

**PREPARATION OF EPIGALLOCATECHIN GALLATE ESTERS
AND EVALUATION OF THEIR ANTIOXIDANT, ANTIVIRAL,
ANTI-INFLAMMATORY AND ANTICANCER EFFECTS**

BY

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Dedicated to my mother Hu Jianming

and

the memory of my father Zhong Jizhi

满纸荒唐言，一把辛酸泪。

都云作者痴，谁解其中味。

ABSTRACT

Epigallocatechin gallate (EGCG), a major green tea polyphenol, has been widely studied and proposed as a functional food ingredient and nutraceutical for therapeutic use. However, EGCG is highly hydrophilic with poor solubility in lipophilic media which hinders its widespread application in foods and affects its absorption *in vivo*, resulting in limited uptake by the cells and bioavailability when ingested orally. Therefore, it is important to alter the hydrophilicity/lipophilicity balance (HLB) of EGCG in order to take better advantage of its multifunctional properties. This work investigated structural modification of EGCG, more specifically esterification with fatty acids of different chain length and degree of unsaturation, as an approach to improving the lipophilicity while maintaining the maximum efficacy or even introducing novel bioactivities not found in the original EGCG. Additional perspectives may exist using the health beneficial omega-3 polyunsaturated fatty acids (PUFA) as acyl donors. For this purpose, lipophilic ester derivatives of EGCG with butyric, stearic, eicosapentaenoic, docosapentaenoic, and docosahexaenoic acids were prepared, and their bioactivities evaluated, including antioxidant activities in different model systems, anti-inflammatory, antiviral and anticancer activities. The results indicated that lipophilization by esterification, especially with omega-3 PUFA, significantly improved the antioxidant and anti-inflammatory activity of EGCG. Moreover, the ester derivatives totally arrested colon cancer in mice, and exhibited an anti-HCV (hepatitis C virus) activity 1700-folds greater than that of the positive control embelin. These findings strongly suggest that the EGCG ester derivatives are of great potential as lipophilic alternatives of the water-soluble EGCG and may serve

as novel functional ingredients for food, cosmetics, and nutraceutical/therapeutical applications and for use in medicine and natural health products.

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LIST OF ABBREVIATION

AA	Arachidonic acid
AAPH	2,2'-azobis (2-aminopropane) dihydrochloride
AC	Aberrant crypt
ACF	Aberrant crypt foci
AOM	Azoxymethane
AP-1	Activator protein
APCI	Atmospheric pressure chemical ionization
BCA	Bicinchoninic acid
BDE	Bond dissociation enthalpy
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CD	Conjugated diene
COX-2	Cyclooxygenase-2
CRC	Colorectal cancer
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
DHASCO	Docosahexaenoic acid single cell oil
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid

DPPH	1,1-diphenyl-2-picrylhydrazyl
DS	Degree of substitution
EC	Epicatechin
ECG	Epicatechin gallate
EDTA	Ethylenediaminetetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
EPR	Electron paramagnetic resonance
GAE	Gallic acid equivalents
GC-MS	Gas chromatography-mass spectrometry
GTP	Green tea polyphenol
HCA	Heterocyclic amine
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLB	Hydrophilic-lipophilic balance
HPLC	High performance liquid chromatography
iNOS	Inducible nitric oxide synthase
IP	Ionization potential
IP	Induction period

LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MLV	Multilamellar vesicle
NF- κ B	Nuclear factor- κ B
NMR	Nuclear magnetic resonance
NO	Nitric oxide
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate buffer solution
PC	Phosphatidylcholine
PF	Protection factor
PG	Propyl gallate
PGE ²	Prostaglandin E ²
PMSF	Phenyl methyl sulfonyl fluoride
PUFA	Polyunsaturated fatty acids
PVDF	Polyvinylidene fluoride
RMCD	Randomly methylated cyclodextrin
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-polymerase chain reaction
SA	Stearic acid
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TAE	Tris-acetic acid-ethylenediaminetetraacetic acid

TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TBHQ	<i>tert</i> -Butylhydroquinone
TCA	Trichloroacetic acid
TE	Trolox equivalents
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMP	1,1,3,3-tetramethoxypropane
TNF	Tumor necrosis factor
UV-DAD	Ultraviolet-diode array detector

CHAPTER 1

Introduction and overview

1 Phenolics

1.1 Classification, chemistry and occurrence

Phenolics are naturally occurring compounds widely distributed in the plant kingdom and important components of human daily diets. They are secondary metabolites synthesized by plants from phenylalanine and to a lesser extent tyrosine, both during normal development and in response to stress conditions (Harborne 1982; Beckman 2000). Phenolics comprise the largest group among the approximately 50,000 secondary plant metabolites (Grassmann *et al.* 2002). They are important constituents of plants with multiple functions from overall fitness regulation to plant defence mechanism against insects, pathogens and extreme environmental conditions. As dietary phytochemicals for humans, phenolics display a broad range of functional and biological activities, depending on their chemical structures. Phenolics vary in their structures from simple phenols to highly polymerized compounds and are characterized by an aromatic ring with one or more hydroxyl group substituents. The aromatic ring(s) may also bear other functional substituents such as esters, methyl ethers and glycosides, and thus contributing to the great diversity of their structures. There are more than 8000 phenolic compounds identified in fruits, vegetables, seeds and related products, which can be divided into several common categories based on their chemical structures. Phenolics are generally classified into simple phenols, phenolic acids, coumarins, stilbenes, flavonoids, lignans and their polymerized counterparts, such as tannins and lignins (Table 1-1). These groups

Table 1-1. Major classes of naturally occurring phenolic compounds.

