Lipophilized Derivatives of Epigallocatechin Gallate (EGCG): Preparations and Bioactivities

By

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ABSTRACT

Green tea polyphenols (GTP) are a major source of dietary phenolics that render a myriad of health benefits. Among GTP, epigallocatechin gallate (EGCG) is dominant and has been considered as being effective in both food and biological systems. However, its application and benefits may be compromised due to limited absorption and bioavailability. In order to expand the application of EGCG to more diverse systems, it may be lipophilized through structural modification.

In this work, lipophilized derivatives of EGCG were prepared by acylation with different chain lengths fatty acyl chlorides such as acetyl chloride, C2:0; propionyl chloride, C3:0; hexanoyl chloride, C6:0; octanoyl chloride, C8:0; dodecanoyl chloride, C12:0; octadecanoyl chloride, C18:0; and docosahexaenoyl chloride, C22:6. The resultant products, mainly tetra-esters, were purified and their bioactivities evaluated, including antioxidant activities in different model systems and anti-glycation activities. The lipophilicity of the esters increased with increasing chain length of the acyl group and also led to the enhancement of their antioxidant properties that were evaluated using assays such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity, oxygen radical absorbance capacity (ORAC) and reducing power of the molecules involved. These findings strongly suggest that the EGCG ester derivatives have great potential as lipophilic alternatives to the water-soluble EGCG.

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LIST OF ABBREVIATION

AAPH	2,2’-Azobis (2-aminopropane) dihydrochloride
AG	Aminoguanidine
AGEs	Advanced glycation endproducts
BDE	Bond dissociation enthalpy
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CD	Conjugated diene
CML	Carboxymethyllysine
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
DHASCO	Docosahexaenoic acid single cell oil
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPH	1,1 -Dipheny1-2-picrylhydrazyl
DS	Degree of substitution
EC	Epicatechin
ECG	Epicatechin gallate
EDTA	Ethylenediaminetetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
EPR	Electron paramagnetic resonance
GAE	Gallic acid equivalents

GC-MS	Gas chromatography-mass spectrometry
GTP	Green tea polyphenol
HCA	Heterocyclic amine
HCC	Hepatocellular carcinoma
HLB	Hydrophilic-lipophilic balance
HPLC	High performance liquid chromatography
IP	Ionization potential
IP	Induction period
LDL	Low density lipoprotein
MGO	Methylglyoxal
NMR	Nuclear magnetic resonance
NO	Nitric oxide
ORAC	Oxygen radical absorbance capacity
OPD	*O*-phenylenediamine
PBS	Phosphate buffer solution
PF	Protection factor
PG	Propyl gallate
PUFA	Polyunsaturated fatty acids
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances

TBHQ	tert-Butylhydroquinone
TCA	Trichloroacetic acid
TE	Trolox equivalents
THF	Tetrahydrofuran
TLC	Thin layer chromatography

CHAPTER 1

INTRODUCTION

Tea, the second most popular beverage worldwide, is produced from the leaves and buds of the plant *Camellia sinensis*. It is a major source of dietary polyphenols and is known to render a myriad of health benefits. These green tea polyphenols (GTP) play a protective role and reduce the risk and pathogenesis of several chronic ailments, especially cardiovascular disease and cancer. They are also responsible for the characteristic colour, flavour and aroma of tea (Cabrera *et al*. 2006). The dry leaves of tea contain approximately 30% (by weight) polyphenols, the majority of which are catechins (flavan-3-ols). Epigallocatechin gallate (EGCG) is the predominant catechin in green tea, followed by epigallocatechin (EGC), but these are less abundant in black tea due to the formation of their condensation products (Zhong *et al*. 2012). EGCG has a four-ring structure with eight hydroxyl groups and is therefore highly hydrophilic. It renders its bioactivities or health effects mainly in aqueous environments or water compartments in body tissues.

EGCG is known as a powerful antioxidant protecting against free radical-mediated oxidative changes both in food and in living organisms. EGCG acts as scavenger of many reactive oxygen/nitrogen species such as superoxide radical anion, peroxyl and hydroxyl radicals, singlet oxygen, nitric oxide and peroxynitrite, among others, which are implicated in human pathogenesis, including inflammation and carcinogenesis. EGCG can trap peroxyl radicals and thus break the chain reaction of free radicals and terminate lipid oxidation. EGCG can also inhibit oxidation by chelating metal ions, such as Fe and Cu ions, which are catalysts of free

radical generation. Antioxidant activity of EGCG depends on many factors, including metal-reducing potential, chelating behaviour, pH, solubility characteristics, bioavailability, and stability in the environment (Luczaj and Skrzydlewska 2005), which are, in turn, determined by the structural features of the molecule. EGCG shows varied antioxidant activity in different model systems, and it is well documented that its activity depends largely on the substrate system under investigation (Wanasundara and Shahidi 1996; He and Shahidi 1997; Zhong *et al*. 2012). Also the inhibitory effects of EGCG against LDL-cholesterol oxidation, DNA scission and liposome oxidation have been observed in various *in vitro* and *in vivo* studies (Huang and Frankel 1997; Ishikawa *et al*. 1997; Hu and Kitts 2001, Zhong *et al*. 2012).

In humans, EGCG may have limited bioavailability due to its physical and chemical characteristics, which influence its rate of absorption through the gastrointestinal tract, metabolism, and elimination from the body. The hydrophilic nature of EGCG may negatively affect its ability to protect lipophilic systems, such as fats, oils, and lipid-based foods or cosmetic formulas and emulsions, as well as in biological environments. In order to expand the effectiveness of these compounds as antioxidants or other functional ingredients in more diverse systems, and to improve the cellular uptake and affinity to lipid particles and membrane and other oxidation-susceptible sites *in vivo*, these compounds may be lipophilized through structural modification. Although when joining two biologically active components, one may expect to see additive, synergistic or antagonistic effects, EGCG was esterified with saturated or polyunsaturated fatty acids in order to enhance the lipophilicity and hopefully improve their antioxidant activities.

Therefore, the objectives of this study were to investigate the effect of structural modification of EGCG on its lipophilicity, antioxidant potential and its functional properties in different food and biological systems.

CHAPTER 2

LITERATURE REVIEW

2.1. Phenolic compounds

Phenolic compounds are secondary metabolites with considerable physiological and morphological importance in plants. These compounds play an important role in growth, reproduction and in providing protection against pathogens and predators as well as oxidative stress under sunlight. They also contribute toward the colour and sensory characteristics and oxidative stability of foods (Shahidi and Naczk 2004; Alasalvar *et al.* 2001).

Phenolic compounds exhibit a wide range of physiological properties associated with health benefits in animals, such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic and cardioprotective effects (Manach, Mazur, and Scalbert 2005; Hertog *et al*. 1993; Parr and Bolwell 2000). The beneficial effects derived from phenolic compounds have been attributed, in part, to their antioxidant activity (Heim *et al*. 2002; Zhong and Shahidi 2011).

Phenolic compounds have one or more hydroxyl groups on the aromatic ring(s) and are derived from phenylalanine or tyrosine, and hence are referred to as secondary metabolites (Harborne 1982; Morello, Shahidi and Ho 2002). Most naturally occurring phenolic compounds are present as conjugates, with mono-, di- or polysaccharides linked to one or more of the phenolic groups, and may also occur as functional derivatives such as esters (Harborne 1989; Harborne, Baxter

and Moss 1999; Shahidi and Naczk 1995). Among dietary phenolic compounds, phenolic acids, flavonoids, and tannins (proanthocyanidins) are most important (King and Young 1999).

Phenolic compounds have a large diversity of structures that include simple phenol molecules, polyphenols such as stilbenes and flavonoids and polymers such as proanthocyanidins or condensed tannins derived from these various groups. Simple phenols include monophenols (*e.g*. cresol) and diphenols (*e.g*. hydroquinones).

2.1.1. Phenolic Acids

Phenolic acids are substituted derivatives of hydroxybenzoic and hydroxycinnamic acids and are the predominant phenolics in plant sources, mainly grains, oilseeds and cereals. These derivatives differ in the pattern of hydroxylation and methoxylation of their aromatic rings (Shahidi and Naczk 2004).

The basic pathway (Figure 2.1) for synthesis of phenolic acids in plants begins from sugars through an aromatic amino acid, mainly phenylalanine, and, to a lesser extent, tyrosine, as noted earlier. The formation of *trans*-cinnamic acid from phenylalanine and *p*-hydroxycinnamic acid from tyrosine are catalyzed by phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL), respectively (Amarowicz *et al.* 2009).

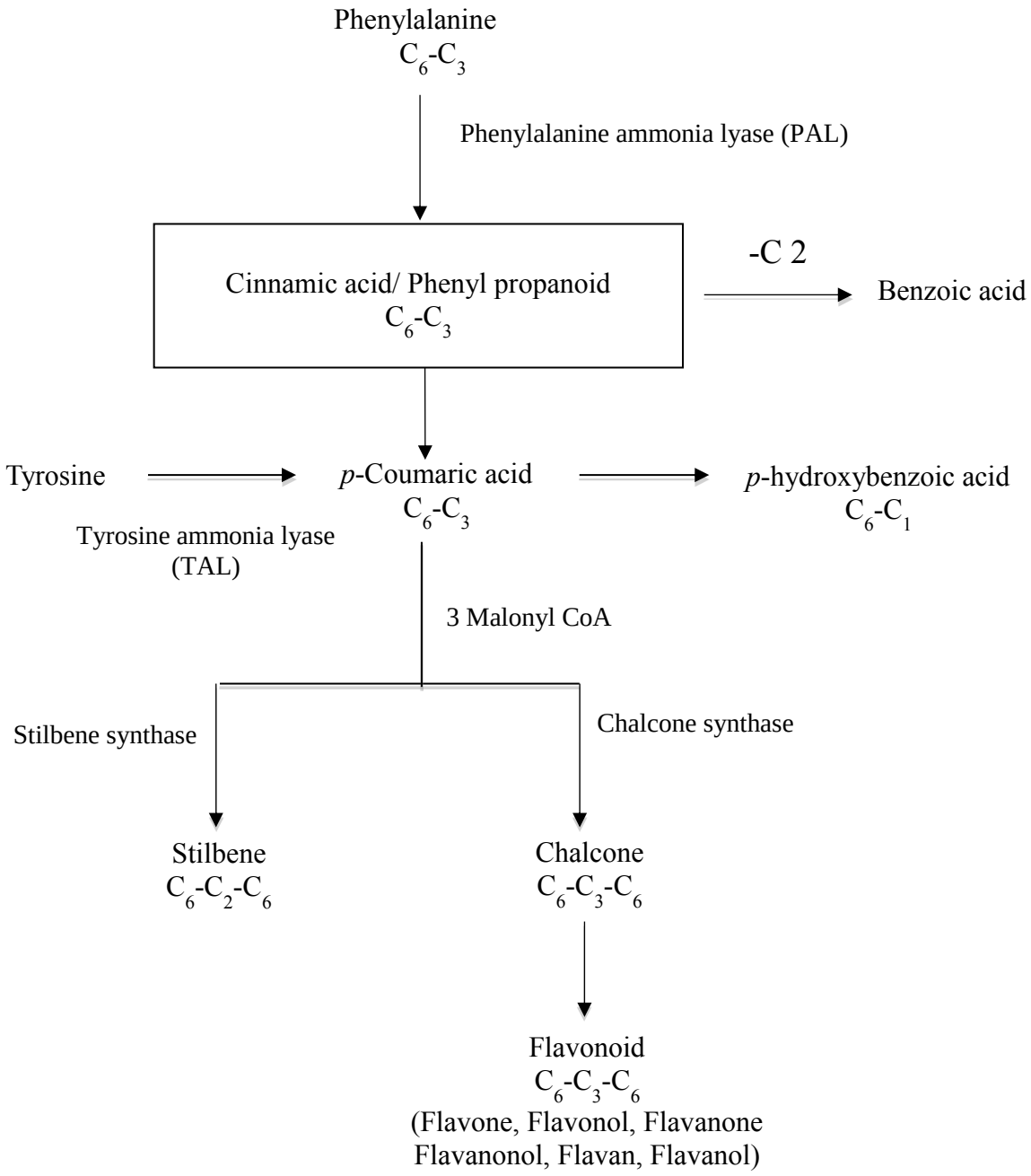


Figure 2.1: Basic pathway for synthesis of phenolic compounds from phenylalanine and tyrosine by the action of phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL), respectively.

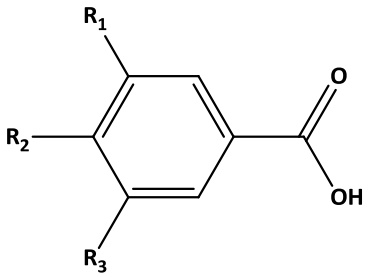
Phenolic acids are the major non-flavonoid phenolics and comprise approximately 30% of total dietary phenolics (Bravo 1998). Other non-flavonoid phenolics such as stilbenes and lignans are less commonly found in the diet. Stilbenes (C_6-C_2-C_6) are present mostly as heartwood constituents of trees, while in smaller amounts are present in the vegetative parts; resveratrol is the most widespread stilbene in nature. Lignans (C_6-C_4-C_6) are often found in seeds and nuts, and are most abundant in flax and sesame seeds.

Hydroxybenzoic acids (Figure 2.2) such as *p*-hydroxybenzoic, vanillic and gallic acids are present in nearly all plants (Shahidi and Naczk 1995; Robbins 2003), but foods from plant sources are generally low in hydroxybenzoic acids (Ssonko and Wenshui 2005). They are mainly found in the bound state in food and are components of complex structures such as hydrolysable tannins and lignin.

Hydroxycinnamic acids (caffeic, ferulic, sinapic, *p*-coumaric and chlorogenic acid) are the most widely occurring phenylpropanoids and are precursors to their cyclic derivatives, the coumarins (Figure 2.3).

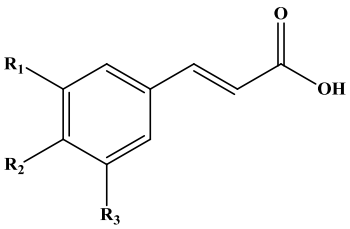
2.1.2. Flavonoids

Among polyphenols, flavonoids are the largest and best studied group that account for 60% of the total dietary phenolic compounds (Harborne and Williams, 2000; Shahidi and Naczk, 2004). Flavonoids are C_{15} compounds with the C_6-C_3-C_6 structure (Harborne and Simmonds, 1964). They are composed of three rings, all of which exhibit various levels



Acid	R_1	R_2	R_3
p-Hydroxybenzoic	H	OH	H
3, 4-Dihydroxybenzoic	H	OH	OH
Vanillic	OCH_3	OH	H
Syringic	OCH_3	OH	OCH_3
Gallic	OH	OH	OH

Figure 2.2: Structures of common benzoic acid derivatives



Acid	R_1	R_2	R_3
p-Coumaric	H	OH	H
Caffeic	H	OH	OH
Ferulic	OCH_3	OH	H
Sinapic	OCH_3	OH	OCH_3

Figure 2.3: Structures of common cinnamic acid derivatives

of hydroxylation and methoxylation (Yao *et al.* 2004). More than 6500 flavonoid structures have been identified and described. The structural variations within the flavonoid subclasses arise from substitution patterns, such as hydroxylation, methoxylation, O-glycosylation, C-glycosylation and covalent addition of prenyl or alkyl groups (Aron and Kennedy 2008). Moreover, the primary substituents (e.g. hydroxyl, methoxyl and glycosyl groups) may themselves be subjected to substitution, such as additional glycosylation or acylation, yielding highly complex structures (Cheynier 2005).

Flavonoids can be divided into seven major groups, namely flavones, flavanones, flavonols, flavanonols, flavanols, isoflavones, and anthocyanidins (Figure 2.4). Flavanones, characterized by a saturated C ring and a 4-keto group, are precursors to all flavonoid structures and are among the most prevalent naturally occurring flavonoids (Fowler and Koffas 2009). Flavonols, containing a 2, 3-double bond and 4-keto and 3-hydroxyl groups in the C ring, and flavanols lacking the double bond and keto group, are the most widespread in plants. Isoflavones, differing from flavones in having their B ring attached at C-3 of the C ring, are present in significant concentrations in soybean and its fermented products. Anthocyanidins with a central C ring are aglycones of anthocyanin pigments in flowers, leaves, fruits and roots of many plants. In addition to the major groups, other flavonoids with slightly varied structures have also been identified. Among these are neoflavonoids, which have a C-4 linkage of the B and C rings, and some minor flavonoids such as chalcones, aurones and auronols (Marais *et al.* 2006). Among the seven major groups of flavonoids, anthocyanins and catechins, known collectively as flavans, because of lack of the carbonyl group in the 3-position and falvan-3-ol and flavan-3,4-diols belong to this category (Shahidi and Naczk, 2004). Flavan-3-ols are found abundantly in green

tea, grapes and blackberries whereas flavanones are exclusively found in citrus fruits in the glycosidic forms (Jaganath and Crozier 2010).

Apart from various vegetables and fruits, flavonoids are found in seeds, nuts, grains, spices and different medicinal plants as well as in beverages, such as wine, tea, and beer (Shahidi and Naczk, 1995). Flavonoids from natural sources often exist as glycosides rather than in the free form, and the glycosyl groups are usually attached to the flavonoid core at the C-3 position of the C ring (Finotti and Di Majo 2003). In general, the leaves, flowers and fruits of plants contain mainly flavonoid glycosides, whereas the woody tissues contain aglycones and the seeds may contain both (Pan and Ho 2008). Some of the most common flavonoid aglycones from dietary sources include aluercetin, a flavonol abundant in onion, apple and tea; catechins, a group of flavanols in tea and several fruits; cyanidin, an anthocyanidin in many red fruits (strawberry, raspberry, black currant, etc.); hesperetin, a flavanone in citrus fruits; and daidzein, the main isoflavone in soybean (Scalbert *et al*. 2005). Distribution of phenolics in plants is not uniform and varies both qualitatively and quantitatively among plant species as well as among tissues.

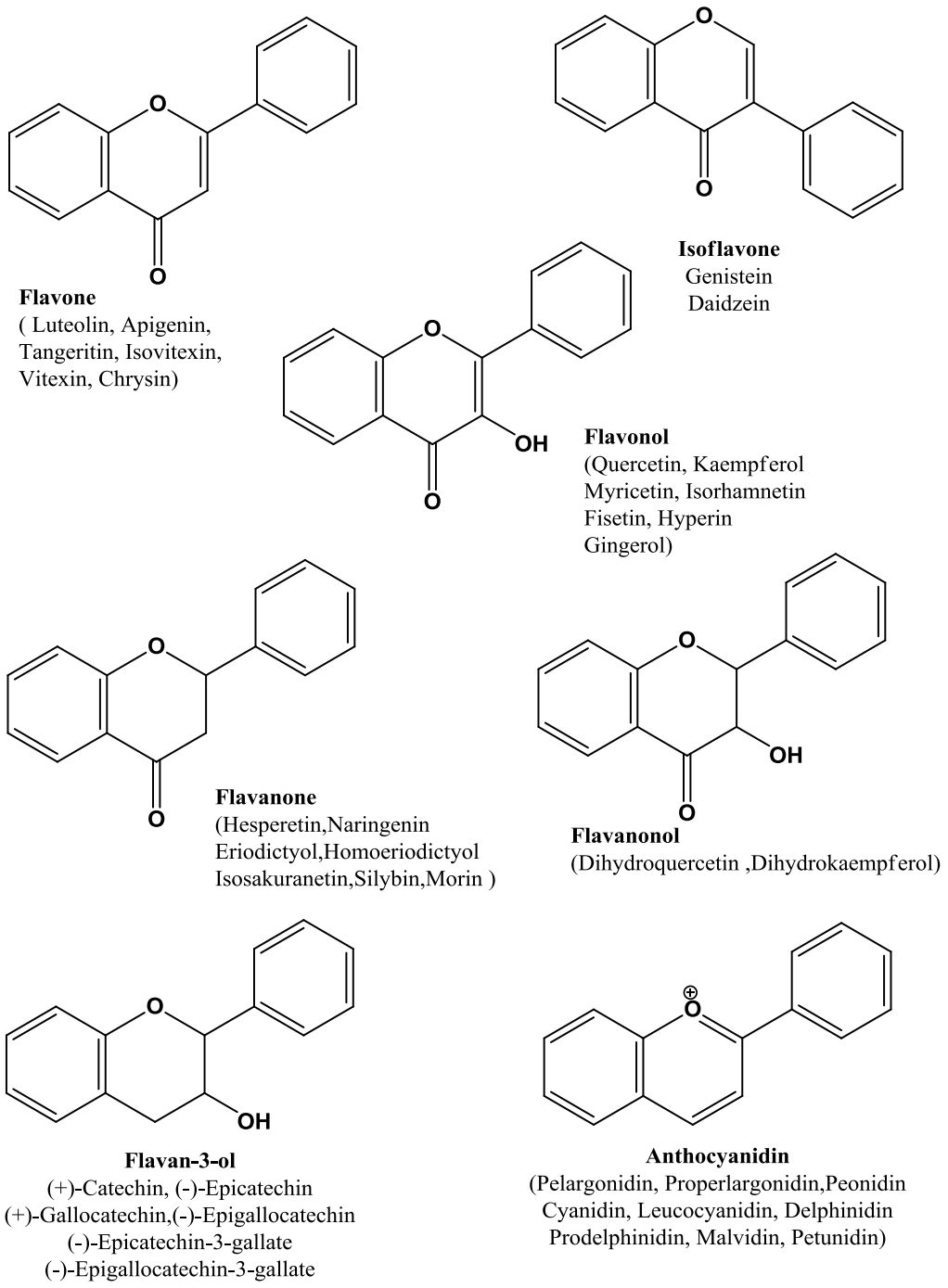


Figure 2.4: Major classes of flavonoids and some individual compounds

Factors affecting phenolic profiles and contents in plants include species and cultivar, cultivation techniques employed, growing and environmental conditions, and maturation stage, among others. Some phenolic compounds may show increased levels under stress conditions such as UV radiation, air pollution, wounding, infection by pathogens and parasites, and exposure to extreme temperatures, among others. For example, the synthesis of stilbenes in grapes and isocoumarins in carrots can be stimulated by various plant injuries (Lafuente *et al*. 1989; Bavaresco *et al*. 1997).

