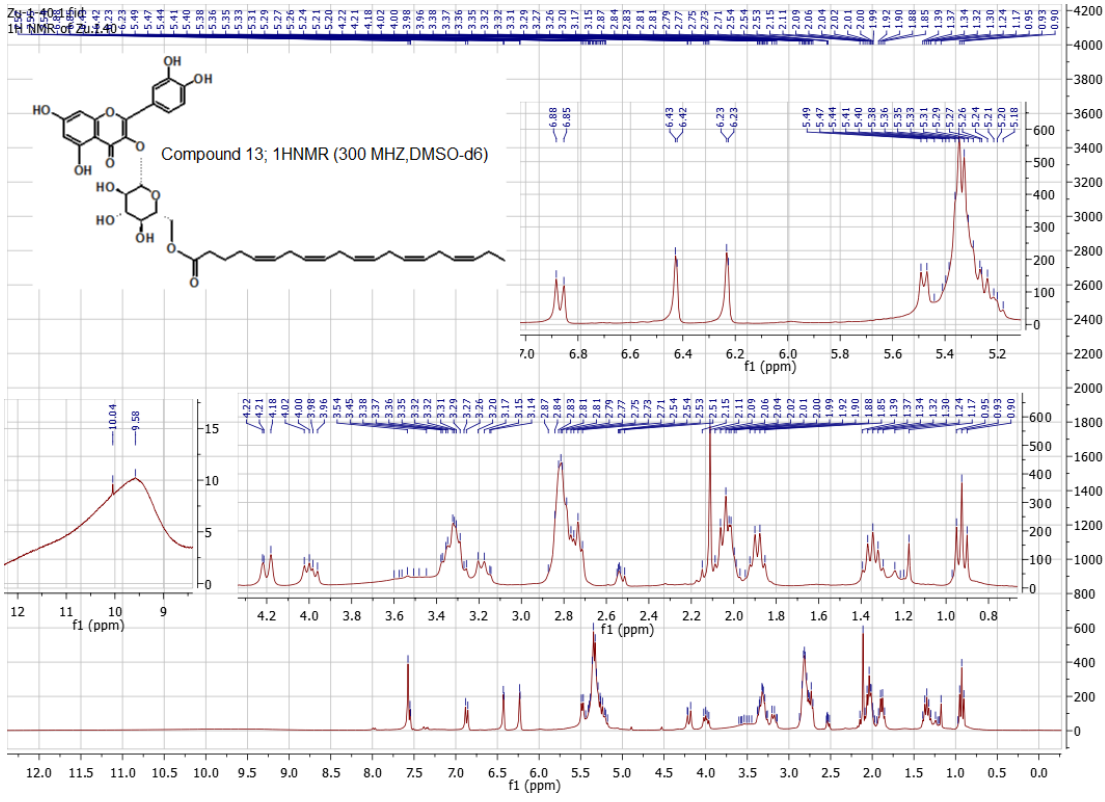
^{1}H NMR for α-linolenic acid derivative of Q3G:



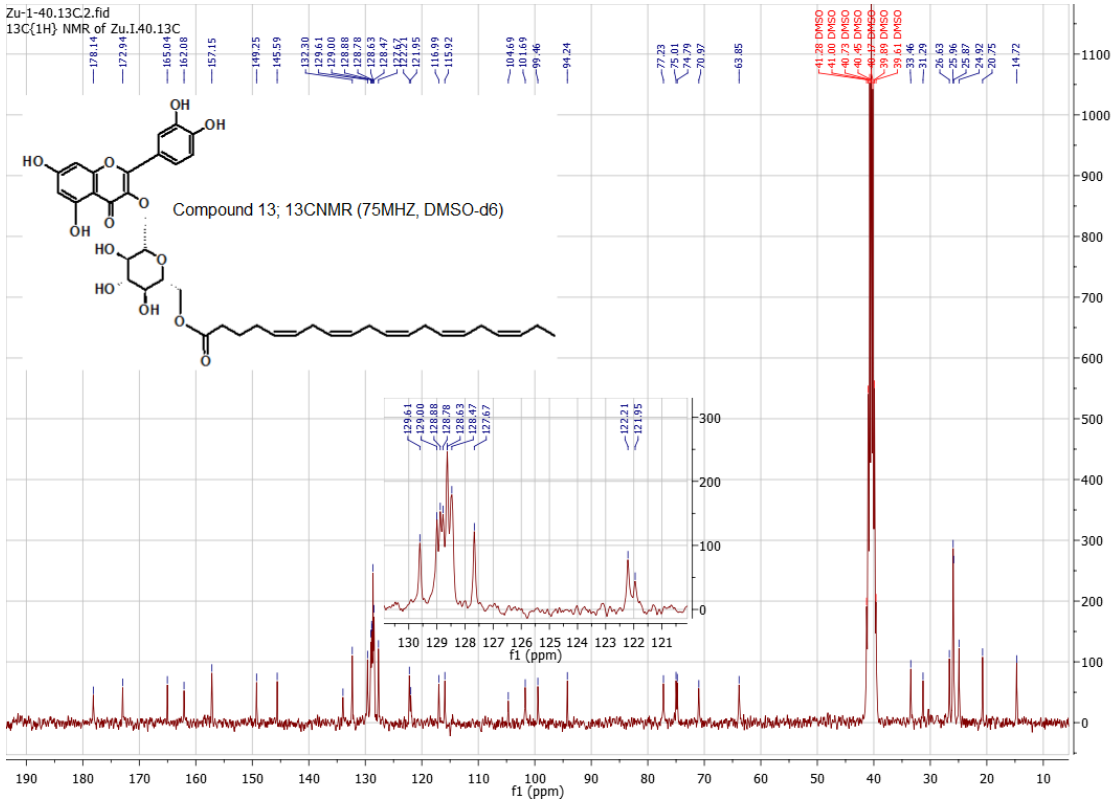
^{13}C NMR for α-linolenic acid derivative of Q3G:



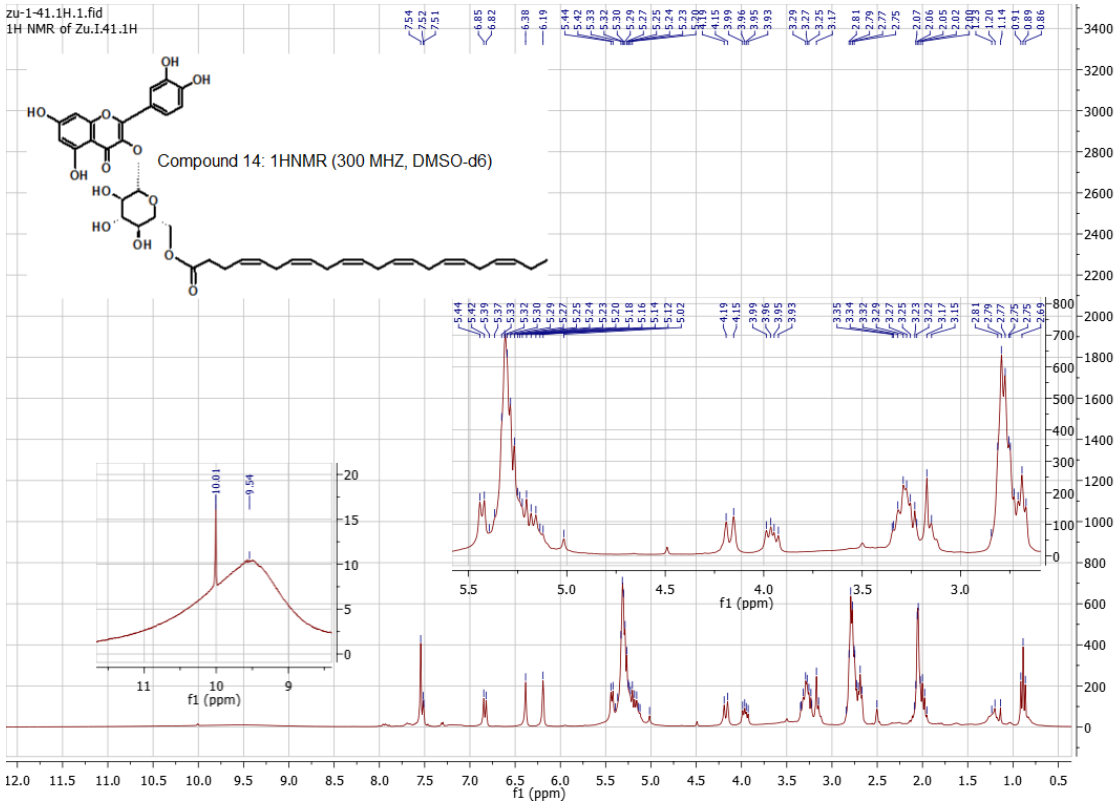
^{1}H NMR for EPA derivative of Q3G:



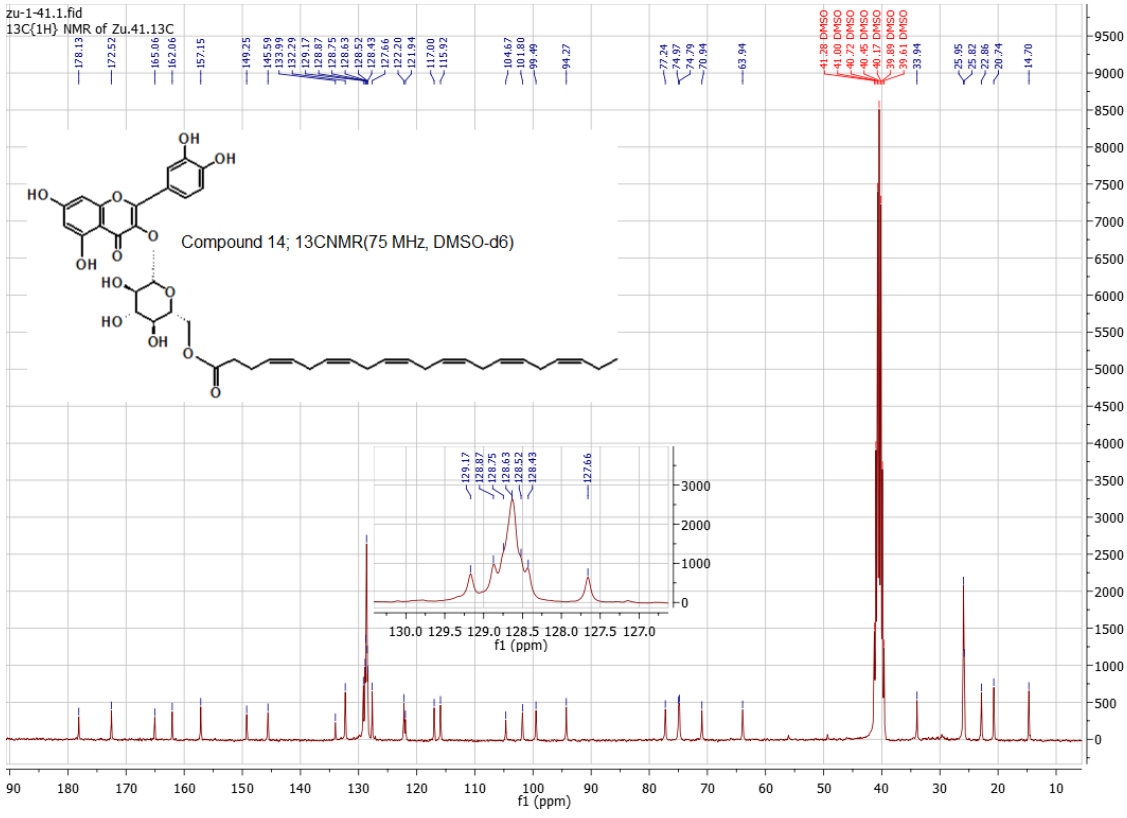
^{13}C NMR for EPA derivative of Q3G:



^{1}H NMR for DHA derivative of Q3G



^{13}C NMR for DHA derivative of Q3G



APPENDIX 2: FATTY ACID COMPOSITION OF THE FISH OIL

Maxxam

Success Through Science

Maxxam Job #: B236086
Report Date: 2012/03/28

Nova Scotia Agricultural College
Client Project #: NSAC
Site Location: TRURO,NS

RESULTS OF ANALYSES OF FOOD

Maxxam ID		MU5562	MU5563	
Sampling Date		2012/01/04	2012/03/08	
	Units	**SUMUDU FISH OIL - BASF**	**SATVIR 7**	**RDL**
C4 Butyric	g/100g	0.006	0.009	0.001
C6 Caproic	g/100g	<0.001	<0.001	0.001
C8 Caprylic	g/100g	<0.001	0.007	0.001
C10 Capric	g/100g	<0.001	<0.001	0.001
C12 Lauric	g/100g	0.053	<0.001	0.001
C14 Myristic	g/100g	3.35	0.115	0.001
C14:1 Myristoleic	g/100g	0.050	<0.001	0.001
C16 Palmitic	g/100g	16.5	0.127	0.001
C16:1 Palmitoleic	g/100g	4.89	0.121	0.001
C18 Stearic	g/100g	4.34	3.63	0.001
C18:1trans	g/100g	0.317	0.211	0.001
C18:1 Oleic	g/100g	13.1	7.76	0.001
C18:2trans	g/100g	0.308	0.212	0.001
C18:2 Linoleic	g/100g	1.77	0.652	0.001
C18:3 Linolenic	g/100g	0.551	1.21	0.001
C18:3 Gamma Linolenic	g/100g	0.149	0.075	0.001
C18:4 Moroctic	g/100g	0.889	0.980	0.001
Conjugated Linoleic Acid	g/100g	0.225	0.678	0.001
C20 Arachidic	g/100g	0.302	0.497	0.001
C20:1	g/100g	0.809	2.26	0.001
C20:2 Eicosadienoic	g/100g	0.263	0.542	0.001
C20:3 Eicosatrienoic	g/100g	0.106	0.423	0.001
C20:4 Arachidonic	g/100g	1.58	2.70	0.001
C20:5 W3 (EPA)	g/100g	5.59	48.3	0.001
C22 :0 Behenic	g/100g	0.207	0.132	0.001
C22:1 Erucic	g/100g	0.655	1.08	0.001
C22:2 Docasadienoic	g/100g	0.018	0.093	0.001
C22:4 Docosatetraenoic	g/100g	0.181	0.081	0.001
C22:5 Docosapentaenoic	g/100g	1.03	1.86	0.001
C22:6 (DHA)	g/100g	22.9	8.62	0.001
C24:0	g/100g	0.153	<0.001	0.001
C24:1 Nervonic	g/100g	0.428	0.134	0.001
Fat (GC/FID)	g/100g	83.2	83.0	0.001

RDL = Reportable Detection Limit

Page 2 of 5

axxam

Success Through Science

xam Job #: B236086
port Date: 2012/03/26

Nova Scotia Agricultural College
Client Project #: NSAC
Site Location: TRURO,NS

RESULTS OF ANALYSES OF FOOD

Maxxam ID		MU5562	MU5563	
Sampling Date		2012/01/04	2012/03/08	
	Units	**SUMUDU FISH OIL - BASF**	**SATVIR 7**	**RDL**
Fat (GC/FID)	g/100g	83.2	83.0	0.001
Saturated Fatty Acids	g/100g	25.6	4.34	0.001
cis-Monounsaturated Fatty Acids	g/100g	19.1	10.9	0.001
cis-Polyunsaturated Fatty Acids	g/100g	33.7	62.9	0.001
Trans-Fatty Acids	g/100g	1.16	0.877	0.001
Omega-3 Polyunsaturated Fatty Acids	g/100g	29.9	58.6	0.001
Omega-6 Polyunsaturated Fatty Acids	g/100g	3.72	4.30	0.001

RDL = Reportable Detection Limit

axxam

Success through Science

axam Job #: B236086
port Date: 2012/03/28

Nova Scotia Agricultural College
Client Project #: NSAC
Site Location: TRURO,NS

RESULTS OF ANALYSES OF FOOD

Maxxam ID		MU5562	MU5563	
Sampling Date		2012/01/04	2012/03/08	
	Units	**SUMUDU FISH OIL - BASF**	**SATVIR 7**	**RDL**
Saturated Fatty Acids	g/100g	25.6	4.34	0.001
cis-Monounsaturated Fatty Acids	g/100g	19.1	10.9	0.001
cis-Polyunsaturated Fatty Acids	g/100g	33.7	62.9	0.001
Trans-Fatty Acids	g/100g	1.16	0.877	0.001
Omega-3 Polyunsaturated Fatty Acids	g/100g	29.9	58.6	0.001
Omega-6 Polyunsaturated Fatty Acids	g/100g	3.72	4.30	0.001

RDL = Reportable Detection Limit