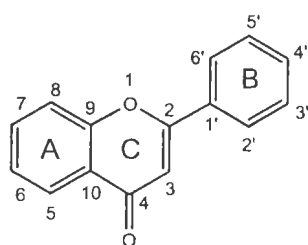
Classes	Basic skeleton	Examples
Simple phenols	C6	Cresol
Phenolic acids		
Hydroxybenzoic acids	C6-C1	<i>p</i> -Hydroxybenzoic acid
Hydroxycinnamic acids	C6-C3	Caffeic acid
Coumarins	C6-C3	Scopolin
Stilbenes	C6-C2-C6	Resveratrol
Flavonoids	C6-C3-C6	Catechin
Lignans	C6-C4-C6	Secoisolariciresinol

can be further classified into many subcategories. Simple phenols include monophenols (e.g. cresol) and diphenols (e.g. hydroquinones). Phenolic acids are mostly either hydroxybenzoic acid (C6-C1) or hydroxycinnamic acid (C6-C3) derivatives with different number and location of hydroxy and/or methoxy substituents on the phenyl ring. Caffeic and ferulic acids are among the most common phenolic acids from dietary sources; the former is found abundantly in fruits and vegetables, most often as esters or other conjugates, and the latter is present in cereals, esterified to hemicelluloses in the cell wall (Scalbert *et al.* 2005). Phenolic acids are the major non-flavonoids 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 (C6-C2-C6) are present mostly as heartwood constituents of trees while in smaller amounts in the vegetative parts, and resveratrol is the most widespread stilbene in nature (Seigler 1998). Lignans (C6-C4-C6) are often found in seeds and nuts, and are most abundant in flax and sesame seeds.

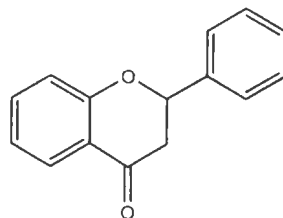
Flavonoids are ubiquitous in plants and account for about 60% of dietary polyphenols (Bravo 1998). Flavonoids exhibit multiple biological activities and have been implicated in the risk reduction of many chronic diseases and health conditions. The structures of flavonoids consist of two aromatic rings linked by three carbons in an oxygenated heterocycle with variable oxidation levels. The fused aromatic ring is referred to as A ring, the phenyl constituent as B ring, and the heterocyclic pyran as C ring. Flavonoids with this common characteristic diphenylpropane (C6-C3-C6) skeleton exhibit a broad variation in structural specificity, which differentiates them into several subclasses. More than 6500 different flavonoid structures have been described (Harborne and Williams

2000), among which 7 major groups are usually distinguished. These include flavones, flavanones, flavonols, flavanonols, flavanols, isoflavones, and anthocyanidins, varying in saturation and oxidation status of the C ring as well as the position of B and C ring linkage (Figure 1-1). 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, which contain 2, 3-double bonds, 4-keto and 3-hydroxyl groups in the C ring, and flavanols lacking the double bonds and keto groups are the most widespread in plants. More than 50% of plants contain quercetin, kaempferol and myricetin in their leaves and flowers (Kartsova and Alekseeva 2008). Isoflavones, differing from flavones in having their B ring attached at C-3 of the C ring, are rich in soybean and its fermented products, and have attracted special interest for their estrogen-like activity. Anthocyanidins with a pyrylium central C ring are aglycones of anthocyanin pigments in flowers, leaves, fruits and roots of many plants.

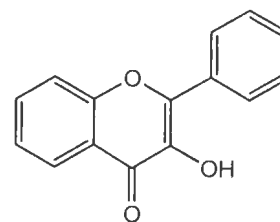
In addition to the major groups, other flavonoids with slightly varied structures have also been identified. Among these are neoflavonoids, which have a C-4 linkage of the B and C rings, and some minor flavonoids such as chalcones, aurones and auronols (Marais *et al.* 2006). Structural variations within the flavonoid subclasses may derive 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 from natural sources often exist as gly-



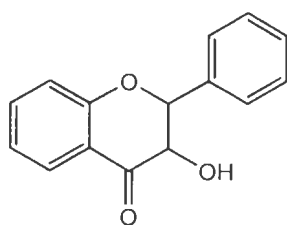
flavone



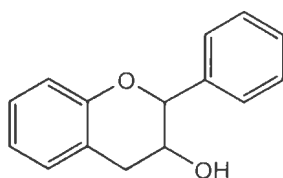
flavanone



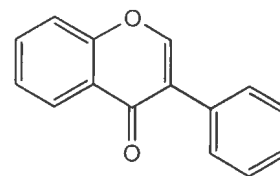
flavonol



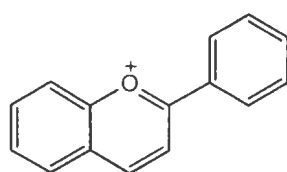
flavanonol



flavan-3-ol



isoflavone



anthocyanidin

Figure 1-1. Basic structures of major flavonoids.

cosides 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, while 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 quercetin, 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; daidzein, the main isoflavone in soybean, among others (Scalbert *et al.* 2005).

Distribution of phenolics in plants is not uniform and varies both qualitatively and quantitatively among plant species as well as tissues. Factors affecting phenolic profiles and contents in plants include plant species and cultivar, cultivation techniques employed, growing conditions and maturation stage, among others. Some phenolic compounds may show increased content under stress conditions such as UV radiation, air pollution, wounding, infection by pathogens and parasites, and exposure to extreme temperatures, etc. 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). When consumed as dietary components for humans, phenolics in plant-derived food are also influenced by processing and storage conditions involved. For instance, peeling, chopping, boiling, microwaving and frying lead to loss of quercetin conjugates of up to 75%, as observed for onions; whereas cold-stored whole parsnips had a 33-fold increase in their furanocoumarin content (Makris and Rossiter 2001).