Stilbenes possess a C_6-C_2-C_6 basic carbon skeleton of 1,2-diphenylethylene structure. Resveratrol (3,5,4′-trihydroxystilbene) (Figure 2.5) is a member of the stilbene family produced in some fruits and occur in both free and glycoside forms (Taruscio *et al.* 2006). In plants, the major form of resveratol is *trans*-resveratol-3-*O*-β-D-glucoside, often referrd to as piceid or polydatin (Jenzen *et al*. 2010).

The hydroxystilbenes are of particular interest in grapes and red wine (Dercks and Creasy, 1989; Celotti *et al*. 1996), with lesser amounts found in berries, red cabbage, spinach and certain herbs (Jaganath and Crozier 2010), and are known to render health benefits to the cardiovascular system and to possess cancer chemopreventive activities.

2.2. Bioactivities and bioavailability

The health promoting effects of fruits, vegetables and related products have largely been attributed to the presence of phenolic compounds which are linked to a lower risk of

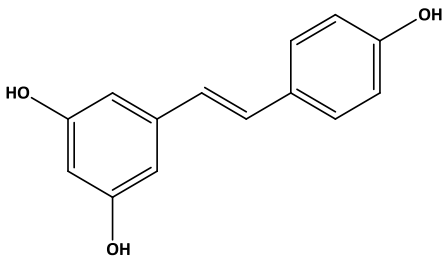


Figure 2.5: Structure of *trans*-resveratrol

many diseases, including inflammation, cardiovascular disease (CVD), cancer, diabetes and neurodegenerative disorders. Evidence from a large body of *in vitro* and *in vivo* studies has shown that phenolic compounds possess antioxidant, anti-inflammatory, antiatherogenic, anticarcinogenic, antidiabetic, anti-allergic, antimicrobial and antiviral activities, among others (Scalbert *et al*. 2005; Aron and Kennedy 2008).

Phenolics are known as powerful antioxidants that inhibit oxidative deterioration of foods and protect against oxidative stress-mediated diseases in the body. They may act as free radical scavengers, singlet oxygen quenchers, metal ion chelators, reducing agents and synergists with other antioxidants, thus inhibiting oxidation of biomolecules and suppressing related pathologies such as inflammation, atherosclerosis and carcinogenesis. Phenolic compounds act as anti-inflammatory agents by down-regulation of cytokines at the expression level and direct inhibition of some pro-inflammatory mediators (Shahidi and Zhong 2009).

A cholesterol lowering effect and inhibition against LDL-cholesterol oxidation contribute to the cardioprotective property of many plant polyphenols. Phenolics have proven to possess anticancer activities. A study by Hertog *et al*. (1994) revealed that flavonoid intake from fruits and vegetables were inversely associated with all-cause cancer risk and cancer of the alimentary and respiratory tracts. Consumption of soybean isoflavones reduced the incidence of breast, urinary tract and colon cancers, and provided protection against coronary heart disease and osteoporosis (Brandi 1997). Genistein, the main isoflavone in soybean with anti-estrogenic

activity, has shown antitumour capabilities supported by many *in vitro*, cellular, animal model and clinical studies (Ravindranath *et al*. 2004).

In addition to their health-promoting ability, some phenolics may exert anti-nutritional properties that are of great importance to both consumers and producers. Phenolics at high intake levels may act as pro-oxidants, mutagens and inhibitors of key enzymes involved in hormone metabolism (Skibola and Smith 1999; Galati *et al*. 2002). Therefore, ingestion of excessively high levels of phenolics may overload the natural barriers or detoxification mechanisms, hence rendering a toxic effect and posing a serious health risk for humans. However, daily intake of phenolics from common food sources produces very low toxicities, if any, because of their low absorption, rapid metabolism and efficient defense mechanism in mammals. The average intake of dietary polyphenols is around 1 g/day, with individual phenolic intakes varying depending on the food pattern. For example, consumption of flavonols has been estimated at 20-25 mg/day in the US (Biesalski 2007).

Due to the low bioavailability of ingested phenolics caused by both poor absorption and rapid elimination in the body, the activities established in *in vitro* experiments may not necessarily reflect their protective effectiveness in living organisms. Cellular uptake differs greatly among phenolics, hence the abundance of a phenolic compound in the diet may not necessarily lead to a high concentration of the compound or its active metabolites in target tissues. Some phenolic compounds may be absorbed in sufficient amounts to render their biological effects and the

presence and concentration of these phenolics and/or their metabolites in blood indicates their potential to cross the intestinal barrier and exert bioactivities *in vivo* (Hooper and Cassidy, 2006).

Absorption of a phenolic compound in the small intestine is influenced by such factors as its molecular size and structure, lipophilicity, solubility and pKa, as well as gastric and intestinal transit time, membrane permeability and pH of the lumen. It is supposed that most phenolics are incorporated into cells by passive transport since no specific transporter has been found in mammalian cells (Nakayama *et al.* 2006). The rate of incorporation is related to the affinity of the phenolic compounds for the cell membrane. Studies have been carried out on the membrane affinities of different phenolics, including gallic and caffeic acid esters, curcuminoids, flavonols, and isoflavones, among others, and it was concluded that the lipophilicity, rigidity and planar structure of phenolic molecules are key in determining their affinity for cell membranes and hence their cellular absorption (Murota *et al*. 2002; Nakayama *et al*. 2006).

Phenolics are conjugated by methylation, sulphation, glucornidation or some combination of the three, resulting in decreased hydrophobicity (Zhong and Shahidi 2011; Ramos 2007). The overall bioavailability of phenolics is the combined effect of cellular uptake, metabolism, distribution and elimination (Manach *et al*. 2005).

2.3. Sources

Phenolics are present abundantly in a wide range of natural food products, especially in fruits, vegetables, legumes, cereals, nuts, herbs and spices, among others. Fruits such as apples, citrus,

berries, grapes and pomegranates are rich sources of phenolics, particularly flavonols (*e.g.* quercetin, kaempferol, myricetin and isorhamenetin), proanthocyanidins (*e.g.* procyanidins and prodelphinidins) and phenolic acids (mainly in the esterified form, *e.g.* gallic, ferulic, sinapic, coumaric, caffeic and chlorogenic acids). Cranberries are an excellent source of anthocyanins (Wang and Stretch 2001; Zuo *et al*. 2002). Phenolics in citrus fruits include mainly cinnamic acid derivatives, coumarins and flavonoids. Stilbenes are the characteristic phenolics present in grape skins, leaves, seeds and stems in monomeric, oligomeric and polymeric forms. Resveratrol is the predominant stilbene located in grape skin (Versari *et al*. 2001).

Vegetables provide a rich source of phenolic compounds. The content and composition of phenolics in various fruits (Shahidi *et al*. 2010), such as peppers, eggplants, tomatoes, bitter melon, and pumpkin, have been reviewed and they were found to contain a wide array of phenolic compounds, including protocatechuic, chlorogenic, coumaric and ferulic acids, and O-glycosides of quercetin and luteolin, among others. Among bulb vegetables, onions are a rich source of flavonoids with quercetin being the most predominant (Galdon *et al*. 2008). Green leafy vegetables such as lettuce, spinach and kale have high levels of flavonoids (Howard *et al*. 2002).

Root (carrots, beets) and tuber (sweet potatoes, potatoes) vegetables contain chlorogenic and caffeic acids as their major phenolic acids, and betalains in beets, in particular, contribute to their colour.

Cereals, legumes, nuts and oilseeds are also recognized as good sources of phenolics. The highest concentrations of phenolic acids and flavonoids are located in the aleurone layer in grains and therefore removal of this layer in milling and pearling processes leads to their loss. In beans, a higher level of phenolics was detected in the hulls (6.7-270 mg catechin equivalents/g extracts) than in whole seeds (4.9-93.6 mg/g extracts) (Madhujith and Shahidi 2005). The major phenolic acids present in bean hulls include vanillic, caffeic, *p*-coumaric, ferulic and sinapic acids. These phenolic acids are also found in wheat bran at higher levels than in its corresponding flour (Liyana-Pathirana and Shahidi 2007).

The predominant phenolic compounds of oilseeds belong to the phenolic acid, coumarin, flavonoid, tannin and lignin groups of compounds. Unlike phenolics in fruits and vegetables that are often in the free or soluble conjugate forms of glucosides, phenolic compounds in grains and oilseeds exist mostly in the insoluble bound form associated with cell wall polyssacharides (Naczk and Shahidi 1989). Naczk and Shahidi (1989) reported that insoluble-bound phenolics contribute some 6-20 % to the total phenolics in canola meal. Wheat and barley also have large proportions of bound phenolics (60 and 75% of the total phenolics, respectively) (Liyanapathirana and Shahidi 2006; Madhujith and Shahidi 2009).

Essential oils of many herbs and spices contain considerable amounts of phenolics, which are directly related to their plant origin (Theissedre and Waterhouse 2000). Essential oils of clove and Spanish red thyme had the highest total phenolic contents. Eugenol, thymol and vanillin were identified as the major phenolic compounds in these essential oils.

Tea leaves and their beverages are rich sources of flavan-3-ols, or catechins, which are responsible for many health benefits associated with tea consumption. The simple catechins in fresh tea leaves undergo enzyme- and/or microorganism-assisted fermentation during tea processing and are converted into more complex polymers. Upon recognition of the multiple bioactivities of phenolics, various plant extracts containing a broad array of phenolic compounds are commercially available and used in the food, cosmetic and pharmaceutical industries. A number of extraction methods have been employed for the preparation of plant phenolic extracts in industry or in the laboratory. These include solvent, solid phase, supercritical fluid and high-pressure liquid extraction, among others (Kartsova and Alekseeva 2008).

2.4. Lipid Oxidation

Lipid oxidation is a major cause of food quality deterioration and generation of off-odours and off-flavours, decreasing shelf-life, altering texture and colour, and decreasing the nutritional value of food (Alamed *et al*. 2009). One of the primary pathways of lipid degradation is that of autoxidation. The process of autoxidation of polyunsaturated lipids in foods involves a free radical chain reaction that is generally initiated by exposure of lipids to light, heat, ionizing radiation, metal ions or metalloprotein catalysts. The enzyme lipoxygenase can also initiate oxidation (Shahidi and Naczk 2004). The classic route of autoxidation includes initiation (production of lipid free radicals), propagation and termination (production of non-radical

products) steps (Shahidi and Wanasundara 1992). A general schematic pathway for autoxidation of polyunsaturated lipids is shown in Figure 2.6.

Allylic hydrogen, especially hydrogen attached to the carbon between two double bonds, is easily removed due to its low bond dissociation energy (Choe and Min 2006). The carbon and hydrogen dissociation energies are the lowest at the bis-allylic methylene position (Wagner, Buettner and Burns 1994). Formation of lipid radicals by hydrogen removal facilitates the double bond adjacent to the carbon radical in linoleic and linolenic acids to shift to a more stable next carbon (Choe and Min 2009). In conjugated diene structures the shifted double bond takes the *trans* form, which is thermodynamically more stable. The lipid radical reacts with triplet oxygen very quickly under normal oxygen pressure and forms lipid peroxyl radical (Zhu and Sevilla 1990). The lipid peroxyl radical abstracts a hydrogen atom from other lipid molecules to form lipid hydroperoxide and another lipid radical. Free radicals then react with each other to form non-radical species and the reaction is terminated (Choe and Min 2009).

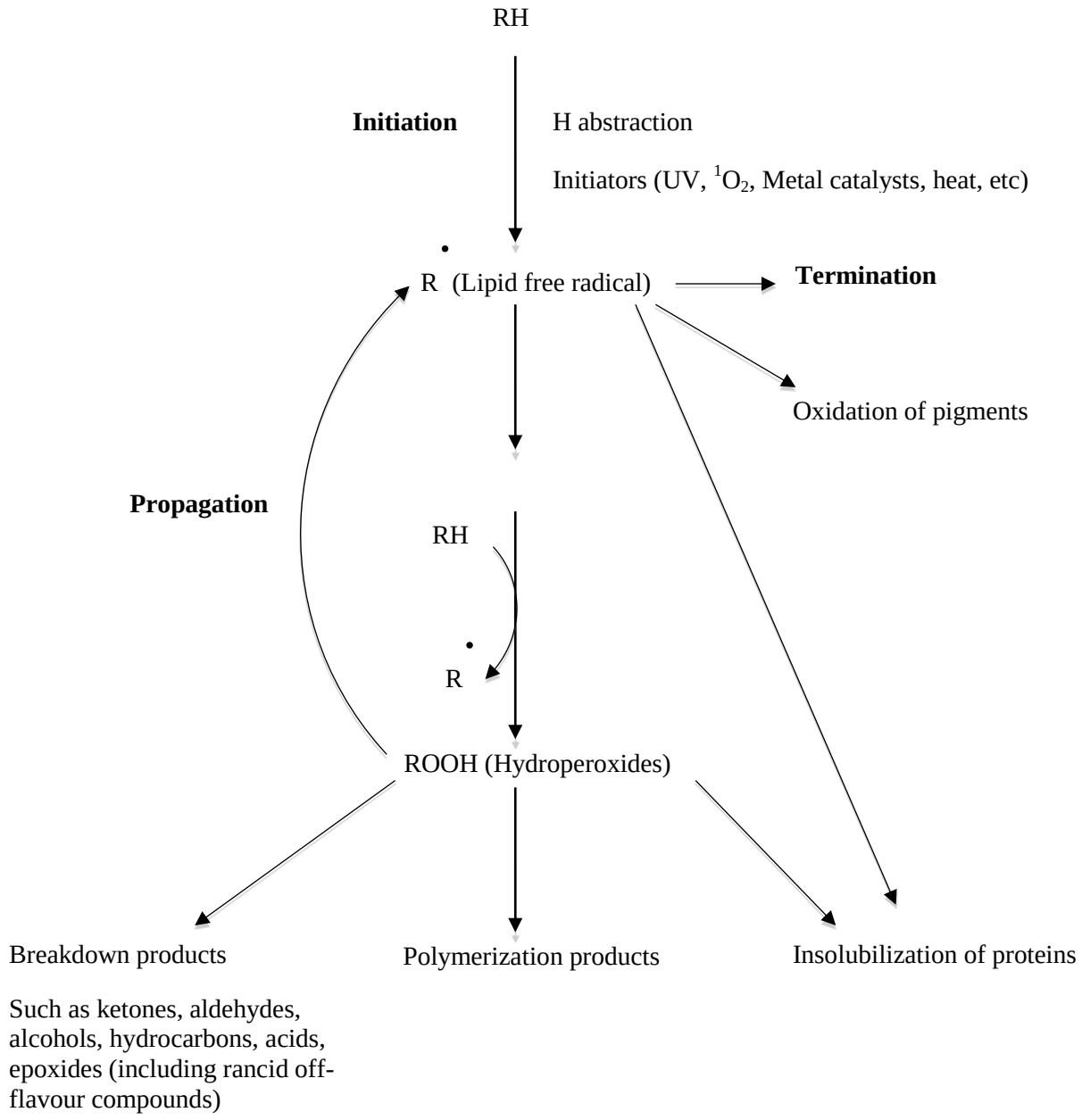
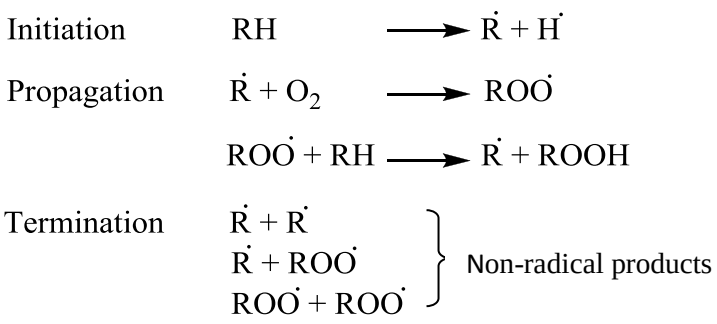


Figure 2.6: General scheme for autoxidation of lipids containing polyunsaturated fatty acids (RH) and their consequences (Adopted from Wanasundara 1992)



Hydroperoxides are the primary products of lipid oxidation, but hydroperoxides, despite their deleterious effects on health, have no effect on flavour quality of foods (Shahidi 1998). However, these unstable molecules decompose readily to form a myriad of products such as aldehydes, ketones, alcohols and hydrocarbons, among others (Shahidi 1998); these impart unpleasant flavors and odours to fats, oils and lipid containing foods. The homolytic cleavage of hydroperoxides (ROOH) between the two oxygen molecules is the most likely hydroperoxide decomposition pathway (Min and Boff 2002). This reaction yields an alkoxyl ($RO^{\bullet}$) and a hydroxyl radical ($^{\bullet}OH$). The alkoxyl radical ($RO^{\bullet}$), which is more energetic than either the alkyl ($L^{\bullet}$) or peroxyl radical ($ROO^{\bullet}$), can enter into a number of different reaction pathways (Figure 2.7). Alkoxyl radicals can attack another unsaturated fatty acid, a pentadiene group within the same fatty acid or the covalent bonds adjacent to the alkoxyl radical. This last reaction is known as the β-scission reaction and is important to food equality as it can cause fatty acids to decompose into low molecular weight, volatile compounds that cause rancidity (Frankel 1985).

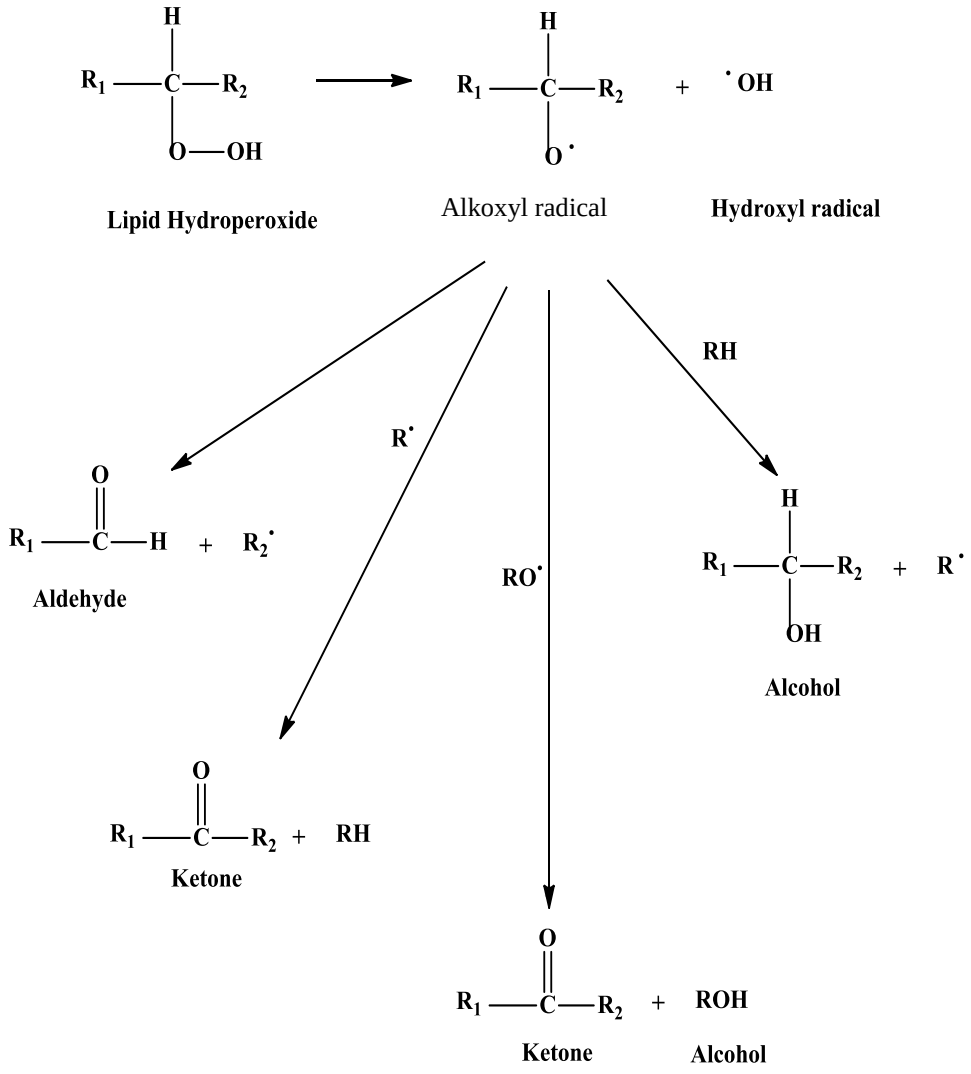


Figure 2.7: Some major secondary products of lipid autoxidation

In the β-scission reaction, the highly energetic alkoxyl radical ($RO^{\bullet}$) is able to abstract a hydrogen from the carbon-carbon bond on either side of the oxygen radical. The decomposition product on the carboxylic acid end of the fatty acid is usually esterified to the glycerol of a triacylglycerol or phospholipid, thus it would not be volatile and therefore would not contribute to rancidity unless it undergoes further decomposition reactions to form low molecular weight compounds. Cleavage of the hydrocarbon chain by alkoxyl radicals on the methyl end of the fatty acid will produce volatile compounds. Upon cleavage of the fatty acid chain, the resulting radicals will interact with a variety of compounds to produce secondary lipid oxidation products such as aldehydes, ketones, alcohols, furans, hydrocarbons and acids.