The phenolic distribution within the plants varies at the tissue, cellular and subcellular levels. Phenolics are generally present at higher levels in the outer layers of plants than in their inner parts (Prez-Ilzarbe *et al.* 1992). At the cellular level, cell walls contain more insoluble phenolics, while soluble phenolics are mainly found in the cell vacuoles compartment (Wink 1997). This tissue and cellular distribution pattern of phenolics takes advantage of their specific characteristics and is believed to contribute to the mechanical strength, thermal stability and cell defence against stress and pathogens as well as regulation of growth and morphogenesis of the plant (Scalbert 1993; Wallace and Fry 1994). Phenolics in plants may act as phytoalexins, antifeedants, antioxidants, attractants for pollinators, and protective agents against UV, among others (Shahidi and Naczki 2004). Although phenolics are ubiquitous, individual compounds may vary and be restricted to specific families and species, thus contributing to the characteristic pigmentation and sensory properties of plant-derived foods. Phenolics may, at least partially, be responsible for the bitterness and astringency, color, odor, and oxidative stability of foods containing them (Naczki and Shahidi 2003). Phenolics are highly reactive compounds and substrates for various enzymes, including polyphenol oxidases, peroxidases, glycosidases and esterases. They undergo a number of chemical and enzymatic reactions during harvesting, processing and storage of the plant material, resulting in a complex mixture of products whose composition and concentration are still poorly understood (Cheynier 2005). The phenolics and their derived products contribute to major sensory characteristics of many plant foods and beverages, and more importantly are associated with numerous health benefits of consumption of fruits, vegetables, nuts and other plant-related foods.

1.2 Bioactivities and bioavailabilities

Consumption of fruits and vegetables has been linked to a lower risk of many diseases, including inflammation, cardiovascular disease (CVD), cancer, diabetes and neurodegenerative diseases. Although most research has focused on their vitamins and other micronutrients, an association between health benefits of plant-rich diets and their bioactive phytochemicals is being established. The health promoting effects of fruits, vegetables and related products have recently been largely attributed to the presence of phenolics. 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. The mechanisms of these biological activities of phenolics and their related health effects have been reviewed (Scalbert *et al.* 2005; Aron and Kennedy 2008).

Phenolics are known as powerful antioxidants by inhibiting oxidative deterioration of foods and protecting against oxidative stress-mediated diseases. They may act as free radical scavengers, singlet oxygen quenchers, metal chelators, reducing agents and excellent synergists with other antioxidants, and thus inhibiting oxidation of biomolecules (e.g. membrane lipids, LDL-cholesterol, proteins and DNA) and suppressing related pathologies such as inflammation, atherosclerosis and carcinogenesis. These compounds are also able to modify physiological and pathological conditions independently from or in combination with antioxidant mechanisms (Biesalski 2007). Phenolics act as anti-inflammatory agents by down-regulation of some inflammatory mediators at expression level and direct inhibition of the activity of the mediator enzymes (Shahidi and Zhong 2009). The cholesterol--lowering effect and inhibition against LDL-cholesterol oxidation

contribute to the cardioprotective property of many plant polyphenols. The French paradox, an inverse relationship between red wine consumption and mortality rates from CVD and certain cancers, has been attributed to the polyphenols present in the skin of grapes, particularly resveratrol, which is also found in peanuts and mulberries (Boehm 2000). Grape anthocyanidins were found to suppress the stomach mucosal injury induced by acidified ethanol, and their antiulcer property was believed to be due to both antioxidant activity and the ability to bind proteins (Saito *et al.* 1998). Phenolics have proven to possess anticancer activities and are considered as good candidates for food-derived drugs. A study on the Zutphen elderly revealed that flavonoid intake from fruits and vegetables was inversely associated with all-cause cancer risk and cancer of the alimentary and respiratory tract (Hertog *et al.* 1994). Quercetin has shown vasoactive and gastroprotective effects, as well as inhibition against heterocyclic amines (HCA)-induced mutagenesis (Alarcon 1994; Kahraman *et al.* 2003). Proanthocyanidins A₂ treatment effectively modulated expression of antioxidant enzymes and decreased UVB-induced skin tumors (Pan and Ho 2008). Isoflavones in soybean exhibit estrogenic activities and may protect against hormone-related cancer and CVD (Adlercreutz and Mazur 1997; Lichtenstein 1998). Consumption of soybean isoflavones reduced the incidence of breast, urinary tract and colon cancers, and provided protection against coronary heart diseases and osteoporosis (Brandi 1997). Genistein, the main bioactive isoflavone in soybean with anti-estrogenic activity has shown antitumor capabilities supported by many *in vitro*, cellular, animal model and clinical studies (Ravindranath *et al.* 2004). Antimicrobial activities of phenolics have also been reported. Cranberry proanthocyanidins can competitively inhibit cellular adherence of uropathogenic strains of P-type *E coli* to

mucosal cells in the urinary tract (Howell 2002). Other bioactivities of phenolics include antiviral, anti-allergic, antidiabetic and analgesic properties, among others (Musci 1986; Nguyen *et al.* 1999; Julfikar Hossain *et al.* 2008).

In addition to their health-promoting capability, some phenolics may exert antinutritional properties that are of great importance to both consumers and producers. Toxicity data on food phenolics, however, is still quite inadequate. Phenolics at high intake levels may act as prooxidants, 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 pose a serious health risk for humans. However, daily intake of phenolics from common food sources produces very low toxicities because of their low absorption, rapid metabolism and efficient defence 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, the consumption of flavonols has been estimated at 20-25 mg/day in the US (Biesalski 2007). The emerging bioactivity data on plant phenolics by various approaches have indicated that the activities established in *in vitro* experiments may not necessarily reflect their protective effectiveness in living organisms. This is due to the low bioavailability of ingested phenolics caused by both poor absorption and rapid elimination in the body. Information on absorption and metabolism of phenolics is still fragmentary and controversial. The structure-activity relationship obtained from cellular studies suggested that the amount of the phenolic compounds incorporated governs the dose-dependency (Nakayama *et al.* 2006). Cellular uptake differs greatly among individual phenolics, and

hence the abundance of a phenolic compound in our 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. The presence and concentration of these phenolics and/or their metabolites in blood indicate 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 (Higuchi *et al.* 1981). It is supposed that most phenolics are incorporated into the 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 compound for the cell membrane. Studies have been carried out on membrane affinities of different phenolics, including gallic and caffeic acid esters, curcuminoids, flavonols, and isoflavones, among others, and have concluded that the lipophilicity, rigidity and planer structure of the phenolic molecule have a key effect on its affinity for cell membrane and hence its cellular absorption (Murota *et al.* 2002; Nakayama *et al.* 2006). In addition to cellular incorporation, the metabolism, distribution and excretion are also important in determining the bioavailability of dietary phenolics. The phenolic metabolites present in blood resulting from digestive and hepatic activity often differ from the native compounds. The food-derived phenolic compounds in their ester, glycosides or polymer forms are either absorbed as such or are hydrolyzed by intestinal enzymes or the colonic microflora to release their aglycones. The phenolic aglycones undergo extensive metabolism in the small intestine and later in the liver and other organs.