However, in most foods there are several pro-oxidative systems that produce free radicals and lipid hydroperoxides besides the classic initiation and propagation steps. Pro-oxidants, which are found in virtually all food systems, are compounds that initiate, facilitate or accelerate lipid oxidation. Many prooxidants are not true catalysts because they are altered during the reaction. For example, ferrous ion is converted to ferric ion during interactions with hydroperoxides, and singlet oxygen is converted to a hydroperoxide upon interaction with unsaturated fatty acids. Hydroperoxides are significant substrates for rancidity because their decomposition produces the low-molecular-weight volatile compounds that have off-odour characters.

Prooxidants can accelerate lipid oxidation by directly interacting with unsaturated fatty acids to form lipid hydroperoxides (*e.g*. lipoxygenases or singlet oxygen) or by promoting formation of free radicals (*e.g*. transition metals or ultraviolet light promoted hydroperoxide decomposition).

The decomposition of hydroperoxides produces additional radicals that could be responsible for the exponential increase in oxidation rates that is seen in many foods. Elevated temperatures, light and many prooxidants can promote the decomposition of hydroperoxides. At high temperatures, hydroperoxides rapidly break down after their formation, as is the case with frying oil; therefore, there is often no hydroperoxide accumulation during use and storage. Light causes hydroperoxide decomposition with rates increasing with decreasing wavelength. Transition metals, which are common contaminants in food, are often introduced via the water and ingredients used in food preparations (Taylor 1987). These reactive metals decompose hydrogen peroxide and lipid hydroperoxides into free radicals through the following redox cycling pathway (Reische *et al.* 2002; Berger and Hamilton 1995):

$$Mn^{n+} + ROOH \rightarrow Mn^{(n+1)+} + RO^{\bullet} + {}^{-}OH$$

$$Mn^{n+} + HOOH \rightarrow Mn^{(n+1)+} + HO^{\bullet} + {}^{-}OH$$

$$Mn^{(n+1)+} + ROOH \rightarrow Mn^{n+} + ROO^{\bullet} + H^{+}$$

Where Mn^{n+} and $Mn^{(n+1)+}$ are transition metals in their reduced and oxidized states, respectively. Hydroxyl radical ($HO^{\bullet}$) is produced from hydrogen peroxide (HOOH), whereas alkoxyl radicals ($RO^{\bullet}$) are produced from lipid hydroperoxides (ROOH). The oxidized state of the metal ion can be regenerated by lipid hydroperoxides (ROOH) in a slow consecutive reaction. The concentration, type and chemical state of the metal the rate of hydroperoxide decomposition.

Copper and iron are common transition metals in foods (Berger and Hamilton 1995); iron is normally found at greater concentration than copper. The type of hydroperoxide species is also important, with the ferrous ion capable of decomposing lipid hydroperoxides about 10 times faster than is hydrogen peroxide (Girotti 1998).

2.4.1. Prevention of Oxidation

Numerous methods have been developed to control the rate and extent of lipid oxidation in foods, but addition of antioxidants is most effective. Antioxidants have become an indispensible group of food additives, mainly because of their unique properties of extending the shelf life of food products without any damage to their sensory or nutritional quality. Historically, gum guaiac was the first antioxidant approved for stabilization of animal fats, especially lard in the 1930s (Nanditha and Prabhasankar, 2009). Halliwell *et al*. (1995) reported that antioxidants are also of interest to biologists and clinicians because they may help to protect the human body against damage by reactive oxygen species (ROS). According to the United States Department of Agriculture (USDA) Code of Federal Regulations, "antioxidants are substances used to preserve food by retarding deterioration, rancidity or discoloration due to oxidation" (Shahidi and Wanasundara, 1992). Antioxidants for use in food systems must be inexpensive, non-toxic and effective at low concentrations, have high stability and capability of surviving processing, no odour, taste or colour of their own, easy to incorporate and have a good solubility in the product (Kiokias, Varzakas and Oreopoulou 2008).

Antioxidants may exist naturally in foods, such as tocopherols, ascorbic acid, and some phenolic compounds, or maybe synthetic compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and *tert*-butylhydroquinone (TBHQ) (Shahidi and Zhong 2007). These synthetic phenolic antioxidants are deliberately added to products in order to prevent or delay lipid oxidation during processing and storage of fats, oils and lipid-containing foods and have been used by the food industry for over 50 years (Saad *et al*. 2007).

Antioxidants act at different levels in the oxidative sequence involving lipid molecules. They may decrease oxygen concentration, intercept singlet oxygen ($^1O_2$), prevent first-chain initiation by scavenging initial radicals such as hydroxyl radicals, bind metal ion catalysts, decompose primary products of oxidation to non-radical species or break the chain reaction in order to prevent continued hydrogen abstraction from substrates (Shahidi 2000, 2002; Shahidi and Naczk 2004). However, many factors can impact the activity of antioxidants, with some antioxidants retarding lipid oxidation under certain conditions but promoting lipid oxidation under other conditions (Huang *et al*. 1994). Antioxidants can be classified according to their mechanism of action as either primary or secondary (Reische *et al*. 2002). However, some substances have more than one mechanism of antioxidant activity and are referred to as multi-functional antioxidants.

2.5. Phenolics as antioxidants

Naturally-occurring antioxidative compounds are flavonoids, phenolic acids, lignans, tocopherols, phospholipids and polyfunctional organic acids, among others. There have been numerous studies on the biological activities of phenolics, which are potent antioxidants and

free radical scavengers (Naczk and Shahidi 2004; 2006; Tung, Wu, Kuo and Chang, 2007, Zhong and Shahidi 2011).

Phenolic compounds in foods originate from one of the main classes of secondary metabolites in plants (Naczk and Shahidi 2004). At low concentration, phenolics act as antioxidants and protect food from oxidative rancidity (Karakaya 2004). Phenolic antioxidants interfere with the oxidation process as free radical terminators and sometimes also as metal chelators. Phenolic compounds are classified as primary antioxidants which are mainly free radical scavengers (FRS) that delay or inhibit the initiation step or interrupt the propagation step of lipid oxidation, thus decreasing the formation of volatile decomposition products (*e.g.* aldehydes and ketones) that cause rancidity (Shahidi, Wanasundara and Amarowicz 1994; Naczk and Shahid 2004; Kiokias, Varzakas and Oreopoulou 2008; Alamed, Chaiyasit, McClements, and Decker 2009; Nanditha and Prabhasankar 2009). Secondary antioxidants are those that chelate pro-oxidant metal ions.

2.5.2. Mechanism of action of phenolic antioxidants

The antioxidant potential of phenolic compounds depends on the number and arrangement of the hydroxyl groups in the molecules of interest (Cao, Sofic and Prior 1997; Sang *et al*. 2002). Phenolic antioxidants (AH) can donate hydrogen atoms to lipid radicals and produce lipid derivatives and antioxidant radicals (Reaction I), which are more stable and less readily available to promote autoxidation (Kiokias, Varzakas and Oreopoulou 2008). The antioxidant free radicals may further interfere with the chain-propagation reactions (Reactions II and III).

$$R^{\bullet} / RO^{\bullet} / ROO^{\bullet} + AH \longrightarrow A^{\bullet} + RH / ROH / ROOH \quad (I)$$

$$RO^{\bullet} / ROO^{\bullet} + A^{\bullet} \longrightarrow ROA / ROOA \quad (II)$$

$$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet} \quad (III)$$

As the bond energy of hydrogen in a free radical scavenger decreases, the transfer of hydrogen to the free radical is more energetically favorable and thus more rapid (McClements and Decker 2007). Any compound that has a reduction potential lower than the reduction potential of a free radical (or oxidized species) is capable of donating its hydrogen atom to that of the free radical unless the reaction is kinetically unfeasible. The phenoxyl radical is stabilized by delocalization of its unpaired electron around the aromatic ring (Figure 2.8), which participates in the termination reaction.

Gorden (1990) reported that substitution at the *para* position with an ethyl or *n*-butyl group rather than a methyl group improves the activity of the antioxidant; however, the presence of chain or branched alkyl groups in this position decreases the antioxidant activity. The stability of the phenoxyl radical is further increased by bulky groups in the 2 and 6 positions as in 2,6-di-*t*-butyl-4-methylphenol (butylted hydroxytoluene; BHT), since these substituents increase the steric hinderance in the region of the radical and thereby further reduce the rate of propagation reactions involving the antioxidant radical (Reactions IV, V, VI).

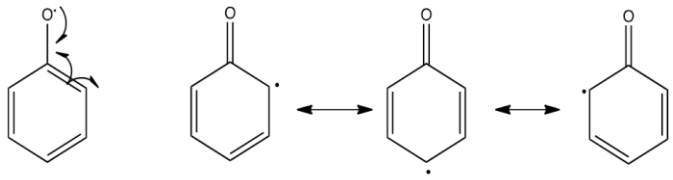


Figure 2.8: Resonance stabilization of phenoxyl radical

$$\dot{A} + O_2 \longrightarrow AOO^{\cdot} \quad \text{(IV)}$$

$$AOO^{\cdot} + RH \longrightarrow R^{\cdot} + AOOH \quad \text{(V)}$$

$$\dot{A} + RH \longrightarrow AH + R^{\cdot} \quad \text{(VI)}$$

The effect of antioxidant concentration on autoxidation rates depends on many factors, including the structure of the antioxidant, oxidation conditions and the nature of the sample being oxidized (Shahidi and Naczk 2004). Often phenolic antioxidants lose their activity at high concentrations and behave as pro-oxidants (Gorden 1990) by involvement in initiation reactions (Reactions VII, VIII).

$$AH + O_2 \longrightarrow \dot{A} + HOO^{\cdot} \quad \text{(VII)}$$

$$AH + ROOH \longrightarrow RO^{\cdot} + H_2O + \dot{A} \quad \text{(VIII)}$$

Phenolic antioxidants are more effective in extending the induction period when added to an oil that has not deteriorated to any great extent. However, they are ineffective in retarding decomposition of already deteriorated lipids. Thus, antioxidants should be added to foodstuffs as early as possible during processing and storage in order to achieve maximum protection against oxidation (Shahidi and Wanasundara 1992). Some phenolic compounds may act as chelators or secondary antioxidants that can bind and thus inactivate or reduce the activity of prooxidant metals (Figure 2.9).

2.6. Tea and tea polyphenols

Tea, a product made from leaves and buds of the plant *Camellia sinensis*, is the second most popular beverage worldwide after water and a major source of dietary flavonoids (Rietveld and Wiseman 2003). Consumed as a daily routine drink, it also confers great health beneficial effects to humans, and therefore is also considered functional beverage and therapeutic aid in many diseases. Tea consumption has been linked to reduced risk of numerous chronic diseases. A large body of in vitro and animal model studies has reported that tea or tea extracts may prevent cancer development, reduce cholesterol levels, decrease the risk of stroke and heart attack, protect against neurodegenerative diseases, and improve dental health, among others (Pham-Huy et al. 2008). Although mixed results from epidemiological and clinical studies have been reported on tea consumption and human health, they were thought to arise, at least in part, from socioeconomic and lifestyle factors which have been ignored, as well as methodology flaws, and at present, tea consumption is recommended (Cabrera et al. 2006).

There are generally three main types of tea in the market depending on the manufacturing process: non-fermented green tea (polyphenol oxidase inactivated by drying and steaming), semi-fermented oolong tea (fresh leaves subjected to a partial fermentation), and fermented black or red teas (leaves undergoing a post-harvest fermentation involving polyphenol oxidase catalyzed oxidation or microorganism assisted fermentation) (Wilson1999; McKay and Blumberg 2002). Of the 2.5 million tonnes of dried tea products manufactured every year, approximately 76-78% is black tea (mainly in Europe, North America and North Africa), 20-22% green tea (mainly in China, Japan and Korea), and 2% oolong tea (popular in China and Taiwan) (Mitscher and Dolby 1998; Wu and Wei 2002).

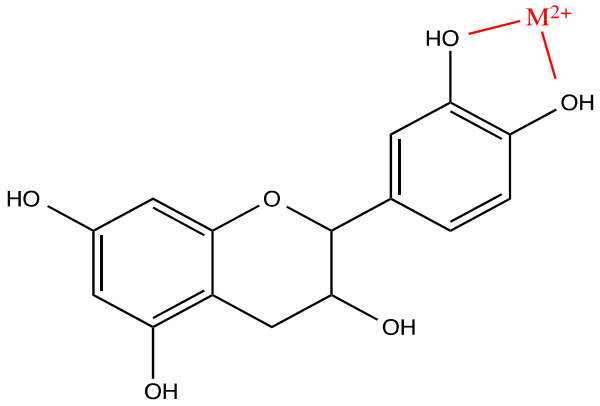


Figure 2.9: Chelation of metal ions by certain phenolic compounds

In addition to the three main tea types, white tea produced from very young leaves and buds of the tea plant by a non-fermentation processing method, has recently been introduced to the market as the most precious Chinese tea.

Although tea consumption has a history of more than 2000 years, scientific research on its chemical composition has only been underway for about three decades. Non-fermented green tea has been reported to contain by dry weight 15-20% protein, 1-4% amino acids, 5-7% carbohydrate, 26% fibre, 7% lipid, 30% phenolic compounds, and other minor components including xanthic bases (*e.g.* caffeine and theophylline), pigments (*e.g.* chlorophyll and carotenoids) and trace elements (*e.g*. fluoride, calcium, magnesium, chromium, zinc, copper, aluminum, selenium, etc.) (Cabrera *et al.* 2006). More than 300 components have been found in the essential oil of green tea, which include aldehydes, alcohols, esters and hydrocarbons (Cabrera *et al.* 2006; Pham-Huy *et al.* 2008). Green tea also contains vitamins B, C and E, which are destroyed in black tea during the fermentation process. The major components with functional properties are xanthic bases which act upon the central nervous system, essential oils facilitating digestion, and polyphenols possessing antioxidant and other bioactivities (Cabrera *et al*. 2006).

Polyphenols, particularly flavonoids, are important constituents of tea. Among all polyphenols in tea, catechins (flavan-3-ols) have been considered the major active compounds responsible for the functional properties of tea. Catechins, namely (-)-epigallocatechin gallate (EGCG, 59% of the total catechins), (-)- epigallocatechin (EGC, 19%), (-)-epicatechin gallate (ECG, 13.6%) and (-)-epicatechin (EC, 6.4%), are the predominant flavonoids present in green tea (McKay and Blumberg 2002).

Catechins contribute to the characteristic bitter and astringent taste of tea. The catechin content of tea varies with climate, season, geographical location, horticultural practices, and variety and age of the plant, as well as processing and the type of products (*e.g*. blended, decaffeinated, instant, etc.). Black tea has a lesser amount of simple catechins than green tea, as these are partially converted *in situ* by polyphenol oxidase to polymerized quinones such as theaflavins and thearubigins. There has been strong evidence showing that green tea polyphenols (GTP) may play a protective role in the risk and pathogenesis of several chronic diseases, especially cardiovascular disease and cancer. GTP have been reported to have cholesterol lowering, insulin activity enhancing immunomodulatory, antioxidant, anti-atherogenic, anticarcinogenic, antimicrobial and antiviral effects, among others (Cabrera *et al*. 2006). GTP are powerful antioxidants *in vitro* and *in vivo* with multifunctional mechanisms. Also, they are able to enhance the actions of many antioxidant enzymes such as glutathione reductase, glutathione-S-transferase and superoxide dismutase in the liver, thus alleviating oxidative stress and accelerating neutralization and elimination of toxic compounds (Sang *et al*. 2005). GTP also exhibit a cardioprotective effect by reducing blood pressure and cholesterol levels, and inhibiting LDL-cholesterol oxidation and atherosclerosis (Arts 2008). Consistent findings have revealed the anti-cancer effect of GTP, which is attributed to their antimutagenic, anticarcinogenic and anti-angiogenic activities (Pham-Huy *et al*. 2008). However, the exact site of action and mechanisms of cancer preventive activity of GTP are not yet clearly understood. **It** is believed that their effectiveness in inducing cell cycle arrest and apoptosis is most significant (Yang *et al.* 2006). Protection against neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease by GTP has also been documented (Weinreb *et al*. 2004).

2.7. Epigallocatechin gallate (EGCG) as an antioxidant

EGCG is the most predominant catechin in tea, and the major component responsible for health effects related to tea consumption. A cup of green tea (2.5 g of leaves in 200 mL of water) may contain 90 mg of EGCG (Wu and Wei 2002). Its level in black tea (1.58%) is lower than in green tea (7.36%) due to the formation of polymerized catechins (Cabrera *et al.* 2006). EGCG displays a wide variety of functional and biological properties, and has attracted much attention as a potential functional food ingredient and pharmaceutical agent. Like many other polyphenols, catechins in tea exhibit antioxidant activity. The antioxidant potential of catechins is dictated by their chemical structures and different mechanisms may apply depending on the reaction environment. EGCG has been shown to possess the highest antioxidant potency among all catechins (Guo *et al*. 1996). The antioxidant activity of EGCG is thought to arise from its structural features, including the trihydroxy (3',4',5'-OH) substitution on the B-ring which allows electron delocalization and confers high reactivity to quench free radicals, the gallate moiety which accounts for increased redox potential and phospholipids/water partition coefficient, and the 5,7- dihydroxylated A-ring which is thought to promote the antioxidant function of EGCG (Caturla *et al.* 2003; Wan *et al.* 2009).

EGCG has been reported to be 25-100 times more potent than the typical antioxidants alpha-tocopherol and ascorbic acid (Mitscher and Dolby 1998). *In vitro* chemical assays have shown the effectiveness of EGCG in scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, reducing ferric ion to ferrous ion and chelating metal ions (Zhu *et al.* 2001; Sun and Ho 2001; Xu *et al.* 2004; Chan *et al.* 2007). In food model systems, EGCG inhibited the bleaching of beta-carotene/linoleate (Amarowicz and Shahidi 1995) and photosensitized oxidation of

conjugated linoleic acid in an oil-in-water emulsion system (Liu and Yang 2008). EGCG was able to protect highly unsaturated marine oils from oxidation to an extent similar to or better than that of BHA, BHT and TBHQ (Wanasundra and Shahidi 1996). EGCG also played a protective role in controlling oxidation of meat lipids. Formation of thiobarbituric acid reactive substances (TBARS) in pork (Shahidi and Alexander 1998) and headspace propanal in fish meat (He and Shahidi 1997) model systems was inhibited by EGCG to a greater extent than that by α-tocopherol. The antioxidant activity of EGCG varies in different model systems, and it is believed that the activity depends largely on the substrate system under investigation (Wanasundara and Shahidi 1996; He and Shahidi 1997).

EGCG exhibits antioxidant efficiency in biological systems. Many researchers have demonstrated that EGCG suppresses oxidation in biological systems and subcellular fractions such as microsome, low-density lipoprotein (LDL) and DNA by scavenging free radicals, including hydroxyl, peroxyl and superoxide anion radicals as well as nitric oxide in living organisms (Kondo *et al.* 1999). EGCG serves as an effective antioxidant in human blood plasma, retarding lipid oxidation and depletion of endogenous lipid solubleantioxidants such as α-tocopherol and beta-carotene (Lotito and Fraga 2000). EGCG can also protect lipids against oxidation in the liver, serum and brain (Skrzydlewska *et al*. 2005).

In addition, EGCG may act indirectly as an antioxidant through its effects on the content and activity of certain enzymes. Rats orally receiving green tea extract containing EGCG showed higher levels of antioxidant enzymes such as glutathione peroxidase and reductase, superoxide dismutase and catalase in the brain (Skrzydlewska *et al.* 2005). Moreover, EGCG inhibits redox enzymes (monooxygenase, cyclooxygenase, lipoxygenase, xanthine oxidase, NADH

oxidase) and therefore suppresses the production of reactive oxygen species (ROS) *in vivo* (Halliwell and Gutteridge 1989; Korkina and Afanasev 1997). EGCG is also capable of protecting erythrocyte membrane-bound ATPases against oxidative stress (Saffari and Sadrzadeh 2004).

2.7.1. Bioavailability and structural modification of EGCG

Bioavailability after oral administration, *i.e.* the rate of absorption through the gastrointestinal tract, metabolism and elimination from the body, has always been an issue for EGCG and other flavonoids. Low oral bioavailability has been observed for tea catechins, possibly due to their bi-directional movement through intestinal epithelial cells (Vaidyanathan and Walle 2003). Moreover, the extensive phase II metabolism during the 1st pass metabolism could lead to serious pre-systemic elimination after oral administration and may contribute to their overall low bioavailability (Zhong et al. 2004). EGCG and other tea catechins, after intestinal absorption, are metabolized and converted to conjugated forms (glucuronides and sulfates), predominantly in the liver, and some are methylated by catechol-O-methyltransferase (Riemersma *et al.* 2001). The conjugation in the intestinal epithelial cells and hepatocytes enhances the hydrophobicity of catechins and hence facilitates their elimination through urine and bile. However, a large portion of EGCG is present in the free form in human plasma and has a longer half-life (3.9 h) in plasma after ingestion compared to EGC (1.7 h), which is mostly in the conjugated form (Van Amelsvoort *et al.* 2001). Breakdown of EGCG by acid hydrolysis occurs mainly in the stomach and by microflora in the gut (Singh *et al.* 2008). EGCG is distributed in body tissues in different storage patterns; for instance, EGCG levels

in rats given green tea polyphenols are higher in the esophagus and large intestine than in other organs, possibly due to poor systematic absorption of EGCG (Cabrera *et al.* 2006). Studies in rats indicated that EGCG is excreted mainly through the bile, whereas EGC and EC are excreted through urine and bile (Cabrera *et al.* 2006). In order to explore the potential of EGCG as a health-promoting agent, more pharmacokinetic details regarding its bioavailability are required.

Lipophilicity is an important factor affecting the efficacy of phenolic compounds as additives in various food systems, and plays a key role in their bioavailability and hence their health effects in living organisms. EGCG has a four-ring structure with eight hydroxyl groups, and is therefore highly hydrophilic. Like many other natural antioxidants, EGCG is more soluble in aqueous media and exerts its bioactivities or health effects mainly in aqueous environments or water compartments in body tissues. The hydrophilic nature of EGCG may pose a restriction on its effectiveness in protecting lipophilic systems, such as fats and oils, lipid-based foods or cosmetic formulas and emulsions, as well as in biological environments. Moreover, poor lipophilicity is associated with the limited absorption of EGCG into the cells through lipid membranes and fast elimination from the body, and therefore compromised bioactivities under physiological conditions. Modification of the molecular structure of EGCG provides a potential means to alter its physico-chemical properties and improve its effectiveness as a bioactive agent in more diverse systems. Many naturally occurring phenolics have been structurally modified for improved lipophilicity and hence expanded applications in more hydrophobic environments (Lue *et al.* 2005; Sabally *et al.* 2005; Lee *et al.* 2006; Giraldo *et al.* 2007). The water-soluble phenolic compounds are converted by chemical or enzymatic

means to their corresponding alkyl esters or ethers. The esters or ethers so prepared with enhanced lipophilicity may show improved liposome incorporation, which offers an advantage to these derivatives in drug delivery and bioavailability in the body over their parent phenolic compounds.

2.7.2. Antiglycation activity of lipophilized epigallocatechin gallate (EGCG) derivatives

Advanced glycation endproducts (AGEs) are produced in the advanced stage of the non-enzymatic reaction between reducing sugars and amino groups. The formation of AGEs begins when the carbonyl functional groups on the open chains of reducing sugars undergo a nucleophilic addition reaction with the amino groups of proteins to form a Schiff base (Ahmed 2005). This group of complex crosslinking compounds can be divided into either fluorescent AGEs such as pentosidine, or non-fluorescent AGEs such as carboxymethyllysine (CML). In addition to a dietary source in which they are generated during food processing and storage, AGEs would also form and accumulate *in vivo* via sugar-protein interaction and cause pathogenic consequences, diabetic complications for example. Therefore, studies on discovering and characterizing effective AGEs inhibitors are valuable in exploring therapeutic approaches to AGEs-associated diseases.

Synthetic AGEs inhibitors, such as the carbonyl scavenger aminoguanidine (AG), suffer from the safety concern of severe side effects and a lack of clinical trial efficacy (Thornalley 2003). Inhibitors from natural sources, therefore, are generating greater research interest given their relative safety and comparable or higher efficacy. The inhibitory mechanisms described so far

include blocking sugar attachment, scavenging reactive carbonyls and radicals, breaking cross-links and so on (Peng *et al*. 2008). The group of phenolic compounds, for example, has been demonstrated in a wide collection of *in vitro* and *in vivo* studies to possess significant inhibitory activity against glycation. Not only can polyphenols inhibit the proceeding of the advanced oxidative glycation stage via their free radical scavenging capacity, they have been demonstrated to directly trap reactive carbonyl species by forming adducts. The expression of the biological functions of polyphenols depends on both the amount consumed and bioavailability, the latter being a limiting factor of applying phenolic AGEs inhibitors as therapeutic agents. Depending on the molecular weight and structural differences, isoflavones and phenolic acids are well absorbed, whereas proanthocyanidins and catechins are poorly absorbed (Han *et al*. 2007).

AGEs are a consequence of high blood glucose in diabetes, and subsequently a cause of other degenerative diabetic conditions. Control of blood glucose is the most important treatment method in diabetes, but is rarely fully achieved, so the use of antiglycation agents to prevent the formation of AGEs may be helpful in preventing damage, and progression of the disease symptoms.

2.7.3. Lipophilized epigallocatechin gallate (EGCG) derivatives and their antioxidant potential in food model systems and biological systems

Antioxidants are also used in health-related areas due to their ability to protect the body against oxidative stress and its consequences. Substances with antioxidant potential are available from a variety of natural sources or as synthetic chemicals.

In vitro assays have shown the antioxidant effectiveness of EGCG in radical scavenging, reduction and metal chelation (Sun and Ho 2001; Xu *et al.* 2004; Chan *et al.* 2007; Cabrera *et al.* 2006; Zhong *et al*. 2012). In food model systems, EGCG has been shown to protect highly unsaturated marine oils as well as fish and meat lipids against oxidation (Wanasundara and Shahidi 1996; He and Shahidi 1997; Shahidi and Alexander 1998; Zhong *et al*. 2012). EGCG also inhibited the bleaching of β-carotene/linoleate (Amarowicz and Shahidi 1995) and photosensitized oxidation of conjugated linoleic acid in an oil-in-water emulsion system (Liu and Yang 2008). EGCG shows varied antioxidant activity in different model systems, and it is well documented that its activity depends largely on the substrate system under investigation (Wanasundara and Shahidi 1996; He and Shahidi 1997; Zhong *et al.* 2012). EGCG, like many other phenolic antioxidants in nature, is soluble in water, while sparingly soluble in more lipophilic media, which poses a restriction to its antioxidant effectiveness in lipophilic systems, such as fats, oils, lipid-based foods or cosmetic formulas and emulsions, as well as in biological environments.

Antioxidant strategies also have been employed in preventing/treating oxidative stress-associated diseases, including cardiovascular disease, cancers, hypertension, diabetes, inflammation and other autoimmune disorders. Inhibitory effects of EGCG against LDL-cholesterol oxidation, DNA scission and liposome oxidation have been reported by various *in vitro* and *in vivo* studies (Huang and Frankel 1997; Ishikawa *et al.* 1997; Hu and Kitts 2001, Zhong *et al.* 2012). However, its antioxidant effectiveness *in vitro* may not necessarily correlate

with that *in vivo*, due to its low bioavailability. EGCG is hydrophilic with poor solubility in lipid, which, at least partially, accounts for its low cellular absorption *in vivo*. It is advantageous to increase the lipophilicity of EGCG for improved cellular uptake and affinity to lipid particles and membrane and other oxidation-susceptible sites.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

Epigallocatechin gallate (EGCG) was supplied by GlaxoSmithK.line Consumer Healthcare (Parsippany, NJ, USA). Acetyl chloride, C2:0; propanoyl chloride, C3:0; hexanoyl chloride, C6:0; octanoyl chloride, C8:0; dodecanoyl chloride, C12:0; and octadecanoyl chloride, C18:0 were purchased from Sigma Aldrich (Oakville, ON). Docosahexaenoic acid (DHA) was prepared from DHA single cell oil (DHASCO) procured from Martek Bioscience Corporation (Columbia, MD). Other chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). All solvents used were purchased from Fisher Scientific Ltd. (Ottawa, ON). The solvents employed were HPLC- or reagent-grade.

3.2. Preparation and purification of EGCG esters

EGCG was acylated with a variety of acyl chlorides as described in Zhong and Shahidi (2011). DHA was prepared by saponification followed by a urea complexation process as described by Wansundara and Shahidi (1999). The saponification was catalyzed by KOH and was carried out in 95% ethanol at 60±3ºC under reflux. Hexane was used to remove the unsaponifiable matter and the pH of the aqueous phase was adjusted to 1 by using 6M HCl. Free fatty acids were extracted into hexane and the solvent was removed using a rotary evaporator. For urea

complexation, the free fatty acids were mixed with a urea solution (20% in 95% ethanol) and allowed to stand for 24 h at 4°C for urea-fatty acid adduct crystallization. The mixture was then filtered to obtain the non-urea complex fraction, the pH of which was subsequently adjusted to 4-5 with HCl. DHA was extracted into hexane and the solvent was removed by evaporation.

Esterification of EGCG was carried out with acyl chlorides at a mole ratio of 1:1 with the exception of EGCG C2:0 and C3:0, which were carried out at 1:2 ratios. Acyl chloride was added dropwise to EGCG dissolved in ethyl acetate. The reaction was carried out in the presence of pyridine, which removed the released HCl from the medium. The mixture was then heated in an oil bath at 50°C under a nitrogen blanket with constant stirring. Upon completion of the esterification, the reaction mixture was cooled to ambient temperature and filtered. The filtrate was then washed three times with distilled deionized water (60°C), and the ethyl acetate layer was collected and passed through a cone of anhydrous sodium sulphate. A dry powder of crude products containing a mixture of EGCG esters (at different degrees of substitution) was obtained by evaporating the solvent.

3.3. Purification and identification of EGCG derivatives

The crude products of EGCG esters were purified by flash column chromatography. EGCG esters were eluted on a silica column with a gradient of hexane/ethyl acetate/formic acid

(90:10:2-40:60:2, v/v/v). Fractions corresponding to each band were collected and solvents removed using a rotary evaporator.

The compositions of the reaction mixture and purified compounds were determined by reversed phase HPLC-MS, using an Agilent 1100 HPLC unit (Agilent Technologies, Palo Alto, CA, USA) with a UV-diode array detector (UV-DAD). Separation was achieved on a C-18 column (Agilent) by gradient elution with methanol/water mobile phase (80:20-100:0, v/v from 0 to 30 min) and fractions were detected at 280 nm. Liquid chromatograph (LC) flow was further analyzed on-line by the MS detector system (LC-MSD-Trap-SL, Agilent) with APCI (atmospheric pressure chemical ionization) in positive mode for identification of each fraction.

The ^{1}H and ^{13}C NMR analyses were carried out for purified EGCG esters in order to identify their molecular structures, *i.e.* the location of fatty acid incorporation into the EGCG molecule. The ^{1}H and ^{13}C spectra were recorded on a Bruker Avance 500 MHz NMR spectrometer (Bruker Biospin Co. Billerica, MA) operating at 500.13 and 125.77 MHz, respectively. The samples were dissolved in dimethyl sulphoxide (DMSO)-d6 containing TMS as internal standard. Signal processing and interpretation were performed with the softwares Topspin 1.3 (Bruker Biospin Co.) and MestRe Nova (Mestrelab Research SL, Santiago De Compostela, Spain) and structure elucidation was accomplished by comparing the chemical shifts of EGCG derivatives with that of the EGCG parent molecule (Zhong *et al*. 2011).

3.4. Determination of lipophilicity

The lipophilicity of EGCG and its derivatives was determined as the octanol-water partition coefficient (*P*) by a shake flask method. A flask containing a mixture of octanol (100 mL) and deionized water (100 mL) was shaken in a water bath at room temperature (22°C) for 24 h. The content was then allowed to stand for 24 h for separation into two phases. Test compounds (0.2 µmol) were dissolved in 5 mL of the pre-saturated octanol phase (upper phase), and the absorbance (A_0) was read at 280 nm. A blank without sample also was prepared. Five millilitres of the pre-saturated water phase (bottom phase) were then added, and the mixture was vortexed for 2 min and allowed to stand for 24 h to allow separation. Absorbance (A_x) of the upper phase was measured. The octanol-water partition coefficient (*P*) was calculated using the following equation:

$P = \log A_x/(A_0 - A_x)$, where blank - corrected absorbance was used.

3.5. Analysis of oxygen radical absorbance capacity (ORAC)

A modified method for assessing lipophilic antioxidants was employed (Huang *et al*. 2002). The assay for EGCG and its derivatives was carried out using a Fluostar Optima plate reader (BMG Labtech, Durham, NC) equipped with an incubator and two injector pumps. A standard curve was prepared with trolox. Trolox (6.25-100µM) and samples (0.25µM)) were dissolved in acetone/water (1:1, v/v). AAPH [2,2'-azobis (2-aminopropane) dihydrochloride] and fluorescein were used as the radical generator and probe, respectively. A Trolox standard or test compound solution (20 µL) was added to each well of a 96-well black microplate, followed by 200 µL of

fluorescein in phosphate buffer solution (0.11 μM). A blank was also run using only phosphate buffer and fluorescein. The plate was incubated at 37°C for 15 min and the machine was programmed to inject 75 μL of AAPH into the wells. The conditions used were as follows: 0.3 s position delay, 8 s orbital shaking before each cycle with 4 mm width, 210 s cycle time, and 25 cycles. Fluorescence was measured at an excitation wavelength of 485 nm and emission of 520 nm. A standard curve was plotted and ORAC values for test compounds were obtained as trolox equivalents (TE) per mole of test compound.

3.6. DPPH radical scavenging capacity (DRSC) using electron paramagnetic resonance (EPR)

The DRSC assay was carried out using the method described by Madhujith and Shahidi (2006), with slight modifications. Two millilitres of 0.3 mM solution of DPPH in ethanol were added to trolox standards (50-300 μM) and test compounds (25 μM) dissolved in ethanol. Contents were mixed well, and after 10 min the mixture was passed through the capillary tubing which guides the sample through the sample cavity of a Bruker e-scan EPR spectrophotometer (Bruker E-scan, Bruker Biospin Co.). The spectrum was recorded after 1 min. The operating parameters for EPR were as follows: 1.86 G modulation amplitude, 2.621 s sweep time, 8 scans, 100.000 G sweep width, 3495 G center field, 5.12 ms time constant, 9.795 GHz microwave frequency, and 86 kHz modulation frequency. The corresponding signal intensity was used to monitor the reduction of the DPPH radical concentration in the presence of test compounds.

3.7. Reducing power

The reducing power of EGCG and its derivatives was determined according to Oyaizu (1986). One millilitre of test compounds (1 mM in 95% ethanol) was mixed with 2.5 mL of a phosphate buffer solution (PBS, 0.2M, pH 6.6) and 2.5 mL of a 1% solution of potassium ferricyanide, $K_3Fe(CN)_6$. The mixture was incubated in a water bath at 50 °C for 20 min. Subsequently, 2.5 mL of a 10% (w/v) solution of trichloroacetic acid were added and the mixture was subsequently centrifuged at 770×*g* for 10 min. Afterwards, 2.5 mL of the supernatant was combined with 2.5 mL of distilled water and 0.5 mL of a solution of ferric chloride (0.1%, w/v). The absorbance of the reaction mixture was read spectrophotometrically at 700 nm; the increased absorbance of the reaction mixture indicates greater reducing power. Ascorbic acid (1 to 6 mM in 95% ethanol) was used as a standard.

3.8. Metal chelation

The metal chelation capacity of the test compounds was measured according to Decker and Welch (1990) with some modifications. The test compounds dissolved in ethanol (0.2 mL, 1 mM) were mixed well with 1.74 mL of ethanol and 0.02 mL of ferrous chloride ($FeCl_2$, 2 mM). To the mixture, 0.04 mL of ferrozine (5 mM) was added, and the reaction mixture was allowed to stand for 10 min for colour development. The absorbance was then read at 562 nm. A blank without ferrozine was used for each compound, as the antioxidant-Fe^{2+} complex gives a colour that might interfere with the absorbance readings. Metal chelation capacity was calculated using

the following equation.

$$\% \text{ chelation} = [1 - (Abs_{sample} - Abs_{blank}) / Abs_{control}] \times 100$$

3.9. Antiglycation activity of lipophilized epigallocatechin gallate (EGCG) derivatives

3.9.1. Materials

Potassium peroxosulphate, bovine serum albumin, glucose (BSA), disodium hydrogen phosphate, methylglyoxal (MGO) (40% aqueous solution), 5-methylquinoxaline (5-MQ), OPD (*o*-phenylenediamine), sodium carbonate, sodium bicarbonate, Tween-20, *p*-nitrophenyl phosphate substrate, PBS (phosphate buffered saline) were all purchased from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal antibody to carboxymethyllysine and alkaline phosphatase conjugated goat polyclonal antibody to rabbit IgG were purchased from Abcam (Cambridge, UK). All solvents and acids used were of analytical or HPLC grade and were obtained from BDH Laboratory Supplies (Poole, UK).

3.9.2. Evaluation of methylglyoxal (MGO) scavenging capacity

The MGO trapping capacity was tested according to the method described by Peng *et al*. (2008). MGO (0.25 mL; 1mM) was incubated with 0.25 mL PBS (blank), 1mM aminoguanidine (positive control) in PBS or 1mM EGCG derivatives in PBS at 37°C for 1h.

The derivatization agent was *o*-phenylenediamine (OPD) and 5-methylquinoxaline was used as internal standard. After the derivatization, MGO was converted to 2-methylquinoxaline with UV absorbance at 315 nm. The Shimadzu (Kyoto, Japan) HPLC system used was composed of a separation module (LC-20AT), an autosampler (SIL-20A), a degasser (DGU-20A3) and a photodiode array detector (SPD-M20A). Separation was conducted on an ACE C18 column (5 μm, 250×4.6 mm, Advanced Chromatography Technologies, Aberdeen, UK). Isocratic elution was performed with 0.5% (*v*/*v*) acetic acid in water – methanol (40:60, *v*/*v*). Flow rate was 0.8 mL/min and the injection volume was 10 μL. The quantity of residual MGO after trapping reaction was indicated by the ratio of peak area of 2-MQ to 5-MQ and the percentage decrease in MGO was calculated as 100* (1- quantity of MGO in sample with AG/EGCG derivatives / quantity of MGO in the blank).

3.9.3. Evaluation of inhibition on fluorescent antiglycation end products (AGEs) and carboxymethyllysine (CML) formation

BSA (2 mg/mL) was co-incubated with 33mM D-glucose in 0.1M pH 7 phosphate buffer at 37°C for 7 days. EGCG derivatives were added to the glycation model at 100 μM. Aminoguanidine was used as a positive control at a concentration of 1mM. After incubation, 100 μL of sample solution were transferred to each well of a 96-well plate and fluorescent AGEs were indicated by fluorescence intensity with an excitation wavelength of 355/40 nm and an emission wavelength of 405/10 nm (Victor X4 Multilabel Plate Reader, Perkin Elmer).

The non-fluorescent glycation product carboxymethyllysine was analyzed by enzyme-linked immunosorbent assay (ELISA) as described by Sun *et al.* (2010) with some modifications. The

solution was diluted in 50mM sodium carbonate buffer (pH 9.5-9.7) and 100 μL were loaded to each well of 96-well polystyrene plate. Antigen coating lasted overnight at 4°C. After coating, wells were washed three times with 200 μL of washing solution (1X PBS with 0.05% Tween-20). Blocking was achieved by adding 300 μL of 0.5 % gelatin solution and incubating for 2 h at 37 °C. The wells were washed 3 times with 200 μL of washing solution before adding 100 μL of rabbit polyclonal antibody to carboxymethyllysine, diluting 500X and incubating for 1 h at 37 °C. After washing 3 times with 200 μL of washing solution, 100 μL diluted alkaline phosphatase conjugated goat polyclonal antibody to rabbit IgG (diluted 1000X) were loaded into each well and the plate was incubated for 1 h at 37 °C. Subsequent to a final washing step, 100 μL of *p*-nitrophenyl phosphate substrate solution were added to each well. Incubation lasted 15 min at room temperature in the dark and absorbance was read at 405 nm using a Victor X4 Multilabel Plate Reader (Perkin Elmer).

3.10. Lipophilized epigallocatechin gallate (EGCG) derivatives and their antioxidant potential in food model systems and biological systems

3.10.1. Materials

Stripped corn oil, BHA, beta-carotene, Tween 40, TBA (thiobarbituric acid), TMP (1,1,3,3-tetramethoxypropane), and TCA (trichloroacetic acid) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). Fresh, lean, ground pork was purchased from a local supermarket. Human LDL and supercoiled plasmid pBR322 DNA were purchased from Sigma-Aldrich Canada Ltd, and SYBR safe gel stain from Invitrogen Corporation (Carlsbad, CA). Other

chemicals were purchased from Sigma-Aldrich Canada Ltd. All solvents used were obtained from Fisher Scientific Ltd (Ottawa, ON).

3.10.2. Antioxidant activity in bulk oil

The antioxidant activities of EGCG derivatives in bulk oil were measured as protection against lipid oxidation in a stripped corn oil model system by the well-established Rancimat method. EGCG or its derivatives was dissolved in ethanol (1 µmol/mL) and 1 mL of it was transferred into a reaction vessel, followed by evaporation of the solvent to dryness under a stream of nitrogen. To each vessel (an empty vessel was used as a control), 3 g of stripped corn oil (devoid of endogenous antioxidants) were added. The evaluation was carried out under accelerated oxidation in a Rancimat apparatus (Metrohm Model 743, Herisau, Switzerland) at 100°C with an air flow rate of 20 L/h. Lipid oxidation in the absence and presence of test compounds was monitored by changes in electrical conductivity arising from the formation of volatile oxidation products. The induction periods (IP, time to reach a sudden increase in oxidation rate) were recorded. The longer the IP, the greater the oxidative stability of the oil and hence the higher was the antioxidant potential of the compound involved. Antioxidant activity was interpreted as protection factor (PF), which was calculated as follows:

$$PF = IP_{sample} / IP_{control}$$

where, IP_{sample} and $IP_{control}$ represent induction periods for the oil with and without test compound, respectively.