Phenolics are conjugated by methylation, sulfation, glucuronidation or a combination resulting in decreased hydrophobicity, which facilitates their urinary and biliary excretion (Ramos 2007). Although the metabolism of phenolics has not yet been fully characterized, related studies have shown great variability in preferential pathways among individuals. Manach *et al.* (2004) reported the intermolecular conjugation of quercetin with albumin, which supports its slow elimination and hence extended its half-life. Moreover, flavonoids may go through an enterohepatic cycle, i.e. be secreted in bile to the duodenum and then reabsorbed, thus evoking a longer half-life (Ramos 2007). The overall bioavailability of phenolics is the combined effects of cellular uptake, metabolism, distribution and elimination. Manach *et al.* (2005) summarized 97 studies of various classes of phenolics, whose bioavailabilities were presented in terms of maximal plasma concentration, time to reach the maximal plasma concentration, the area under the plasma-concentration-time curve, and the elimination half-life, etc. The intake of 50 mg of aglycone equivalents resulted in plasma concentration of total metabolites ranging from 0 to 4 $\mu\text{mol/L}$, and the relative urinary excretion ranging from 0.3 to 43% of the ingested dose, depending on the phenolic species. Among the phenolic compounds studied, gallic acid and isoflavones were best absorbed, followed by catechins, flavanones and quercetin glucosides, but with different kinetics. Proanthocyanidins, galloylated tea catechins and anthocyanins were among the least absorbed phenolics.

Dietary phenolics may also undergo biotransformation by gut microflora prior to their absorption. Although there are potentially thousands of different phenolic compounds in the diet, they are often transformed to a much smaller number of metabolites. This biotransformation may lead to modulation of the bioactivities of phenolics. It is therefore

suggested that health effects of dietary phenolics should be attributed to both their bioactive metabolites and modulation of the intestinal bacterial population. Research has been conducted to identify microorganisms involved and transformation pathways of particular phenolic groups. Gut microflora that could participate in metabolism of phenolics, particularly flavonoids, include *Bacteroides*, *Clostridium*, *Eubacterium*, *Ruminococcus*, *Eggertheilla*, *Streptococcus*, *Lactobacillus*, and *Bifidobacterium* genera (Kim *et al.* 1998). Meanwhile, phenolics with antimicrobial activities may inhibit the growth of certain microorganisms in the gut. This 2-way phenolics-microflora interaction has been discussed in a recent review by Selma *et al.* (2009).

1.3 Sources, preparation and analysis

Phenolics are present abundantly in a wide range of natural sources, 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, especially flavonols (e.g. quercetin, kaempferol, myricetin and isorhamnetin), proanthocyanidins (e.g. procyanidins and prodelphinidins) and phenolic acids (mainly in esterified form, e.g. gallic, ferulic, sinapic, coumaric, caffeic and chlorogenic acids). The total phenolic content in apple ranged from 1000 to 6000 mg/kg fresh weight (fw) in most varieties, and may be over 10,000 mg/kg fw in some cultivars (Sanoner *et al.* 1999; Gorinstein *et al.* 2001). Blueberries contain substantial amounts of anthocyanins (127-1973.4 mg cyaniding 3-glucoside equivalents/kg fw), among which 3-glucosides, 3-galactosides and 3-arabinosides of cyanidin, delphinidin, peonidin, petunidin, and malvidin were isolated and identified (Mazza and Miniati 1993). Cranberries are another excellent source of

anthocyanins, with the total contents ranging from 180 to 656 mg/kg fw in its fruits (Wang and Stretch 2001). High levels (1g/kg fw) of phenolic acids were also present in cranberries (Zuo *et al.* 2002). Phenolics in citrus fruits include mainly cinnamic acid derivatives, coumarins and flavonoids. Flavanone glycosides such as naringin, neoeriocitrin and hesperidin comprise 50-80% of the total flavonoids in citrus fruits (Kanes *et al.* 1992). Polymethoxylated flavones in citrus peel are unique phenolic compounds found in citrus species (Ortuno *et al.* 2002). 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 as well as in wilting berries (Versari *et al.* 2001). Pomegranates are rich in hydrolysable tannins, particularly the gallagyl type tannins (e.g. punicalagin), which are present in pomegranate juice at levels of 1500-1900 mg/L (Gil *et al.* 2000). In addition to the raw fruits mentioned above, the fermentation products from fruit material also display a variety of phenolic compounds, as observed for different types of wines and fruit vinegars (Shahidi *et al.* 2008).

Vegetables provide a rich source of phenolic compounds. The content and composition of phenolics in various vegetable groups have been reviewed (Shahidi *et al.* 2010). Fruit vegetables such as peppers, eggplant, tomatoes, bitter melon, and pumpkin are reported 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 one ($\geq 95\%$ of the total flavonoids) (Galdon *et al.* 2008). Root (carrots, beets) and tuber (sweet potatoes, potatoes) vegetables contain chlorogenic and caffeic

acids as major phenolic acids and betalains contributing to the color of beets. Green leafy vegetables such as lettuce, spinach and kale have high levels of flavonoids. They are present in spinach leaves at 807-2241 mg/kg fw (Howard *et al.* 2002). Phenolics are also found in flowery (broccoli and artichoke) and stem (asparagus) vegetables at varying levels and compositions.