3.10.3. Antioxidant activity in oil-in-water emulsion

The antioxidant activities of EGCG derivatives in an oil-in-water emulsion were determined by a modification of the β-carotene bleaching assay (Amarowicz and Shahidi 1995). The ability of test compounds to decrease oxidative loss of β-carotene in a β-carotene/linoleic acid emulsion was evaluated as described below. β-Carotene (10 mg) was dissolved in chloroform (10 mL) and an aliquot (1.2 mL) of this solution was transferred into a round-bottom flask containing linoleic acid (40 mg) and Tween 40 (400 mg). A blank without β-carotene was also prepared (40 mg of linoleic acid + 400 mg of Tween 40). After removal of chloroform under a stream of nitrogen, 100 mL of oxygenated distilled water were added to the flask and the mixture was stirred vigorously. Aliquots (4.5 mL) of the above emulsion were transferred into a series of tubes containing 0.5 mL of the test compounds (1 mM in ethanol) or ethanol as the control. A blank mixture (without β-carotene) was prepared for each sample. The tubes were vortexed and the zero time absorbance was read immediately at 470 nm. The tubes were kept in a water bath at 50°C with gentle shaking, and measurement of absorbance was continued over a 105-min period at intervals of 15 min. Blank-corrected absorbance was obtained and plotted against time to produce the kinetic curve of β-carotene bleaching. The antioxidant activity of test compounds in protecting against β-carotene/linoleic acid oxidation was calculated using the following equation:

$$AA\% = [1- (A_0-A_t) / (A_0°-A_t°)] \times 100$$

where Ao and At are corrected absorbance values for test samples measured at zero time and after incubation, respectively and $A_0°$ and $A_t°$ are corrected absorbance values for the control at zero time and after incubation, respectively.

3.10.4. Antioxidant activity in a muscle food

A pork model system was employed for assessing the antioxidant effectiveness of EGCG derivatives. The meat model systems were prepared as described by Shahidi and Alexander (1998). Fresh ground pork (40 g) was mixed with deionized water (10 g) in a Mason jar. Samples and the reference antioxidant compound (BHA) dissolved in ethanol were added to meat at a level of 80 µmol/kg (14, 36, 50, 54, 68, 74, 95, 122, and 136 ppm for BHA, EGCG, EGCG-C2, EGCG-C3, EGCG-C6, EGCG-C8, EGCG-C12, EGCG-C18, and EGCG-C22, respectively). A control without any antioxidant was also prepared. The contents were thoroughly mixed and cooked at 80°C in a thermostated water bath for 40 min with intermittent stirring. The cooked meat was cooled to room temperature and homogenized with a Polytron PT 3000 (Brinkmann Instruments, Rexdale, ON) homogenizer. The homogenate was then transferred into plastic bags and stored at 4°C for 14 days. Meat samples were taken on days 0, 3, 5, 7, and 14 for measurement of oxidation in terms of TBARS (thiobarbituric acid reactive substances) formation. TBARS values were determined as described by Shahidi and Hong (1991). A series of TMP (1,1,3,3-tetramethoxypropane) standard solutions at different concentrations was mixed with thiobarbituric acid (TBA) in screw-capped tubes and heated in a boiling water bath for 45 min. After cooling on ice, the absorbance was recorded at 532 nm and a standard curve was constructed (absorbance versus concentration). For TBARS in the cooked meat model system, 1 g of meat was mixed with 2.5 mL of trichloroacetic acid (TCA, 10 % w/v) in a centrifuge tube, followed by addition of 2.5 mL of TBA reagent. The mixture was centrifuged at 3000x*g* for 10 min and the supernatant was filtered. The filtrate was heated in a boiling water bath for 45 min and the absorbance was measured at 532 nm after cooling to room temperature. TBARS values in meat samples were obtained using the standard curve as µmol malonaldehyde equivalents/kg

of meat. Antioxidant activity was calculated as percentage inhibition of TBARS formation by test compounds.

3.10.5. Inhibition against copper-induced LDL-cholesterol oxidation

The inhibitory activity of EGCG and its derivatives against copper-induced LDL-cholesterol oxidation was determined as described by Lebeau *et al.* (2000). Human LDL solution was dialyzed overnight in PBS (10 mM, 0.15M NaCl, pH 7.4) at 4°C under a nitrogen blanket prior to the test. Sample solutions were prepared by dissolving the test compounds in ethanol (10 µM). Aliquots (10 µL) of sample solutions were transferred into a set of Eppendorf tubes, and the solvent was evaporated to dryness under a stream of nitrogen. To each tube, 0.1 mL of PBS was added, followed by vortexing for 1 min. An aliquot (0.8 mL) of the dialyzed and diluted LDL solution (0.125 mg/mL) was added to each tube, and the contents were mixed well and incubated at 37 °C for 15 min. The reaction was initiated by the addition of 0.1 mL of $CuSO_4$ (20 µM, previously incubated at 37°C). A blank containing only the sample without LDL or $CuSO_4$ was prepared for each test compound. After incubation of the reaction mixture at 37°C for 22 h, the conjugated dienes formed as a result of oxidation were measured spectrophotometrically at 234 nm. Antioxidant activities of test compounds were expressed as percentage inhibition of conjugated diene formation, which was calculated as follows:

$$\% \text{ Inhibition} = 100 \text{ X } (Abs._{control} - Abs._{sample}) / (Abs._{control} - AbS._{native\ LDL})$$

where the control had LDL and $CuSO_4$ without antioxidants, and native LDL contained LDL only. All absorbances for samples were blank-corrected.

3.10.6. Inhibition against DNA scission

The antioxidant activities of EGCG and its derivatives against DNA scission were determined as described by Hiramoto *et al.* (1996) with some modifications. DNA strand breaking was induced by hydroxyl and peroxyl radicals and monitored by gel-electrophoresis. For hydroxyl radical-induced DNA oxidation, 2 µL of test compounds dissolved in ethanol were added to an Eppendorf tube and the solvent was evaporated under a stream of nitrogen. To the tube, 2 µL of distilled, deionized water were added followed by thorough vortexing for 1 min. The following reagents were then added to the tube in the order stated: 2 µL of PBS (pH 7.4), 2 µL of supercoiled pBR322 DNA, 2 µL of H_2O_2 and 2 µL of Fe_2SO_4. The mixture (10 µL) containing 10 µM of the test compounds, 0.1 M PBS, 10 µg/ml DNA, 0.2 mM H_20_2 and 0.1 mM Fe_2S0_4 (final concentration/assay) was incubated at 37°C for 1 h. For peroxyl radical-induced DNA oxidation, a lower sample concentration was used with 4 µL of AAPH [2, 2'-azobis (2-aminopropane) dihydrochloride]. The reaction mixture contained 1µM test compounds, 0.1 M PBS, 10 µg/mL DNA and 7 mM AAPH. A blank (DNA only) and a control (DNA+ free radicals) were also prepared for both assays. After incubation, 1 µL of loading dye (0.25% bromophenol blue / 0.25% xylene cyanol / 50 % glycerol) was added, and the entire mixture was loaded onto an agarose gel. The agarose gel was prepared by dissolving 0.7 % (w/v) agarose in Tris-acetic acid-EDTA (ethylenediaminetetraacetic acid) buffer (TAE, pH 8.5) and stained with SYBR safe gel stain. Gel electrophoresis was performed at 80V for 90 min in TAE buffer using a horizontal submarine gel electrophoresis apparatus. The bands were visualized under UV light and a GelDoc apparatus equipped with a Sony digital camera photographed the images. The images were analyzed using Alpha Ease stand-alone software (Alpha Innotech Co., San

Leandro, CA, USA) and the intensity or density of the bands (obtained from the densitometer) was used as an indicator of the concentration of the native (supercoiled) and nicked DNA fractions.

Antioxidant activity was calculated as DNA retention according to the following equation:

$$\% \text{ Retention} = 100 \times (\text{native DNA sample}/\text{native DNA blank})$$

where, DNA sample and DNA blank are normalized concentrations of native supercoiled DNA in total DNA for sample groups and blank, respectively.

3.11. Statistical analysis

Statistical analysis was performed for all samples used in the experiments. All determinations were replicated three times and mean values and standard deviations reported. Analysis of variance (one-way ANOVA) was performed and the mean separations were performed by Tukey's HSD test ($P<0.05$) using SPSS 16.0 for Windows.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Synthesis of EGCG ester derivatives

There are several strategies for improving the functional and biological properties of EGCG through structural modification. Introduction of alkoxyl groups and peracylation have been shown to increase lipid membrane permeability and the chemical/metabolic stability of EGCG, thereby improving bioactivities under physiological conditions (Tanaka *et al.* 1998; Lam *et al.* 2004, Zhong and Shahidi 2012). Esterification by chemical means, with less selectivity and specificity, has been reported to result in higher degrees of substitution (DS) in the products than in those catalysed by enzymes.

More lipophilic derivatives of EGCG were prepared via reaction with various acyl chlorides of different chain lengths. The yields of the crude products (each series of EGCG polyesters, tetra ester and higher) were 67.3, 66.4, 74.3, 65.4, 56.9, 45.4 and 28.7 % for C2, C3, C6, C8, C12, C18 and C22 esters, respectively. The long-chain PUFA showed a lower rate of incorporation, possibly because of its non-linear (bent) structure that rendered steric hindrance for the acylation reaction. The electrophilic acyl group may react with a different number of hydroxyl groups located in different positions depending on the nature of the electrophile, the reaction conditions and the catalyst employed (Ballesteros *et al.* 2007). In this study, esters of EGCG with high degree of substitution (DS) were formed during acylation under the conditions employed and the composition of the reaction mixture varied depending on the ratio of the starting materials (EGCG/fatty acid). Esters with higher DS (7-8) were produced at lower ratios of EGCG/fatty

acid (<1) (data not shown). As the degree of substitution increased, the lipophilicity increased while hydrolysis by lipolytic enzymes decreased due to steric hindrance (Akoh and Swanson 1990), hence higher membrane permeability and metabolic stability of the esters. According to the results from HPLC-MS analysis, esterification of EGCG with fatty acids (at 1:1 ratio) yielded predominantly tetraesters (DS 4) in all crude products except for C2 and C3 esters, but in different proportions. The chain length of the fatty acids had an effect on their incorporation into the EGCG molecule.

4.2. Identification of EGCG derivatives

The crude EGCG ester products were separated into different fractions by flash column chromatography, and the major products corresponding to tetraesters were collected and subjected to lipophilicity and antioxidant activity assessment tests. The esters were identified by HPLC-MS; the mass spectrum for C6:0 is shown in Figure 4.1. For the teteraester of C6:0**,** the molecular ion peak detected was at m/z 850.91, representing [M^+ ion of the EGCG-tetrahexanoate, which might result from loss of a hydrogen atom from the [$M+H^+$ or its migration to the fragments. The presence and abundance of [M^+ in relation to [$M+H^+$ in APCI-MS have been reported to be dependent on the mobile phase in the LC as well as the H atom affinity of the analyte. The peaks at m/z 752.8, 654.6, 556.5 and 458.4 showed one or multiple mass loss of 98.1 from the molecular ion, representing the ions of [$M-98.1^+$, [$M-2x98.1^+$, [$M-3x98.1^+$ and [$M-4x98.1^+$, respectively.

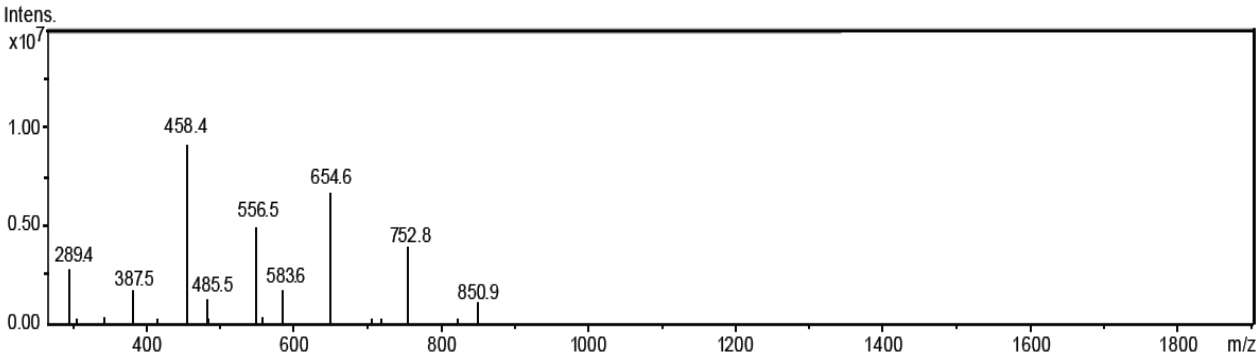


Figure 4.1: Mass spectrum of epigallocatechin gallate (EGCG) tetrahexanoate

The peaks at m/z 583.6, 485.5 and 289.4 corresponding to [M-2x98.1-169$^+$, [M-3x98.1-169$^+$ and [M-4x98.1-169$^+$, respectively, originated from additional cleavage of the gallic acid group (mass 169). Based on these molecular ions and fragments in the mass spectrum, the identity of the ester was confirmed to be EGCG tetrahexanoate.

Similarly, all other tetraesters were identified and confirmed. Mass spectra of these are shown in Figure 4.2.

4.3. Structure elucidation of EGCG derivatives

The location of fatty acid incorporation into the EGCG molecule was determined using ^{1}H and ^{13}C NMR by comparing the chemical shifts of the derivatives with the parent EGCG molecule, as EGCG has eight hydroxyl groups that are capable of acylation with fatty acids. The chemical shifts detected for EGCG in both ^{1}H and ^{13}C NMR were very close to those reported in the literature (Valcic *et al.* 1999; Zhong and Shahidi 2010). The presence of alkyl protons and carbon provided evidence for incorporation of fatty acids to the EGCG molecule.

The chemical shifts of protons in the derivatives compared with those in EGCG showed a downfield shift for all proton signals of EGCG (Table 4.1), which is in accordance with the findings of Islambekov *et al.* (1976) who demonstrated that acetylation of catechin led to a downfield displacement of all the proton signals. Since there is no proton

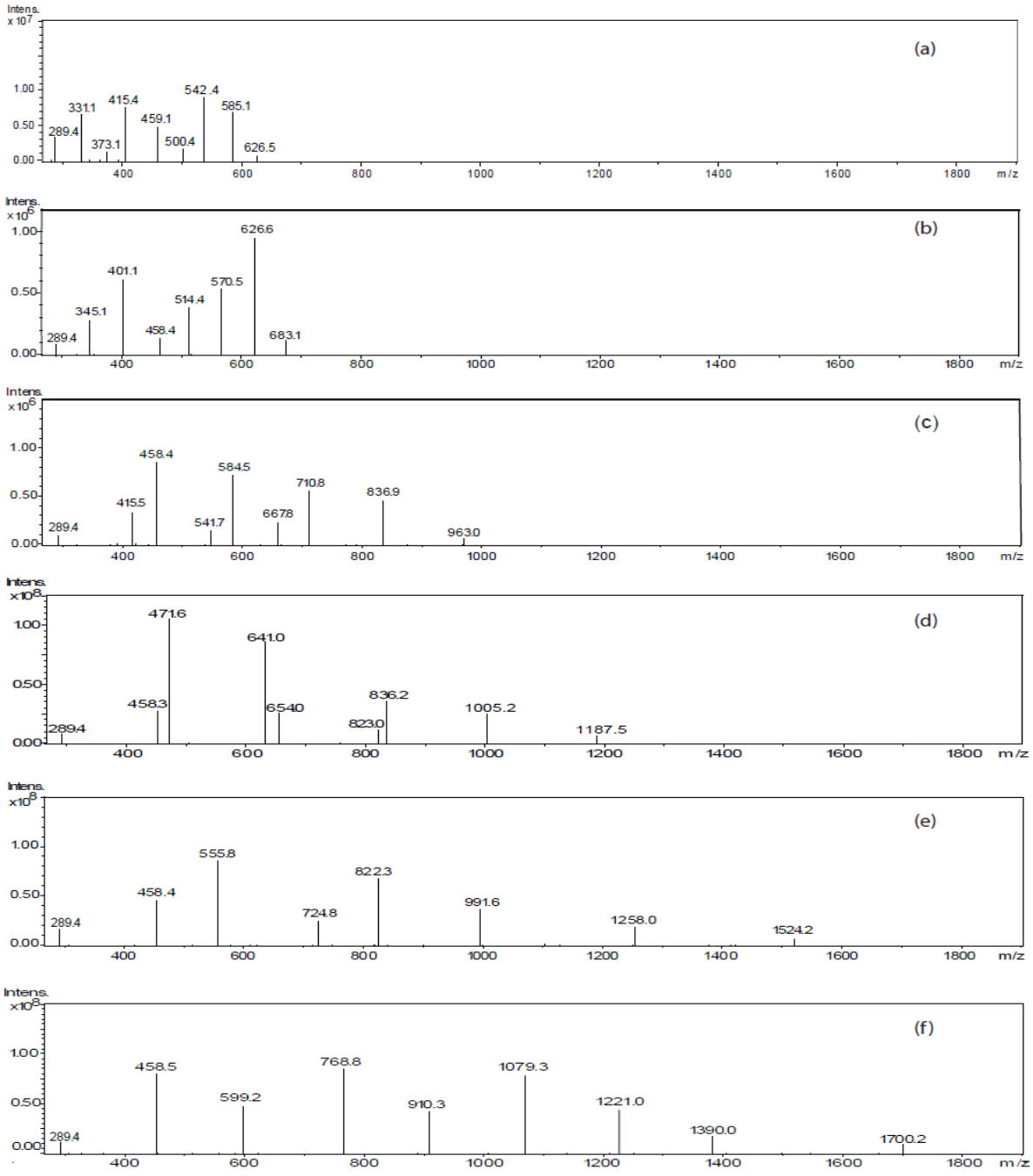


Figure 4.2: Mass spectra of epigallocatechin gallate (EGCG) tetraesters

EGCG-C2, (b) EGCG-C3, (C) EGCG-C8, (d) EGCG-C12,(e)EGCG-C18,

(f) EGCG-C22

attached to the carbon bearing the hydroxyl substituent in the aromatic rings of EGCG, large downfield shifts (δ ~1.0-1.2 ppm) for geminal protons were not detected. Chemical shifts of protons in all three derivatives followed the same trend. H-2' and H-6' showed a downfield shift of δ ~ 0.09 in comparison with the parent EGCG molecule, indicating the occurrence of acylation in the B ring. Downfield shifts of H-2, H-3, and H-4 in the C-ring decreased in the order of H-2>H-3>H-4, due to the increasing distance from the acylation site in the B ring. Acylation sites were tentatively assigned to the D ring as well based on the downfield shifts of H-2'' and H-6'' (δ ~0.04-0.05 ppm), which appeared to be smaller than those of H-2' and H-6' in the B-ring, possibly because of the ester bond in the gallic acid moiety. Only minor shifts were found for H-6 and H-8 in the A-ring, suggesting that the A ring was not the acylation site. Based on the ^{1}H NMR results, a tentative conclusion can be reached that the fatty acids were incorporated in the B- and D-rings of the EGCG molecule.

The specific positions of hydroxyl groups being acylated were further confirmed by ^{13}C NMR. The general strategy established by Yoshimoto *et al.* (1980) was employed for structure elucidation of the EGCG derivatives. As described by Yoshimoto *et al.* (1980), acylation of a hydroxyl group of the substrate resulted in a downfield shift of the 0-acylated carbons and an upfield shift of the neighbouring carbon. As presented in Table 4.1, a large downfield shift (δ ~ 4.25-4.58 ppm) was found for C-3' and C-5 ', indicating these might be the positions of acylation. The remarkable upfield shift (δ ~ 0.72-3.20 ppm) observed for C-4', the carbon adjacent to both C-3' and C-5', suggested the presence of a free hydroxyl group at C-4' and also further confirmed acylation of C-3' and C-5'.

Table 4.1: ^{1}H and ^{13}C chemical shift (δ ppm) of epigallocatechin gallate (EGCG) and EGCG-3', 5', 3'', 5''-O-tetrahexanoate

C/H position	EGCG		EGCG-C6	
	^{13} C	^{1} H	^{13} C	^{1} H
2	77.66	4.96	77.59	4.97
3	69.14	5.36	69.18	5.39
4	26.86	2.65	26.81	2.67
		2.93		2.96
5	156.8		156.77	
6	95.46	5.83	95.45	5.83
7	158.62		158.64	
8	96.68	5.93	96.71	5.94
9	157.61		157.63	
10	98.5		98.47	
1'	129.71		129.61	
2'	106.61	6.39	106.65	6.48
3'	146.52		150.62	
4'	133.47		131.77	
5'	146.42		150.72	
6'	106.61	6.39	106.65	6.48
1''	120.42		120.44	
2''	109.81	6.82	109.41	6.86
3''	146.76		151.26	
4''	139.64		137.14	
5''	146.76		151.26	
6''	109.78	6.80	106.54	6.86
COO	166.38		166.36	

The other neighbouring carbons (e-2' and e-6') of the acylation site did not show any upfield shift, possibly due to the steric conformation of the fatty acids incorporated, which is believed to affect the chemical shifts. It has been demonstrated that ^{13}C chemical shifts may be determined by more complex stereochemical factors, whereas electronic influences dominated chemical shifts of ^{1}H (Ranganathan and Balaram 1984). Similarly, the positions of acylation in the D-ring were assigned to e-3" and e-5", based on the downfield shift of e-3" and e-5" and upfield shift of e-4". Absence of a downfield or upfield shift for carbons in the A-ring implied that hydroxyl groups in the A ring were not acylated, which is in agreement with the ^{1}H NMR results. Thus, structural characterization of the EGCG derivatives was conclusive based on the combined ^{1}H and ^{13}C NMR, and the compound was identified as EGCG-3', 5', 3", 5"-O- tetrahexanoate, as shown in Figure 4.3.

4.4. Lipophilicity

The EGCG derivatives were evaluated for their lipophilicity in terms of their octanol-water partition coefficients (*P*). Higher *P* values indicate higher lipophilicity of the compound. Acylation with fatty acids resulted in increased lipophilicity, as esters showed higher *P* values than the parent EGCG molecule (Table 4.2). EGCG-DHA had the highest *P* value, due to the presence of the DHA, which has the longest hydrocarbon side chain.

4.5. Radical scavenging capacity

The antioxidant activities of EGCG and its esters were evaluated as scavenging capacity against DPPH radical, a stable hydrophobic radical frequently used in antioxidant assessments. All ester derivatives displayed a higher scavenging activity against DPPH than did the parent compound EGCG (Figure 4.4).