Cereals, legumes, nuts and oilseeds are recognized as good sources of phenolics. The highest concentrations of phenolic acids and flavonoids are located in the aleurone layer in grains and seeds, and therefore removal of this layer in milling and pearling processes leads to loss of phenolics. 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). Major phenolic acids present in bean hulls include vanillic, caffeic, *p*-coumaric, ferulic and sinapic acids. These phenolic acids were also found in wheat bran at higher levels than in its corresponding flour (Liyana-Pathirana and Shahidi 2007). Anthocyanidins such as delphinidin, cyanidin and procyanidins B2, C1, C2 and X were identified in red, brown and black bean hull extracts (Madhujith *et al.* 2004). Soybeans contain significant amounts of isoflavones (472-4200 mg/kg), which are of much interest because of their special health effects (Wang and Murphy 1994). The predominant phenolic compounds of oilseeds belong to the phenolic acid, coumarin, flavonoid, tannin and lignin groups of compounds. Flaxseed, for example, contains 350-710 mg/kg of total flavonoids and 3700 mg/kg of secoisolariciresinol, a major lignan of flaxseed (Oomah *et al.* 1996; Mazur *et al.* 1996). Unlike the 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 contributed some 6-20% to the total phenolics in canola meal. More recent studies showed that both wheat and barley had large proportions of bound phenolics (60 and 75% of the total phenolics, respectively) (Liyanapathirana and Shahidi 2006; Madhujith and Shahidi 2009). It has been shown that colonic fermentation of such material, which survives gastrointestinal digestion, may lead to the release of some of the bound phenolics and hence exert their unique health benefits in the colon after absorption.

Herbs and spices are loaded with high concentrations of phenolics, which are responsible for the medicinal properties of many herbs and their extracts. However, the compositional data on phenolics in herbs and spices are incomplete. Nakatani (2003) reported a number of phenolic compounds derived from herbs and spices. These include several phenolic diterpenes from rosemary and sage, phenolic carboxylic acids and glycosides from oregano and marjoram, ginerol-related compounds and diarylheptanoids from common ginger, biphenyls and flavonoids from thyme, phenolic amides from pepper and chilli peppers, carbazoles from curry leaves, lignans from Papua mace and polymethoxylated phenols from *Alpinia*. Zheng and Wang (2001) studied 27 culinary herbs and 12 medicinal herbs for their phenolic compounds. The total phenolic content for the culinary herbs ranged from 260 to 17510 mg gallic acid equivalents (GAE)/kg fw. Among the identified phenolic compounds, rosmarinic acid was predominant in *Salvia officinalis*, *Thymus vulgaris*, *Origanum × majoricum*, and *P. longiflora*, whereas quercetin and kaempferol were the major aglycones in *Ginkgo biloba* leaves. Essential oils of many herbs and spices were demonstrated to contain considerable amounts of phenolics, which

are directly related to their plant origin (Theissedre and Waterhouse 2000). Essential oils of clove and Spain red thyme were among those with highest total phenolic contents (1567 and 972 mg/ml, respectively). 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 preparation of plant phenolic extracts in industry or laboratories. These include solvent, solid phase, supercritical fluid extraction and high-pressure liquid extraction, among others (Kartsova and Alekseeva 2008). In addition to extraction from natural plant sources, some high-value phenolic compounds are also prepared by chemical synthesis and plant cell cultures as well as biosynthesis by microorganisms. Production of phenolics, particularly flavanones, using microorganisms through heterologous protein expression has been discussed by Fowler and Koffas (2009). Separation of phenolics may be necessary when one or more specific compounds are of interest in various plants/food materials and biological fluids (e.g. urine, plasma, blood serum, saliva). Chromatography such as HPLC and TLC, and electrophoresis such as capillary zone electrophoresis and micellar electrokinetic chromatography are the main physicochemical methods for separation of phenolics (Kartsova and Alekseeva 2008).

2 Phenolics as antioxidants for food and health

2.1 Lipid oxidation

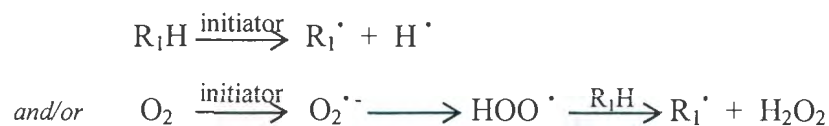
Oxidation is a major cause of quality deterioration of products and negatively affects the integrity of biological systems. It occurs in foods during harvesting, processing and storage, giving rise to the development of off-flavors, loss of essential fatty acids, fat-soluble vitamins and other bioactives, and even formation of potentially toxic compounds, thus making the lipid or lipid-containing foods unsuitable for consumption. Lipids in living organisms may also undergo oxidation during normal aerobic metabolism or upon exposure to other oxidizing agents (Beckman and Ames 1998). Oxidation *in vivo* has destructive cellular effects and has been associated with pathophysiology of numerous diseases and health conditions including inflammation, atherosclerosis, cancer and aging, among others (Dalton *et al.* 1999; Davies 2000; Kruidenier and Verspaget 2002; Floyd and Hensley 2002).

Lipids are susceptible to oxidation in the presence of catalytic systems such as light, heat, enzymes, metals, metalloproteins and microorganisms, leading to complex processes of autoxidation, photooxidation, thermal, and enzymatic oxidation, most of which involve free radicals and/or other reactive species as the intermediate (Vercellotti *et al.* 1992; Shahidi 2000). Autoxidation is the most common process among all and is defined as the spontaneous reaction of lipids with atmospheric oxygen through a chain reaction of free radicals. The process can be accelerated at higher temperatures as in thermal oxidation, with increased content of polar matters, foaming, color and viscosity, as observed in deep frying (Perkins 1992). Photooxidation involves excitation of a photosensitizer and energy transfer to lipid molecules or oxygen. Oxidation can also be catalyzed by certain enzymes

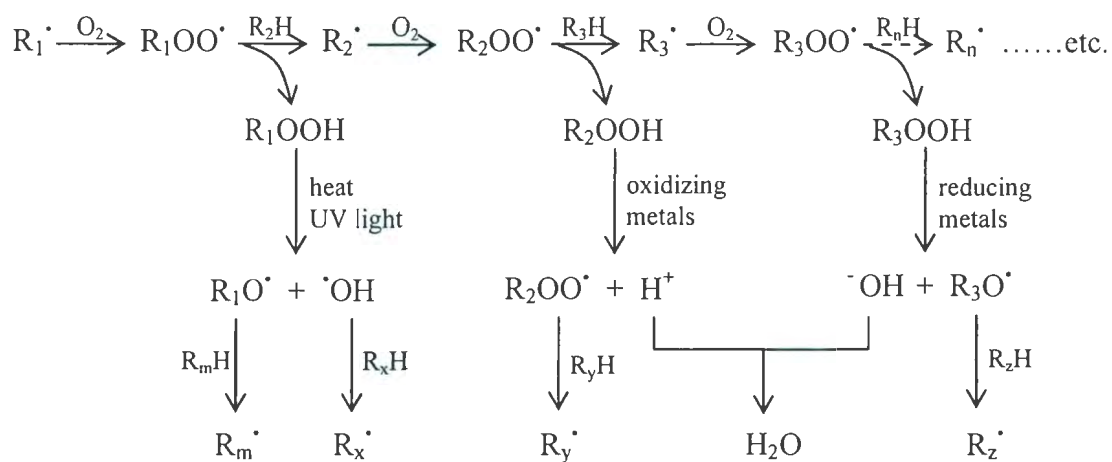
such as lipoxygenases. Unsaturated fatty acids are the major reactants affected by such reactions, whether they are present as free fatty acids, simple alkyl esters, acylglycerols or phospholipids. The polyunsaturated fatty acids (PUFA) in membrane phospholipids and cholesterol, especially low density lipoprotein (LDL)-cholesterol, are the major target substrates of oxidation *in vivo*, causing irreversible cellular and tissue damage.

It has been widely accepted that lipid autoxidation occurs via a free radical chain mechanism that proceeds through three distinct stages of initiation, propagation, and termination, leading to a series of complex chemical changes (Shahidi and Zhong 2005). A simplified scheme explaining the mechanism of autoxidation is given in Figure 1-2. Unsaturated lipid molecules, in the presence of initiators such as heat, light/ionizing radiation and metal ions/metalloproteins, lose a hydrogen atom and produce free radicals. Lipid radicals subsequently react with oxygen to form peroxy radicals, which act as the chain carriers of the rapid progressing reaction by attacking a new lipid molecule. This self-propagating and self-accelerating reaction may be repeated several thousand times until no hydrogen source is available or the chain is interrupted, for example, by antioxidants (deMan 1999). Oxidation normally proceeds very slowly at the initial stage, until it reaches a sudden increase after an induction period. The initiation process is quite complex and not yet fully understood; however, it is believed to involve removing of a hydrogen atom from the lipid molecule. The loss of hydrogen atom takes place most readily at the carbon next to the double bond in the olefinic fatty acids, due to a lower C-H bond energy. The bond energies for bisallylic, allylic and alkyl hydrogens are 75, 88 and 101 kcal/mol, respectively, which explains why highly unsaturated fatty acids are more susceptible to oxidation than their saturated or less saturated counterparts (Erickson

Initiation:



Propagation:



Termination:

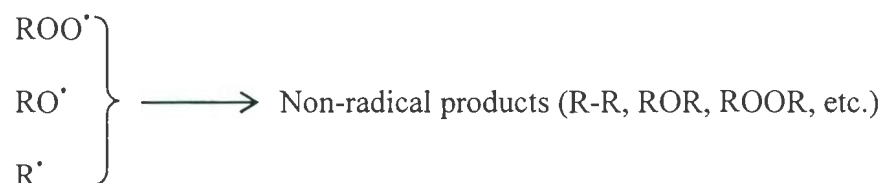
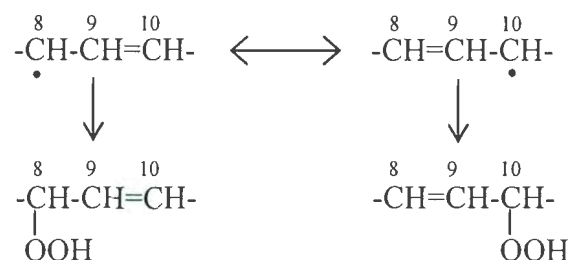


Figure 1-2. Lipid autoxidation pathways.

2002). The lipid free radicals generated during initiation and propagation are stabilized by radical resonance (as shown below), which also leads to shift of double bonds and *cis-trans* isomerization.



Conjugated dienes and trienes are produced, due to the rearrangement of the methylene-interrupted double bonds in PUFA, and are used as an index of oxidation (Shahidi and Zhong 2005). During propagation, lipid hydroperoxides are produced as primary products of oxidation. They are unstable and break down to a wide range of secondary oxidation products, including aldehydes, ketones, alcohols, hydrocarbons, volatile organic acids and epoxy compounds, among others, some of which have undesirable odors with very low threshold values. Meanwhile, alkoxyl (RO \cdot), peroxy (ROO \cdot), hydroxyl (\cdot OH) and new lipid radicals (R \cdot) are generated from decomposition of hydroperoxides, and further participate in the chain reaction of free radicals. In the termination stage of oxidation, radicals neutralize each other through radical-radical coupling or radical-radical disproportionation to form stable non-radical products, including a variety of polymeric products (Erickson 2002).

Free radicals play a key role as important intermediates in the chain reaction of lipid oxidation. Free radicals are unstable, highly reactive and energized molecules with one or

more unpaired electron(s). They capture electrons from other compounds to gain stability. Examples of oxygen-derived free radicals include superoxide anion, hydroxyl, hydroperoxyl, peroxy, and alkoxyl radicals, all of which are referred to as reactive oxygen species (ROS). Superoxide anion ($O_2^{\cdot-}$), which is formed from the single-electron reduction of molecular oxygen through a variety of sources under both physiological and pathophysiological conditions, is a primary ROS. Approximately 2-5% oxygen consumed by a cell is reduced to oxygen radicals (Floyd and Hensley 2002). Superoxide anion can be neutralized by superoxide dismutase (SOD) to yield hydrogen peroxide; the latter is a known non-radical ROS and, although less reactive than superoxide anion, has a longer lifetime and a higher diffusibility and can cross biological membranes freely (Tylicki *et al.* 2003). Moreover, it can react in Fenton reaction with reduced metal ions such as Fe^{2+} and Cu^+ to form hydroxyl radical ($\cdot OH$), which is considered as the most harmful ROS (Lubec 1996). Other ROS include hydroxyalkenals, singlet oxygen and ozone, among others. Nitrogen dioxide and peroxynitrite anion formed from the interaction of nitric acid with superoxide anion are also strong oxidants, and together with nitric oxide (NO) are known as reactive nitrogen species (RNS). Singlet oxygen is generated photochemically from molecular oxygen and is usually involved in photooxidation processes. Table 1-2 summarizes the reactive species involved in food and biological oxidation. Excessive ROS/RNS generated during oxidation may attack, in principle, all cellular and extracellular components, including cholesterol, proteins, enzymes, lipoproteins and DNA, leading to cell disruption and tissue damage.