Table 4.2: Lipophilicity of epigallocatechin gallate (EGCG) and esters

Compound	Lipophilicity (Octanol-water partition coefficient, *P*)
EGCG	0.38 ± 0.06^{d}
EGCG-C2	0.43 ± 0.04^{a}
EGCG-C3	0.45 ± 0.06^{a}
EGCG-C6	0.55 ± 0.38^{a}
EGCG-C8	0.70 ± 0.38^{b}
EGCG-C12	0.77 ± 0.38^{b}
EGCG-C18	1.07 ± 0.38^{c}
EGCG-C22	1.38 ± 0.3^{c}

Values (mean ± SD of three replicates) with different letters were significantly different at $P<0.05$.

(a) EGCG-C2, EGCG tetraethanoate
(b) EGCG-C3, EGCG tetrapropanoate
(c) EGCG-C6, EGCG tetrahexanoate
(d) EGCG-C8, EGCG tetraoctanoate
(e) EGCG-C12, EGCG tetradodecanoate
(f) EGCG-C18, EGCG tetraoctadecanoate
(g) EGCG-C22, ,EGCG tetradecosahexaenoate

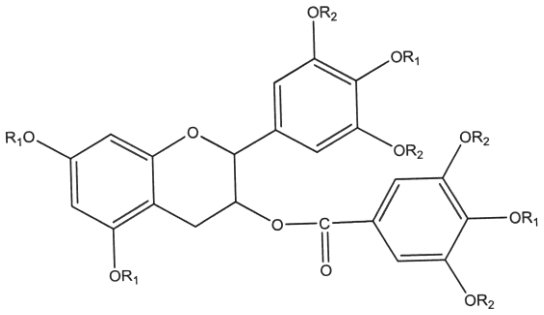


Figure 4.3: Chemical structure of EGCG-3',5',3",5"-0- tetrahexanoate

$R_1 = H,\ R_2 = CO(CH_2)_4CH_3$

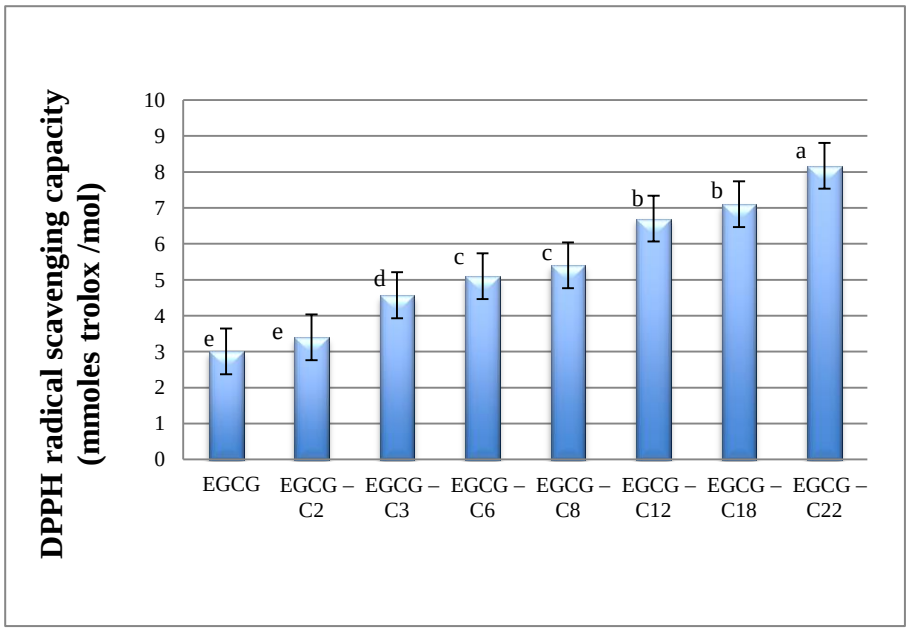


Figure 4.4: DPPH radical scavenging capacity of epigallocatechin gallate (EGCG) esters

(a) EGCG-C2, EGCG tetraethanoate (b) EGCG-C3, EGCG tetrapropanoate
(c) EGCG-C6, EGCG tetrahexanoate (d) EGCG-C8, EGCG tetraoctanoate
(e) EGCG-C12, EGCG tetradodecanoate (f) EGCG-C18, EGCG tetraoctadecanoate
(g) EGCG-C22, EGCG tetradecosahexaenoate

The radical scavenging capacity of EGCG was significantly enhanced by incorporation of fatty acid side chains, which was attributed to the increased lipophilicity and hence greater accessibility/affinity of the derivatives to the lipophilic DPPH radical or compared to the parent EGCG molecule. However, since DPPH radical is a stable artificial free radical that is not found in nature, the antioxidant activity estimated from DPPH scavenging may not necessarily reflect the real situation in food and biological systems, where radicals are generally unstable with very short lifetime. In order to have a better understanding of their antiradical activities in biological environments, the EGCG derivatives were also evaluated for their capability to scavenge peroxyl radicals, measured as oxygen radical absorbance capacity (ORAC). The ORAC assay utilizes a biologically relevant radical source and has been established as a standard method for assessing the activity of hydrophilic antioxidants. In this study, a modified ORAC method for lipophilic antioxidants proposed by Huang *et al*. (2002) was employed, which introduces acetoacetone/water as the solvent and RMCD as a solubility enhancer.

The ORAC values (Table 4.3) of the derivatives were lower than that of EGCG, indicating their greater hydrogen atom donating capability under the test conditions. The decreased number of hydroxyl groups in EGCG derivatives appeared to negatively affect the antioxidant activity and the substituents on the phenol ring played an essential role in

the efficacy of phenolic antioxidants via both electronic and steric effects. Electron donating groups at the *ortho* and *para* positions are able to lower the activation energy for hydrogen abstraction, and thus enhancing the hydrogen donating capability of the

Table 4.3: Antioxidant activity of epigallocatechin gallate (EGCG) and esters

Values (mean ± SD of three replicates) in the same row with different letters were significantly different at $P<0.05$.

Antioxidant activity of EGCG and EGCG esters								
	EGCG	EGCG-C_2	EGCG-C_3	EGCG-C_6	EGCG-C_8	EGCG-C_{12}	EGCG-C_{18}	EGCG-C_{22}
ORAC (mol trolox eq /mol)	112 ±1.1[a]	68.1 ±0.7[b]	63.9 ±1.0[b]	53.3 ±0.8[c]	54.5 ±0.9[c]	49.1 ±1.8[d]	42.8 ±0.8[e]	41.1 ±1.5[e]
DPPH radical scavenging capacity (mmoles trolox /mol)	3.0 ±0.3[e]	3.4 ±0.2[e]	4.6 ± 0.5[d]	5.1 ± 0.1[c]	5.4 ± 0.1[c]	6.7 ± 0.3[b]	7.1 ±0.3[b]	8.2 ±0.3[a]
Reducing power (mmol ascorbic eq/mol)	18.1 ±0.4[a]	0.71 ±0.09[e]	0.89 ±0.04[e]	3.1 ±0.2[d]	3.6 ±0.6[d]	8.1 ±0.7[c]	12.2 ±0.4[b]	14.1 ±0.2[b]
Metal Chelation (%)	10.1 ±0.3[e]	13.9 ±0.09[d]	15.6 ±0.2[d]	25.1 ±0.6[c]	26.8 ±0.4[c]	36.1 ±0.2[b]	38.4 ±0.4[a]	38.7 ±0.2[a]

(a) EGCG-C2, EGCG tetraethanoate (b) EGCG-C3, EGCG tetrapropanoate
(c) EGCG-C6, EGCG tetrahexanoate (d) EGCG-C8, EGCG tetraoctanoate
(e) EGCG-C12, EGCG tetradodecanoate (f) EGCG-C18, EGCG tetraoctadecanoate
(g) EGCG-C22, EGCG tetradecosahexaenoate

antioxidant (Singh *et al.* 2005; Wright *et al*. 2001). In the EGCG derivatives, the O-acylation replaced the strong electron donating hydroxyl group with a weaker electron donating ester group, which would compromise the hydrogen donating capability of the molecule. The substitution would also hinder the formation of moderately strong hydrogen bond between adjacent hydroxyl groups in the EGCG molecule. These intramolecular hydrogen bonds contribute to stabilization of the molecule and increased bond dissociation enthalpy (BDE) of the -OH bond, *i.e.* resistance to hydrogen atom dissociation (Wright *et al*. 2001). *Ortho* substitution of EGCG might result in enhanced hydrogen atom donating capacity by reducing intramolecular hydrogen bonds and BDE.

4.6. Reducing power

Antioxidants may inhibit oxidation through single electron transfer. The antioxidant can deactivate a free radical or reduce an oxidant by donating an electron and forming an antioxidant radical cation, followed by rapid and reversible deprotonation. The antioxidant radical formed is then stabilized by electron delocalization (resonance), as in the hydrogen atom donation mechanism. The ability of an antioxidant to act as an electron donor, or its reducing power, is determined by the ionisation potential (IP) of the compound and is strongly solvent-dependent. The reducing power of EGCG and its derivatives was measured as Fe^{3+}- Fe^{2+} transformation mediated by the test compounds and expressed as ascorbic acid (a known reducing agent) equivalents. The results showed that EGCG exhibited greater reducing power than its esters (Table 4.3).

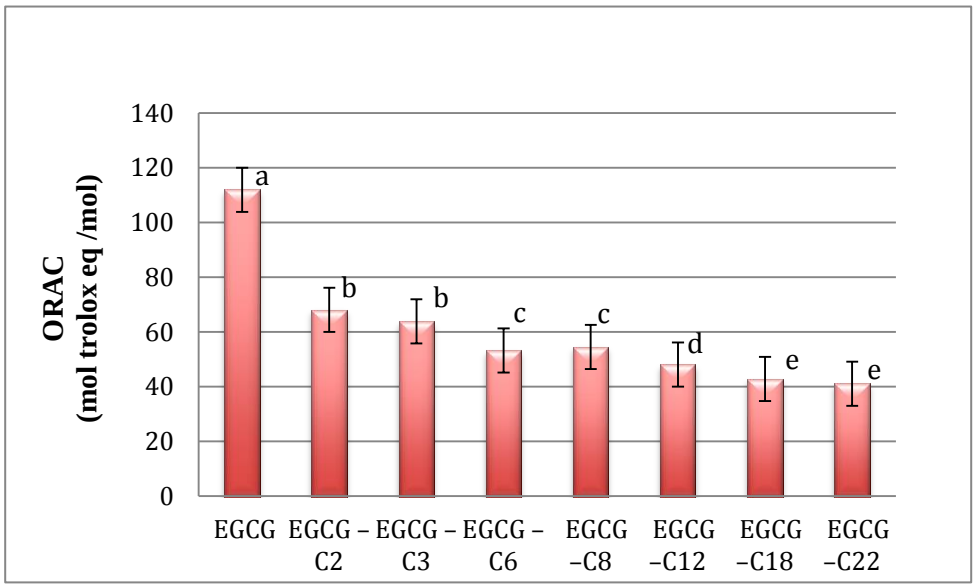


Figure 4.5: Oxygen radical absorbance capacity (ORAC) of epigallocatechin gallate (EGCG) esters

(a) EGCG-C2, EGCG tetraethanoate
(b) EGCG-C3, EGCG tetrapropanoate
(c) EGCG-C6, EGCG tetrahexanoate
(d) EGCG-C8, EGCG tetraoctanoate
(e) EGCG-C12, EGCG tetradodecanoate
(f) EGCG-C18, EGCG tetraoctadecanoate
(g) EGCG-C22, EGCG tetradecosahexaenoate

Acylation with long chain fatty acids led to dramatic increase of reducing power (Figure 4.6) EGCG gave the highest value (Table 4.3). The electron donating capability of antioxidants is dependent on the IP, which may be altered by structure modification. Substituents on the phenol ring may change not only the BDE, but also IP. The presence of electron-donating substituent groups can stabilize the phenol radical cation, hence lowering the IP and enhancing the electron donating capability.

In contrast, electron-donating substituents such as esters increase IP and decrease the reducing power of the antioxidant. As a result, acylated EGCG with ester side chains showed lower reducing power than the original molecule. The opposite trend was observed for ORAC, although phenyl substitution influences the O-H bond dissociation energy (BDE) in a similar way, possibly because the effect of the electron-withdrawing side chain on hydrogen atom donation was counteracted by other factors, such as hydrogen bonding and steric changes (Wright *et al*. 2001).

Solubility also affects the effectiveness of antioxidants, especially their reducing power, since the electron transfer mechanism is strongly solvent dependent due to solvent stabilization of charged species (Wright *et al*. 2001). EGCG derivatives with enhanced lipophilicity have poor solubility in aqueous media and hence compromised activity under the hydrophilic test environment. Moreover, they might have formed micelles in

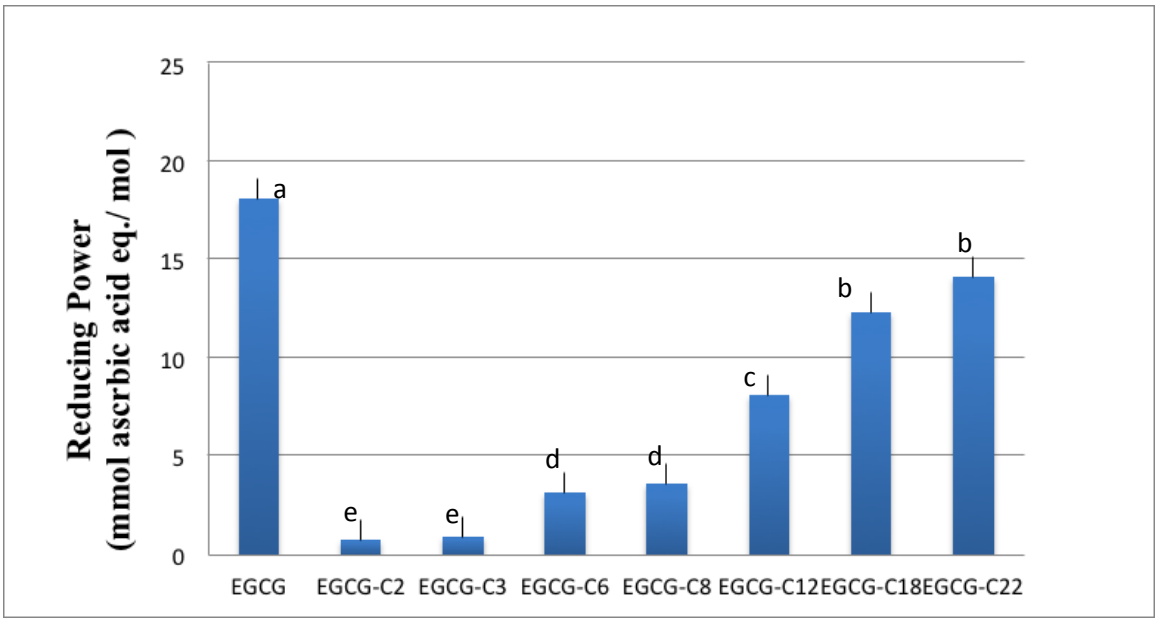


Figure 4.6: Reducing power of epigallocatechin gallate (EGCG) esters

(a) EGCG-C2, EGCG tetraethanoate
(b) EGCG-C3, EGCG tetrapropanoate
(c) EGCG-C6, EGCG tetrahexanoate
(d) EGCG-C8, EGCG tetraoctanoate
(e) EGCG-C12, EGCG tetradodecanoate
(f) EGCG-C18, EGCG tetraoctadecanoate
(g) EGCG-C22, EGC -tetradecosahexaenoate

the assay solution with the B- and D-rings bearing long fatty acid side chains being buried in the hydrophobic core, leaving only A-ring as the site available for antioxidant action. Similar changes in reducing power caused by acylation have been found for rutin esters (Lue *et al*. 2010).

4.7. Metal chelation

Transition metals are extremely reactive and act as catalysts for lipid oxidation by generating free radicals. They can react with hydrogen or lipid peroxides and produce hydroxyl or peroxyl radicals, as in Fenton reaction. Metal chelators such as many polyphenols can bind to metal ions and form stable complexes with reduced redox potential, thus suppressing the pro-oxidant effect of metal ions.

Metals play important roles in health. For example, iron is essential in oxygen transport, respiration and the activity of many enzymes. However, transition metals are extremely reactive and act as catalysts for lipid oxidation by generating free radicals, such as hydroxyl or peroxyl radicals, as in Fenton reaction. Iron- or copper-induced LDL oxidation has been implicated in atherosclerosis and other cardiovascular diseases. Metal chelators such as many polyphenols can bind to metal ions and form a stable complex with reduced redox potential, thus suppressing the prooxidant effect of the metal ions. EGCG is a known metal chelator due to its vicinal trihydroxy structure, in which the oxygen atoms act as electron donors to form bonds with the electrophilic metal ions. In this study, the chelation capacities of EGCG and its ester derivatives for ferrous ion were evaluated. All test compounds exhibited ferrous ion chelation activity, ranging from 10

to 39% (Figure 4.7; Table 4.3). The highest chelation activity was found for EGCG-DHA, indicating that the PUFA ester of EGCG was more potent as a metal chelator than the parent EGCG and its saturated esters. Metal chelation capability has been related to geometric features of the chelator-metal complex (*e.g*. ionic diameter, ring size, conformation, *etc.*) (Hassan 1992), and can therefore be positively or negatively influenced by any structural modifications that may lead to geometric alterations of the complex. The greater metal chelation capability found for EGCG-PUFA esters is possibly due to steric changes resulting from the bent structure of the PUFA chain that favors the stability of the antioxidant-Fe^{2+} complex.

4.8. Trapping capacity of methylglyoxal (MGO)

In the intermediate stage of protein glycation, Schiff bases and Amadori rearrangement products undergo a series of degradation reactions to produce reactive carbonyl intermediates, including methylglyoxal (MGO) and glyoxal (GO). Extra sources of these dicarbonyls are sugar autoxidation and lipid peroxidation. The comparatively higher reactivity of these reactive carbonyls than reducing sugars in attacking amino groups justifies them as important precursors leading to AGEs formation. An increase in carbonyl concentration has been found to result from hyperglycemia and represents a stressful event in diabetic development (Sang *et al*. 2007). Therefore, trapping of these carbonyls by pharmaceutical agents should be an effective strategy to alleviate the "carbonyl stress" and thereafter slow down the pathogenic progress of diabetic complication

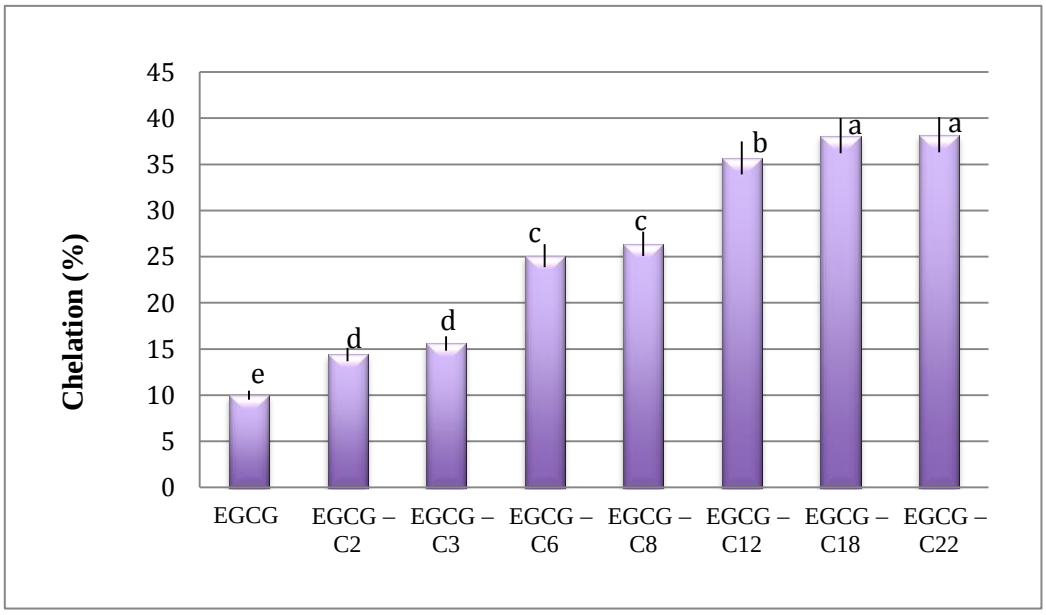


Figure 4.7: Metal chelation of epigallocatechin gallate (EGCG) ester derivatives

(a) EGCG-C2, EGCG tetraethanoate (b) EGCG-C3, EGCG tetrapropanoate
(c) EGCG-C6, EGCG tetrahexanoate (d) EGCG-C8, EGCG tetraoctanoate
(e) EGCG-C12, EGCG tetradodecanoate (f) EGCG-C18, EGCG tetraoctadecanoate
(g) EGCG-C22, EGCG tetradecosahexaenoate

EGCG is among the newly identified carbonyl trapping agents of natural plant origin. The trapping efficiency of MGO at a mole ratio of 3:1 (EGCG/MGO) could be as high as 90% within 5 min under physiological conditions (pH 7.4, 37°C) but the reactivity was lost under acidic conditions (pH 4) (Sang *et al*. 2007). Further studies on the mechanism elucidated that the trapping capacity relied on the A-ring with positions 6 and 8 as major active sites, and EGCG possessed higher affinity for MGO than lysine and arginine, forming mono- or di-MGO adducts (Sang *et al*. 2007).