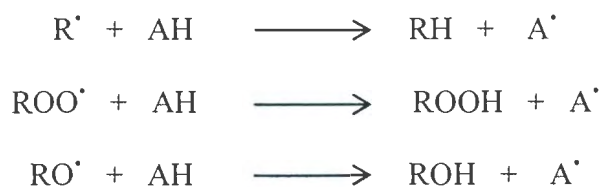
Table 1-2. Major reactive species in food and biological systems.

Free radicals	Non-radicals
<i>Reactive oxygen species (ROS)</i>	
Superoxide anion ($O_2^{\cdot-}$)	Hydrogen peroxide (H_2O_2)
Hydroxyl ($\cdot OH$)	Hydrochlorous acid ($HOCl$)
Alkyl ($R\cdot$)	Singlet oxygen (1O_2)
Alkoxyl ($RO\cdot$)	Ozone (O_3)
Peroxyl ($ROO\cdot$)	Hydroxyalkenals
Hydroperoxyl ($HOO\cdot$)	
<i>Reactive nitrogen species (RNS)</i>	
Nitric oxide ($\cdot NO$)	Peroxynitrite ($ONOO\cdot$)
Nitrogen dioxide ($NO_2\cdot$)	Alkyl peroxynitrite ($LOONO$)
	Dinitrogen trioxide (N_2O_3)
	Nitrous acid (HNO_2)

2.2 Phenolics as antioxidants

Antioxidants have been used by food manufacturers world-wide for stabilizing food lipids and thus preventing quality deterioration and increasing shelf-life of the products. In the health-related areas antioxidants are used for health promotion due to their ability to protect the body against oxidative damage. Antioxidants are substances that when present at low concentrations compared to that of an oxidizable substrate markedly delay or prevent its oxidation. Antioxidants that fit in this definition include free radical scavengers, singlet oxygen quenchers, inactivators of peroxides and other ROS, metal ion chelators, quenchers of secondary oxidation products, and inhibitors of pro-oxidative enzymes, among others (Shahidi and Zhong 2007). Antioxidants may exert their inhibitory effect against oxidation via different mechanisms and with varied activities. They may be broadly classified based on their mode of action into primary antioxidants which break the chain reaction of oxidation by scavenging free radical intermediates, and secondary antioxidants which prevent or retard oxidation by deactivation of oxidation initiators/accelerators or regeneration of primary antioxidants.

Phenolics are the major group of primary antioxidants, and their antioxidant activity arises from their unique structures, which render a superior electron donating capability over many other compounds due to the stable resonance hybrids of the phenyl ring. Phenolic compounds are able to neutralize free radicals by donating a hydrogen atom, as shown below.



The resultant phenolic radicals are stabilized by delocalization of the unpaired electron around the phenol ring to form stable resonance hybrids (Reische *et al.* 2002). These radicals have low reactivity and generally do not initiate formation of new radicals, thus breaking the chain-reaction of free radical propagation (Nawar 1996). Moreover, the phenolic radicals so formed can further scavenge free radicals by participating in the termination of oxidation.



Therefore, phenolic antioxidants can trap two lipid radicals by donating a hydrogen atom to one radical and receiving an electron from another radical to form stable non-radical products (Young and Woodside 1999).

Phenolic compounds may also act as secondary antioxidants that prevent or retard oxidation by suppressing the oxidation promoters, including metal ions, singlet oxygen, pro-oxidative enzymes and other oxidants. Phenolics, as reducing agents, are capable of reducing lipid peroxides and related oxidants through redox reactions, and are also referred to as oxygen scavengers. Metal ions as catalysts of oxidation reaction by producing free radicals through electron transfer (as shown below) may be chelated by some polyphenols.



Polyphenol metal chelators can decrease the prooxidant effect of metal ions by forming a thermodynamically stable complex and reducing their redox potentials (Reische *et al.* 2002). Some phenolic compounds are also able to quench singlet oxygen by a mixture of physical and chemical processes, as reported for some 2,4,6-trisubstituted phenolic compounds (Gorman *et al.* 1984). Some other phenolic antioxidants catalyze decomposition of hydroperoxides into non-radical species, or absorb UV radiation thus protecting lipids from UV-induced photooxidation. Phenolic compounds may exert their antioxidant activity by one or a combination of different mechanisms. The operative or dominant mechanism in a particular situation determines to a great extent the kinetics and hence the activity of the antioxidant (Antolovich *et al.* 2002).

The effectiveness of phenolics as antioxidants is influenced by numerous factors, including their chemical structures, concentration, temperature, type of oxidation substrate and physical state of the system as well as presence of prooxidants and synergists (Yanishlieva-Maslarova 2001). The structural feature of the phenolic compounds determine their reactivity towards free radicals and other ROS and hence their antioxidant activity. The antioxidant efficiency of phenolics varies with their concentration, necessitating the use of optimal concentrations in order to achieve the greatest efficiency. It is important to note that, like many other antioxidants, phenolics may exhibit prooxidant effects at higher concentrations (Koskas *et al.* 1984; Cillard and Cillard 1986). Activity of antioxidant substances may be affected by the system environment. For example, the activity of antioxidants in bulk oils is different from that of oil-in-water emulsions (Porter 1993). A major proportion of naturally occurring phenolic antioxidants are water-soluble and renders their antioxidant activity mainly in

aqueous environments or water compartments in body tissues. Tocopherols or vitamin E, however, are among the very few fat-soluble phenolic antioxidants, and are vital for oxidative stability of lipids in food as well as biological systems.