In the current study when EGCG was co-incubated with MGO at a mole ratio of 1:1, a nearly 70% decrease in the amount of MGO was observed after a 1-h incubation (Figure 4.8). Consistent with previous findings, the trapping capacity of EGCG over MGO was much higher than that ofAG (Sang *et al*. 2007), which led to a 44% reduction in the amount of MGO in the current study. Lipophilized EGCG derivatives with relatively short-chain fatty acids (EGCG-caproic acid and EGCG- caprylic acid) showed slightly higher MGO trapping potential than EGCG. Elongating the esterified fatty acid chain to 12 and 18 carbons reduced the trapping capacity of EGCG to similar values as for AG. Esterification to DHA resulted in a substantial decrease in MGO trapping activity.

The lipophilized EGCG derivatives were tetraesters with acylation by fatty acids on positions 3 and 5 of the B- and D-rings (Zhong *et al*. 2011). The active sites for binding MGO were elucidated to be positions 6 and 8 on the A-ring (Sang *et al*. 2007). Therefore, fatty acids did not occupy the active positions for MGO trapping but acylation with bulky long-chain fatty acids

likely imposed steric effects by blocking the MGO-binding path so as to prevent the formation of MGO-EGCG adducts, which possibly explains the lower trapping efficacy of EGCG derivatives with 12 and 18 carbon saturated fatty acids and with DHA. However, it is unclear how the 6 and 8 carbon saturated fatty acids esterification assisted in the trapping of MGO. The phenomenon might be associated with electronic density re-distribution on the aromatic rings of EGCG induced by fatty acid incorporation.

4.9. Inhibitory activity on advance glycation end-products (AGEs) formation

The antiglycation activity of EGCG is well-documented and it is believed to be mediated by multiple mechanisms including free radical scavenging and reactive carbonyl trapping (Nakagawa *et al*. 2002; Sang *et al*. 2007). As discussed above, esterification with fatty acids potentially influenced the free radical scavenging and MGO trapping capacity of the parent EGCG. In addition, the gallate moiety attached to C-3 position of C-ring was indicated to play an important role in glycation inhibition (Nakagawa *et al*. 2002). Hence, it is expected that lipophilized EGCG derivatives with structural modification of the gallate moiety would possibly differ from the parent EGCG in antiglycation activity.

The *in vitro* antiglycation activity of lipophilized EGCG derivatives was evaluated based on their inhibition of total fluorescent AGEs and non-fluorescent carboxymethyllsine (CML) formation with the glucose/BSA model. The parent EGCG molecule exhibited antiglycation activity at a concentration of 100 μM with around 60 and 70% inhibition of fluorescent AGEs and non-fluorescent CML, respectively. As shown in Figures 4.9 and 4.10, it was found that EGCG derivatives esterified with acetic acid and saturated fatty acids with 6, 8, 12 and 18 carbons

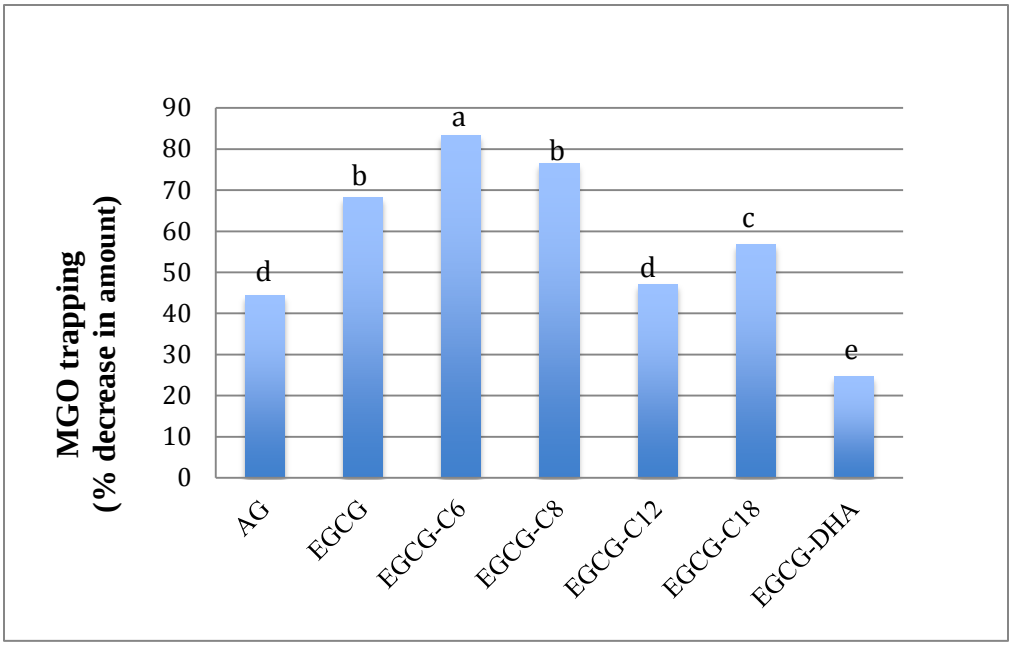


Figure 4.8: Trapping capacity of methylglyoxal (MGO) of epigallocatechin gallate (EGCG) ester derivatives

(a) AG, Aminiguanidine
(b) EGCG-C6, EGCG tetrahexanoate
(c) EGCG-C8, EGCG tetraoctanoate
(d) EGCG-C12, EGCG tetradodecanoate
(e) EGCG-C18, EGCG tetraoctadecanoate
(f) EGCG-DHA, EGCG tetradecosahexaenoate

showed slightly higher inhibitory activity compared AG against fluorescent AGEs formation together with similar CML reduction. Propanoic acid acylation led to insignificant difference on inhibitory rate of both fluorescent AGEs and CML generation while DHA substitution resulted in a slight decrease in CML reduction potential and obvious interference on parent EGCG's prevention of fluorescent AGEs production.

4.10. Antioxidant activity in bulk oil

The effectiveness of antioxidants is influenced by a number of factors, including their structural features, concentration, temperature, type of oxidation substrate and physical state of the system, as well as the presence of pro-oxidants and synergists (Yanishlieva-Maslarova 2001). The chemical structure of an antioxidant determines its reactivity towards free radicals and other reactive oxygen species (ROS) and is also affected by the system environment. For example, the activity of antioxidants in bulk oils is different from that in oil-water emulsions (Porter 1993). In order to investigate the effect of structural modification on the antioxidant activity of EGCG, the lipophilized derivatives were evaluated for their antioxidant efficacy in a bulk oil model system.

Stripped corn oil devoid of endogenous antioxidants was employed for assessing antioxidant efficiency of EGCG derivatives in bulk oil. The oil was heated and aerated as experienced during deep fat frying, and lipid oxidation was accelerated with increased content of polar compounds, foaming, color and viscosity (Perkins 1992).

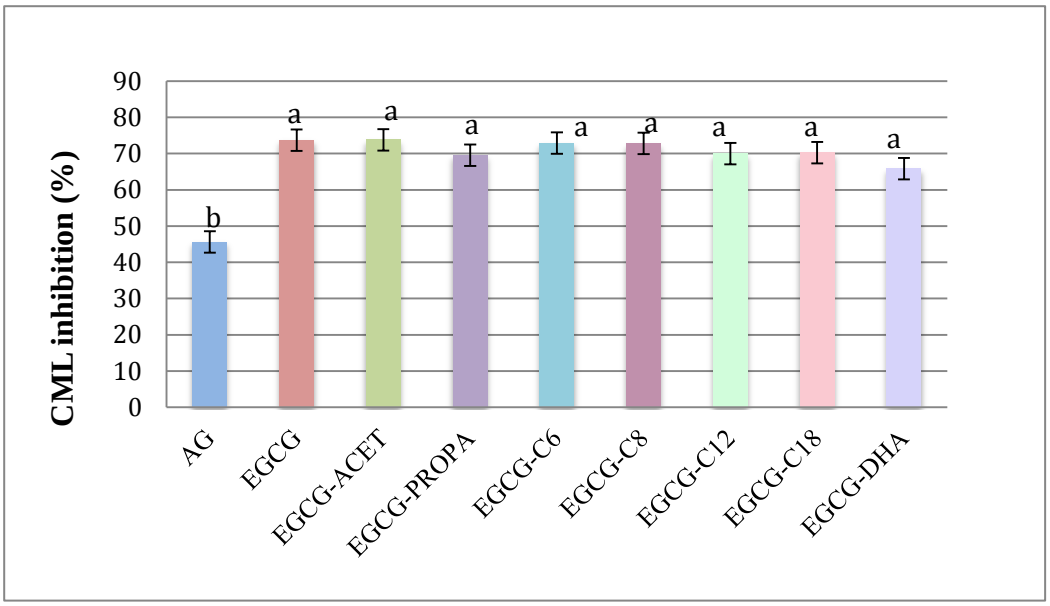


Figure 4.9: Inhibition of non-fluorescent carboxymethyllsine (CML) formation of epigallocatechin gallate (EGCG) ester derivatives

(a) AG – Aminiguanidine
(b) EGCG-ACET, EGCG tetraethanoate
(c) EGCG-Propa,EGCG-tetrapropanoate
(d) EGCG-C6, EGCG tetrahexanoate
(e) EGCG-C8, EGCG tetraoctanoate
(f) EGCG-C12, EGCG tetradodecanoate
(g) EGCG-C18, EGCG tetraoctadecanoate
(h) EGCG-C22, EGCG tetradecosahexaenoate

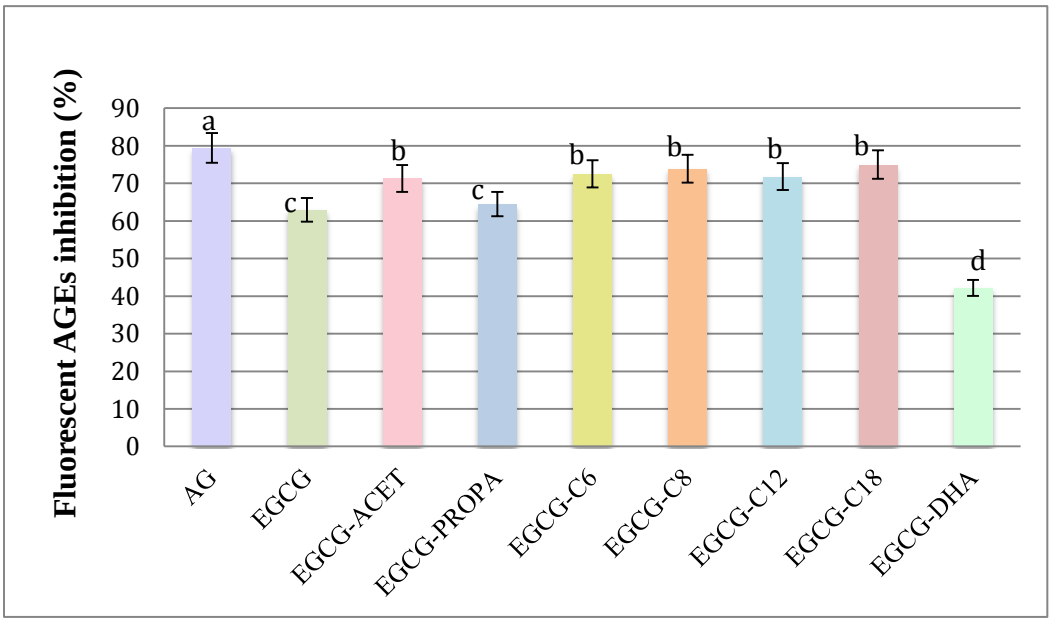


Figure 4.10: Inhibition of total fluorescent advanced glycation end products (AGEs) of epigallocatechin gallate (EGCG) ester derivatives

(a) AG – Aminiguanidine
(b) EGCG-ACET, EGCG tetraethanoate
(c) EGCG-Propa, EGCG-tetrapropanoate
(d) EGCG-C6, EGCG tetrahexanoate
(e) EGCG-C8, EGCG tetraoctanoate
(f) EGCG-C12, EGCG tetradodecanoate
(g) EGCG-C18, EGCG tetraoctadecanoate
(h) EGCG-C22, EGCG tetradecosahexaenoate

Volatile flavour and odour components such as organic acids were formed and detected in the rancimat method as an indicator of oxidation.

The calculated protection factor (PF) of EGCG and its derivatives as affected by their molar concentrations (converted from ppm) are shown in Figure 4.11. EGCG showed an increase in antioxidant activity in the oil (0.5-1 µmol / g of oil) whereas the antioxidant efficiency of its ester derivatives decreased with chain length at EGCG-C12, which had the highest antioxidant activity among all derivatives, tested. EGCG-C2 and C3 acted as antioxidants (PF>1) at the lowest concentration, and had no effect on it. Oxidative stability of the oil (PF=l) when the concentration increased, C2 exerted a slightly prooxidative effect at the highest concentration (PF<l).

With the exception of EGCG-C2 and EGCG-C3 all other derivatives including EGCG exhibited greater antioxidant efficacy at the higher concentration (Figure 4.11). In the case of EGCG, the observed effect was possibly due to its poor solubility in the oil, which may be explained by the interfacial phenomenon of hydrophilic antioxidants in bulk oil. The partially soluble hydrophilic antioxidants tend to be oriented at the air-oil interface where surface oxidation occurs, and therefore protect the system from oxidative changes (Frankel *et al.* 1994). The interfacial phenomenon also lends support to the antioxidant "polar paradox" that in food systems of low surface-to-volume ratio (*e.g.* bulk oils) polar antioxidants with high hydrophilic-lipophilic balance (HLB) are more effective than nonpolar lipophilic antioxidants (Porter 1993; Frankel *et al*.1994

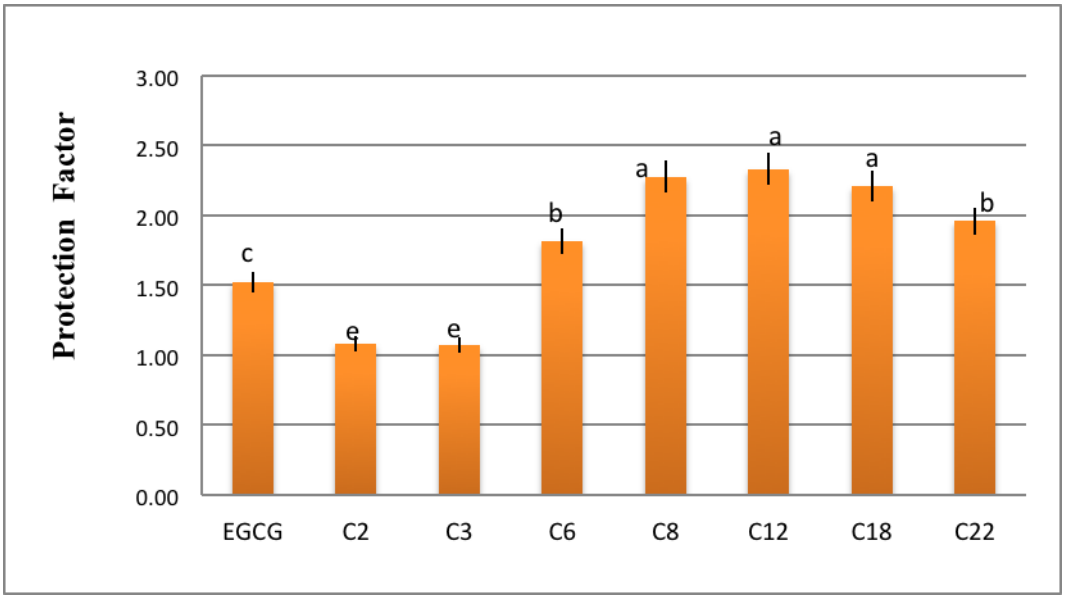


Figure 4.11: PF (protection factor) of epigallocatechin gallate (EGCG) and its acyl derivatives with different carbon chain length (C2-C22) in stripped corn oil at 0.5 µmol/g of oil

(a) EGCG-C2, EGCG tetraethanoate
(b) EGCG-C3, EGCG tetrapropanoate
(c) EGCG-C6, EGCG tetrahexanoate
(d) EGCG-C8, EGCG tetraoctanoate
(e) EGCG-C12, EGCG tetradodecanoate
(f) EGCG-C18, EGCG tetraoctadecanoate
(g) EGCG-C22, EGCG tetradecosahexaenoate

However, the hydrophilic antioxidant EGCG and its lipophilic derivatives at the same mole concentration (0.5 µmol/g of oil) showed a different trend in their antioxidant activity in the stripped corn oil. The compound EGCG-C12 was most effective compared with other compounds in inhibiting oxidation. There was increased antioxidant efficiency when chain length increased and then it reversed itself with an increase in chain length beyond C12. Recent studies suggest that nonpolar antioxidants are not always more active than their polar homologues, nor are polar antioxidants more active than their nonpolar homologues (Viskupicova *et al*. 2010, Chen *et al*. 2010; Laguerre *et al*. 2011a; Aissa *et al*. 2012) which is contradictory to the polar paradox. These authors demonstrated that the relationship between antioxidant activity and hydrophobicity was not as linear as postulated by the polar paradox. For a homologous series, antioxidant activity increased with increasing alkyl chain length until a threshold was reached and beyond which any further chain elongation would lead to an activity reversal. This nonlinear behaviour was termed the "cut-off" effect and was observed in various systems, whether they were living cells or lipid dispersions. The common structural trait may be the lipid-water interface, and the common property may be the cut-off effect at a critical chain length. Both of these assumptions may be incorrect, because data show that under certain conditions and with some compounds, the effectiveness order goes in one way or the other with higher lipophilicity exerting greater antioxidant efficiency than EGCG. More research is needed for confirmation of this hypothesis. With respect to compounds EGCG-C2 and C3, which had slightly prooxidative activity, the short-chain-length of the molecules might have compromised their oxidation inhibitory effect at this concentration by contributing to the formation of lipid oxidation products, because they are highly susceptible to oxidation.

In summary, the lipophilic EGCG ester derivatives with better fat-solubility showed antioxidant activity comparable to the parent EGCG molecule, except for EGCG-C2 and EGCG-C3, and may serve as potential candidates for possible application in bulk oil systems, although with an increase in the chain length there was an increase in antioxidant capacity up to a certain chain length and then the effect was reversed. The concentration of EGCG and its derivatives added to oil should be closely monitored, since they may have compromised efficacy as antioxidants or may even exert a prooxidant activity at higher concentrations.

4.11. Antioxidant activity in an oil-in-water emulsion

Antioxidants behave in different manners when used in different media. Hence, their efficacy in bulk oil may not necessarily reflect that in an oil-in-water (oil/water; o/w) emulsion. Moreover, it has been recognized that high surface-to-volume ratio emulsions are the natural conditions, whereas low surface-to volume bulk lipid is more like an artifact that is less common in foods and biological systems. Therefore, it is important to also include information on effectiveness of an antioxidant in and oil/water emulsion for a more comprehensive assessment of antioxidant activity (Zhong and shahidi 2012).

In this work, the antioxidant activity of EGCG and its lipophilic derivatives in an oil-in-water emulsion system was detected by monitoring the bleaching of β-carotene as affected by test compounds. The decolouration of the pigment, β-carotene, is a free radical-mediated phenomenon resulting from oxidation of linoleic acid in the emulsion which gives rise to

formation of free radicals and hydroperoxides. The presence of antioxidants can minimize the loss of β-carotene during the coupled oxidation of linoleic acid and β-carotene in an emulsified aqueous system. The results indicate that EGCG and its derivatives inhibited bleaching of β-carotene by 13.3 – 54.5 % over the 105 min incubation period (Table 4.4). Lower rates of absorbance decay were observed for emulsions containing test compounds compared to the control, as shown in Figure 4.12. When comparing the antioxidant activity of EGCG and its derivatives, compound EGCG-C12 was the most effective in retaining β-carotene in the first 45 min. The antioxidant activity of test compounds increased with increasing chain length up to C12, and then a further increase in chain length reversed the effect. These findings lend support to studies carried out by Panya *et al*. (2010), Laguerre *et al*. (2011b), and Bayrasy *et al*. (2013) that the antioxidant "polar paradox" theory is contradictory. The theory states that nonpolar antioxidants are more effective than polar ones in emulsions, indicating an opposite trend from that in bulk oil.

4.12. Antioxidant activity in a muscle food

Lipids are not only important components of bulk oils such as cooking oil and emulsions such as salad dressings and margarines. They are also incorporated into processed whole tissue foods such as in many fast foods and are responsible to a large extent for their quality deterioration. The lipids in processed whole tissue foods are characterized by a high surface-to-volume ratio and are usually considered a very polar medium (Porter 1993).

Table 4.4. : Inhibitory effect of epigallocatechin gallate (EGCG) and its derivatives against β -carotene bleaching and TBARS formation in cooked pork

	Inhibition %	
	β -carotene bleaching [1]	TBARS formation in cooked pork [2]
EGCG	13.27 ±0.75[e]	78.45±1.65 [c]
C2	20.53 ±0.50[d]	66.77±0.78 [d]
C3	22.24 ±0.45[d]	65.43±0.65 [d]
C6	40.63 ±0.21[c]	88.78±0.45 [a]
C8	48.25 ±0.34[b]	89.65±1.23 [a]
C12	54.49 ±0.75[a]	90.75±0.43[a]
C18	47.26 ±0.24[b]	89.45±2.45 [a]
C22	46.66 ±0.22[b]	84.22±1.38 [b]
BHA	-	82.76±1.56 [b]

Values (mean ± SD of three replicates) in the same column with different letters were significantly different at $P<0.05$.

1 Inhibition (%) calculated at the end of the incubation (105 min)

2 Inhibition (%) calculated at the end of storage (day 14)

(a) EGCG-C2, EGCG tetraethanoate (b) EGCG-C3, EGCG tetrapropanoate
(c) EGCG-C6, EGCG tetrahexanoate (d) EGCG-C8, EGCG tetraoctanoate
(e) EGCG-C12, EGCG-tetradodecanoate (f) EGCG-C18, EGCG tetraoctadecanoate
(g) EGCG-C22, EGCG tetradecosahexaenoate

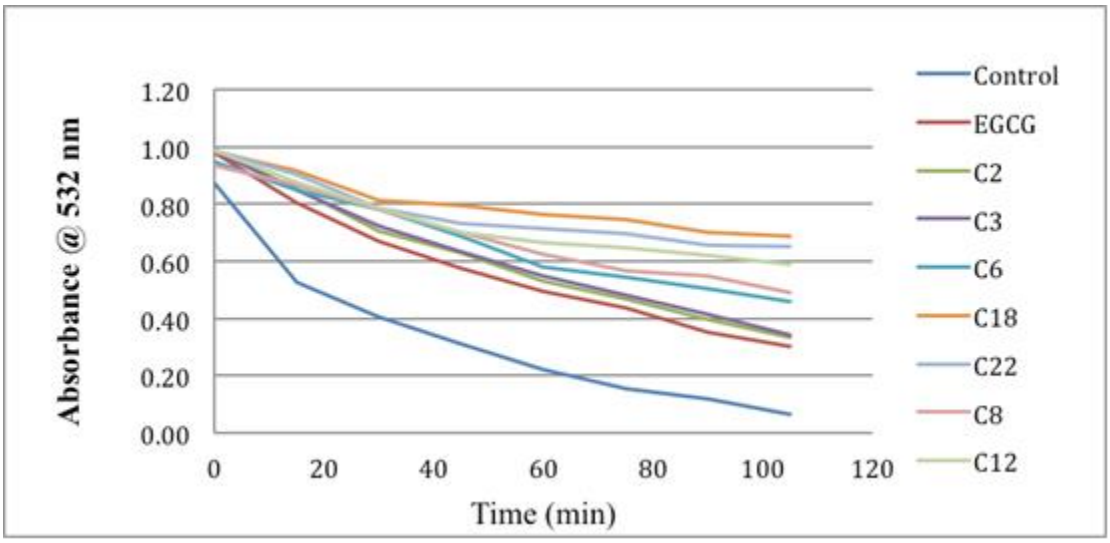


Figure 4.12: β-Carotene bleaching as affected by epigallocatechin gallate (EGCG) and its derivatives

(a) EGCG-C2, EGCG tetraethanoate (b) EGCG-C3, EGCG tetrapropanoate
(c) EGCG-C6, EGCG tetrahexanoate (d) EGCG-C8, EGCG tetraoctanoate
(e) EGCG-C12, EGCG-tetradodecanoate (f) EGCG-C18, EGCG tetraoctadecanoate
(g) EGCG-C22, EGCG tetradecosahexaenoate

These foods, in general, require a larger amount and different types of antioxidants due to their predisposition to oxidation during precooking and long storage time under high surface-to-volume conditions (Porter 1993). Cooked ground muscle foods provide an excellent model for assessing the effectiveness of antioxidants in thermally processed whole or modified tissue foods, where heating causes rapid oxidation of lipid and meat flavour deterioration as well as development of "warmed-over flavour" (Rubin and Shahidi 1988).

In a comminuted pork model system, iron released from heme moieties acts as a catalyst of lipid oxidation in cooked meat. It has been shown that the rate of iron release from the porphyrin ring depends on the time, intensity and temperature of cooking (Frankel *et al.* 2000). Free, non-haem iron catalyzes the oxidation of polyunsaturated fatty acyl components. It indicated that phenolic extracts of all five tested cranberry genotypes may act as effective chelators of free iron ions, as well as scavengers of peroxyl radicals formed during initiation and propagation steps in lipid oxidation.

In a cooked pork model system, EGCG showed antioxidant activity higher than other catechins and typical food antioxidants such as BHT (butylated hydroxytoluene) and α-tocopherol (Shahidi and Alexander 1998). Zhong *et al.* (2012) showed the effect of lipophilized derivatives of EGCG compared to EGCG in terms of antioxidant activity.

In this work, by monitoring the formation of secondary oxidation products as represented by TBARS values and as affected by test compounds and a reference antioxidant BHA (butylated hydroxyanisole), the antioxidant activity of EGCG and its derivatives was determined in a

cooked pork model system. During cold storage (4°C), all meat samples showed increasing concentrations of TBARS with time as a result of lipid oxidation (Figure 4.13). The groups with added EGCG and its derivatives had significantly greater antioxidant efficacy than their parent EGCG molecule. This trend was in accordance with that observed in the β-carotene bleaching assay, which correlated well with the lipophilicity of the compounds. Lipophilicity of antioxidants appeared to be positively associated with their activity in cooked muscle food. There was no significant increasing trend in antioxidant activity with chain length like in β-carotene bleaching assay. All derivatives possessed antioxidant activity comparable to that of BHA, a commonly used antioxidant in foods. The results suggest that lipophilic EGCG derivatives with enhanced antioxidant activity may serve as potential antioxidants in cooked muscle foods, resulting in better oxidative stability and longer shelf life of the products.

4.13. Inhibition against copper-induced LDL-cholesterol oxidation

A large body of literature has shown that EGCG and other tea catechins can inhibit oxidative modification of LDL-cholesterol (Yamanaka *et al*. 1997; Ishikawa *et al*. 1997; Nakagawa *et al*. 1999; Trevisanato and Kim 2000; Gomikawa and Ishikawa 2002), which plays a key role in the pathogenesis of atherosclerosis and heart disease (Sajilata *et al*. 2008).

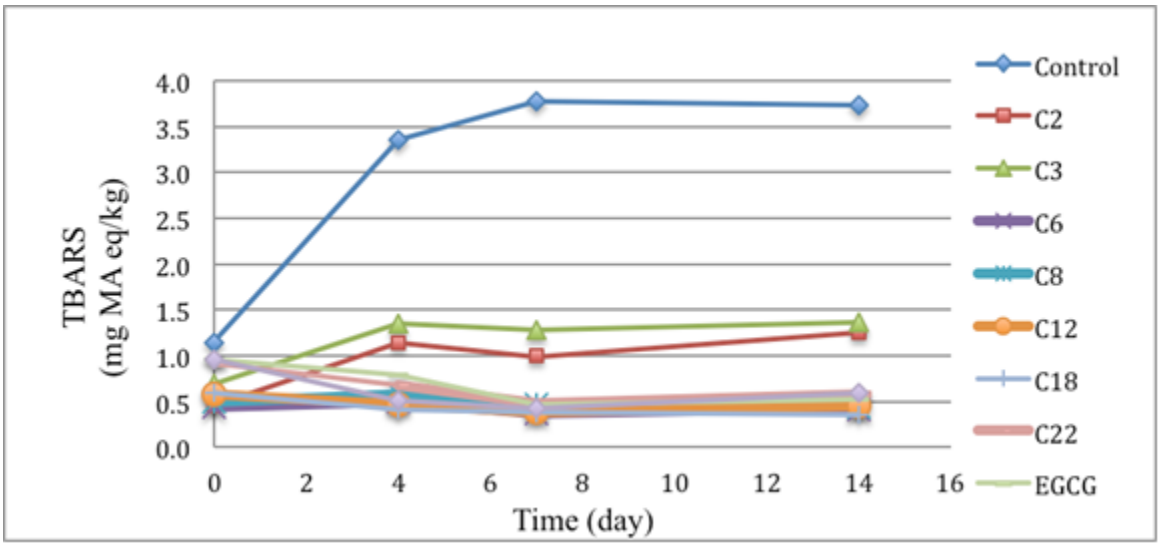


Figure 4.13: TBARS formation in cooked pork as affected by epigallocatechin gallate (EGCG) and its derivatives

(a) EGCG-C2, EGCG tetraethanoate (b) EGCG-C3, EGCG tetrapropanoate
(c) EGCG-C6, EGCG tetrahexanoate (d) EGCG-C8, EGCG tetraoctanoate
(e) EGCG-C12, EGCG tetradodecanoate (f) EGCG-C18, EGCG tetraoctadecanoate
(g) EGCG-C22, EGCG tetradecosahexaenoate

The mechanisms proposed for catechins and other polyphenol antioxidants to inhibit LDL oxidation include scavenging free radicals and other ROS, chelating prooxidant metals, and binding with the apo-lipoprotein B, which promotes the access of antioxidant to the lipids and prevents approaching of oxidation catalysts (Satue-Gracia *et al*. 1997).

In this study, the antioxidant activity of EGCG and its derivatives in inhibiting Cu^{2+} induced LDL-cholesterol oxidation was evaluated and the results are shown in Figure 4.14. After 22 hours of incubation, LDL was oxidized to varying extents depending on the presence and the type of antioxidants employed. Both EGCG and its derivatives were able to inhibit LDL oxidation, possibly through chelation of copper ions and scavenging of free radicals generated.

All the compounds were more effective than the parent EGCG molecule in inhibiting the formation of conjugated dienes. Also there was increased inhibition with increasing of chain length up to EGCG- C12 and then it decreased with chain length. The improved inhibitory activity of the derivatives against LDL oxidation may be due to their greater lipophilicity.

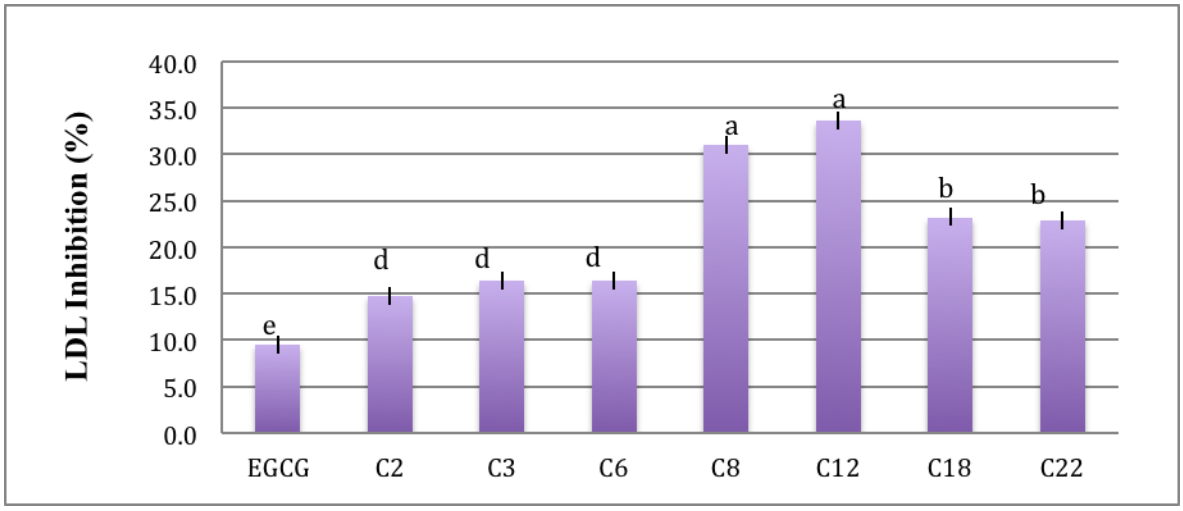


Figure 4.14: Inhibition (%) of epigallocatechin gallate (EGCG) and ester derivatives against copper induced LDL-cholesterol oxidation

(a) EGCG-C2, EGCG tetraethanoate (b) EGCG-C3, EGCG tetrapropanoate
(c) EGCG-C6, EGCG tetrahexanoate (d) EGCG-C8, EGCG tetraoctanoate
(e) EGCG-C12, EGCG tetradodecanoate (f) EGCG-C18, EGCG tetraoctadecanoate
(g) EGCG-C22, EGCG tetradecosahexaenoate

4.14. Inhibition against DNA scission

Oxidative stress in cells caused by ROS such as hydroxyl and peroxyl radicals also leads to DNA damage, which is implicated in mutagenesis and carcinogenesis, among other pathological processes. Oxidative damage to DNA may occur at both the phosphate backbone and the nucleotide bases, resulting in a wide variety of modifications, including strand scission, sister chromatid exchange, DNA-DNA and DNA-protein cross-links, and base modification (Davies 1995). Hydroxyl radical, generated under physiological conditions from hydrogen peroxide (product of superoxide anion dismutation) in the presence of transition metals (known as the fenton reaction), is considered a major source of biologically relevant ROS responsible for oxidative damage of DNA, especially mitochondrial DNA (Perron *et al.* 2008). Peroxyl radicals have also been shown to be involved in inducing DNA strand breakage and base modification (Rodriguez *et al.* 1999). A number of polyphenols have been studied for their effects on DNA oxidation, and both antioxidant and prooxidant activities have been reported. EGCG, for example, has shown both inhibiting and stimulating effects on DNA scission (Hiramoto *et al.* 1996). The dual action of EGCG (inhibitor or stimulator) on DNA oxidation has been found to be concentration dependent (Hiramoto *et al.* 1996; Ohashi *et al.* 2002), and may render either protective or harmful effects to living organisms. EGCG as a prooxidant may have mutagenic effects on normal cells, but may also be beneficial because of its cytotoxic and apoptotic effects on tumor cells (Chen *et al.*1998; Hadi *et al.* 2000).

In this study, the effects of EGCG and its derivatives on hydroxyl and peroxyl radicals induced DNA strand scission were evaluated. As shown in Figures 4.15 and 4.16 (lane 2), the supercoiled plasmid DNA was converted into nicked open circular forms through single

strand cleavage in the presence of hydroxyl and peroxyl radicals. The linear form was not observed, suggesting the absence of double strand breakage under the test conditions. Hydroxyl radical as oxidizing agent was generated from hydrogen peroxide in the presence of Fe^{2+}, which was then converted into Fe^{3+}. EGCG and its derivatives exhibited inhibitory effects against hydroxyl radical-induced DNA scission, possibly through a combination of radical scavenging and ferrous ion chelation. As shown in Table 4.5, the lowest DNA retention was observed for the control (no test compounds added), and presence of EGCG and its derivatives enhanced the retention of native supercoiled DNA.

The ester derivatives of EGCG were more effective than EGCG in protecting DNA against scission, with EGCG-C12 being the most potent antioxidant among all. This may be due to the cumulative factors of lipophilicity, steric features (DNA binding affinity), hydroxyl radical scavenging and metal chelation capacity, all of which could play a role in the overall antioxidant efficacy of the test compounds. The reducing power of EGCG and its derivatives may also be involved, in a negative manner, as reported for many other polyphenols (Hu and Kitts 2001; Azam *et al*. 2004). EGCG with the highest reducing power among all test compounds (results shown in Table 4.3), may reduce ferric ion produced in the Fenton reaction to its more reactive ferrous form, thus stimulating the generation of hydroxyl radical and compromising its overall protection against DNA scission.

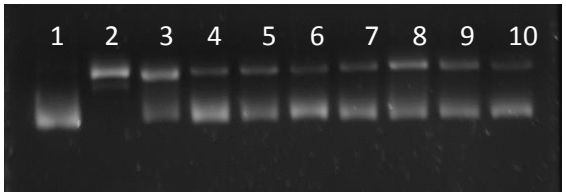


Figure 4.15: Hydroxyl radical induced DNA scission

Lane 1: Blank (DNA only); Lane 2: Control (DNA and H_2O_2 $FeSO_4$); Lane 3: EGCG ; Lane 4: EGCG-C2; Lane 5 EGCG-C3; Lane 6: EGCG-C6; Lane 7: EGCG-C8; Lane 8: EGCG-C12; Lane 9: EGCG-C18, Lane 10: EGCG-C22

(a) EGCG-C2, EGCG tetraethanoate (b) EGCG-C3, EGCG tetrapropanoate
(c) EGCG-C6, EGCG tetrahexanoate (d) EGCG-C8, EGCG tetraoctanoate
(e) EGCG-C12, EGCG tetradodecanoate (f) EGCG-C18, EGCG tetraoctadecanoate
(g) EGCG-C22, EGCG tetradecosahexaenoate

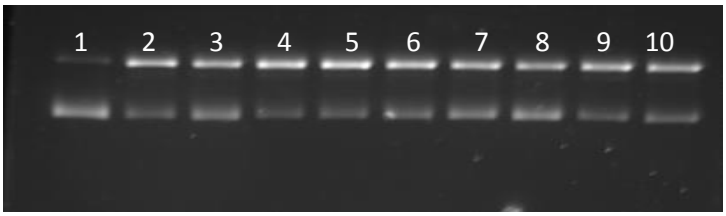


Figure 4.16: Peroxyl radical induced DNA scission

Lane 1: Blank (DNA only); Lane 2: Control (DNA and AAPH); Lane 3: EGCG; Lane 4: EGCG-C2; Lane 5 EGCG-C3; Lane 6: EGCG-C6; Lane 7: EGCG-C8; Lane 8: EGCG-C12; Lane 9: EGCG-C18, Lane 10: EGCG-C22

(a) EGCG-C2, EGCG tetraethanoate (b) EGCG-C3, EGCG tetrapropanoate
(c) EGCG-C6, EGCG tetrahexanoate (d) EGCG-C8, EGCG tetraoctanoate
(e) EGCG-C12, EGCG tetradodecanoate (f) EGCG-C18, EGCG tetraoctadecanoate
(g) EGCG-C22, EGCG tetradecosahexaenoate

EGCG at certain concentrations, where its stimulating effect dominates over scavenging effect on hydroxyl radical, has been reported to accelerate oxidative damage of DNA (Hiramoto *et al.* 1996). This may partially explain the lower antioxidant efficacy of EGCG compared to that of its ester derivatives.

Incubation of supercoiled DNA with peroxyl radical (generated from AAPH) also resulted in single strand breakage of the DNA (Figure 4.16). Addition of EGCG and its derivatives significantly improved the oxidative stability and retention of native DNA, which ranged from 55.75 to 90.02% (Table 4.5). However, the test compounds showed a nearly opposite trend of antioxidant efficacy to that in hydroxyl radical induced DNA scission as the ester derivatives were less effective in inhibiting DNA scission than the parent EGCG molecule. The degree of DNA oxidation varied depending on the nature of the catalysts/pro-oxidents involved (e.g. free radical species, presence of metal ions), leading to different and distinct antioxidant mechanisms and activity of the test compounds in inhibiting DNA scission.

Table 4.5: DNA retention (%) in hydroxyl and peroxyl radical-induced oxidative scission

Compound	DNA retention (%)	
	Hydroxyl radical	Peroxyl radical
Control	27.87 ±0.45[e]	23.27 ±0.75[e]
EGCG	43.27 ±1.75[c]	81.45±1.65 [c]
C2	29.53 ±0.50[e]	56.77±0.78 [d]
C3	30.28 ±0.95[e]	55.43±0.65 [d]
C6	34.43 ±0.21[d]	68.78±0.45 [a]
C8	48.27 ±0.34[b]	69.65±1.23 [a]
C12	55.49 ±0.85[a]	70.75±0.43[a]
C18	40.26 ±1.24[c]	78.45±2.45 [a]
C22	36.65 ±0.22[d]	64.22±1.38 [b]

Values (mean ± SD of three replicates) in the same column with different letters were significantly different at $P<0.05$.

(a) EGCG-C2, EGCG tetraethanoate (b) EGCG-C3, EGCG tetrapropanoate
(c) EGCG-C6, EGCG tetrahexanoate (d) EGCG-C8, EGCG tetraoctanoate
(e) EGCG-C12, EGCG tetradodecanoate (f) EGCG-C18, EGCG tetraoctadecanoate
(g) EGCG-C22, EGCG tetradecosahexaenoate

CHAPTER 5

CONCLUSIONS AND RECOMMENDATION FOR FUTURE RESEARCH

This study investigated the lipophilization of the water-soluble epigallocatechin gallate (EGCG) for its expanded application and improved bioefficiency in food and natural health products. EGCG was esterified with fatty acids of different chain lengths under selected reaction conditions, tetraesters were dominant in the crude products which contained a mixture of EGCG esters with different degrees of substitution. The structures of the isolated tetraesters were identified by MS, and ^{1}H and ^{13}C NMR.

The ester derivatives showed enhanced lipophilicity as expected, and were evaluated for their bioactivities using a number of *in vitro, ex vivo* and *in vivo* tests. The bioactivities examined included antioxidant activity determinations using chemical assays, food and biological model systems.

The EGCG derivatives exhibited excellent antioxidant activity in various *in vitro* chemical assays, including oxygen radical absorbance capacity (ORAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, reducing power, and metal ion chelation capability. EGCG derivatives effectively inhibited lipid oxidation in a bulk oil (stripped corn oil), β-carotene-linoleate emulsion) and a muscle food (pork) model system. Again, the derivatives tested exhibited antioxidant activities similar or superior to that of the EGCG. The ester derivatives, as potential health-promoting agents, were assessed in several biological systems for their antioxidant activity. LDL-cholesterol oxidation was inhibited by the EGCG derivatives to a greater extent than the parent molecule, suggesting their anti-atherogenic effect and, hence, cardioprotective potency. The derivatives also inhibited hydroxyl and peroxyl radical-induced

DNA scission, the free radical-mediated oxidative damage involved in mutagenesis and carcinogenesis. Overall, the results from this study suggest that esterification with long-chain fatty acids can serve as a useful tool to enhance the lipophilicity of EGCG, and that EGCG ester derivatives can be used as lipophilic alternatives to the bioactive EGCG with expanded applications and improved bioefficacy. Moreover, improved bioavailability can be expected as a result of increased liposome membrane affinity and hence enhanced cellular absorption *in vivo*. Thus, the use of EGCG esters as functional food ingredients and pharmaceuticals for health promotion and disease risk reduction is suggested.

However, further investigations need to be carried out on the bioactivities of the EGCG derivatives *ex vivo* and *in vivo* using cell line and animal models, followed by human clinical studies. Meanwhile, scale-up production of ester derivatives of EGCG and other green tea catechins as well as crude tea extracts and evaluation of the economy of the process must be explored. Use of other omega-3 fatty acids as well as selected omega-6 fatty acids can also be considered for their potential, health benefits.

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