There has been growing interest in antioxidant effectiveness of phenolics. Individual phenolic compounds have been isolated and assessed as potential food additives, nutraceuticals and functional food ingredients. Moreover, various plant extracts containing a broad array of phenolic compounds have been extensively investigated for their composition, antioxidant efficiency and potential role in the food, cosmetic and pharmaceutical industries. In addition to naturally occurring phenolics, synthetic antioxidants bearing phenol structure have been used for different purposes, and these include several common synthetic antioxidants used by the food industry, namely butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and *tert*-butylhydroquinone (TBHQ). However, use of synthetic antioxidants is now limited owing to the growing concern over their potential carcinogenic effects (Wattenberg 1986; Sherwin 1990).

3 Epigallocatechin gallate (EGCG)

3.1 Tea and tea polyphenols

Tea, a product made from leaf and bud of the plant *Camellia sinensis*, is the second most popular beverage world wide after water and a major source of dietary flavonoids (Rietveld and Wiseman 2003). Not only consumed as a daily routine drink, it also confers great health beneficial effects to human, and therefore is also considered as functional food 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 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).

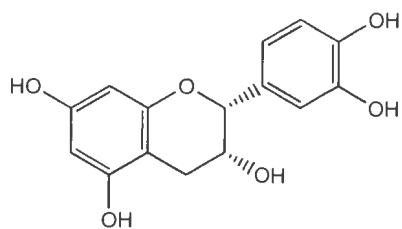
There are generally three types of tea in the market depending on the manufacturing process: non-fermented green tea (polyphenol oxidase inactivated by drying and steaming), semi-fermented oolong tea (fresh leaves subjected to a partial fermentation), and fermented black or red teas (leaves undergoing a post-harvest fermentation involving polyphenol oxidase catalyzed oxidation or microorganism assisted fermentation) (Wilson 1999; McKay and Blumberg 2002). Among the 2.5 million tonnes of dried tea products manufactured every year, approximately 76-78% are 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). In addition to the three main tea types, white tea produced from very young leaves and buds of the tea plant by an unfermented 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% proteins, 1-4% amino acids

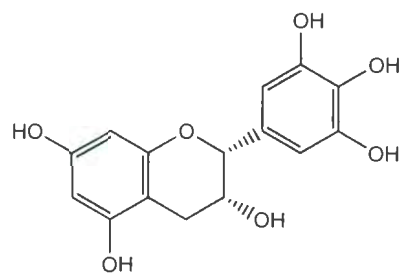
and 5-7% carbohydrates, 26% fiber, 7% lipids, 30% phenolic compounds, and other minor components including xanthic bases (e.g. caffeine and theophylline), pigments (e.g. chlorophyll and carotenoids), and trace elements (e.g. fluoride, calcium, magnesium, chromium, zinc, copper, aluminum, selenium, etc.) (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 processing. The major components with functional properties are xanthic bases acting upon 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 (Figure 1-3), 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).

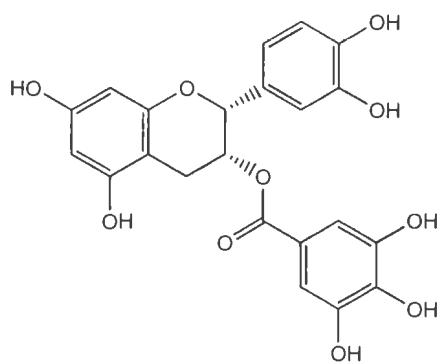
These catechins contribute to the characteristic bitter and astringent taste of tea. Catechin content in tea varies with climate, season, geographical location, horticultural practices, variety and age of the plant as well as processing and type of the products (e.g. blended, decaffeinated, instant, etc). Black tea has a lesser amount of simple catechins than green tea. The catechins in fresh tea leaves, during black tea preparation, are partially converted *in situ* by polyphenol oxidase to polymerized quinones such as theaflavins and thearubi-



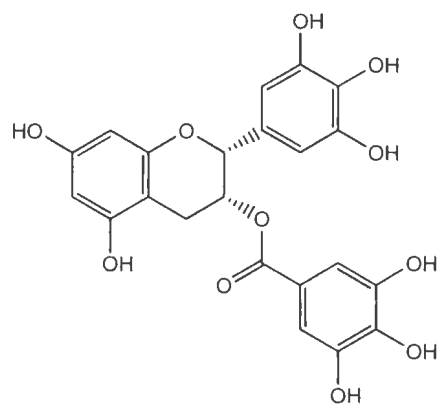
epicatechin (EC)



epigallocatechin (EGC)



epicatechin gallate (ECG)



epigallocatechin gallate (EGCG)

Figure 1-3. Chemical structures of the major catechins in green tea.

gins (USDA 2003). Manufactured bottled tea drinks also contain epimers of the catechins such as (+)-C, (-)-CG, (-)-GC, (-)-GCG, which are produced during the sterilization step (Murakami *et al.* 2006). In addition to catechins, other phenolic compounds are also found in tea as minor components, including gallic acid, chlorogenic acid, caffeic acid and flavonols such as kaempferol, myricetin and quercetin (USDA 2003).

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. Meanwhile, 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 the 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 antiangiogenic 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 are the most significant (Yang *et al.* 2006). Protection against neurodegenerative diseases such as Parkinson's and Alzheimer's diseases by GTP have also been documented (Weinreb *et al.* 2004). On the

other hand, anti-nutritional effects of GTP are noted, in particular, in reducing the digestibility of carbohydrates, proteins and lipids, whose digestive enzymes (e.g. α -amylase, pepsin, trypsin and lipase) are inhibited by the polyphenols (Arts 2008). Health effects of tea and tea polyphenols have been reviewed (Cabrera *et al.* 2006; Pham-Huy *et al.* 2008; Arts 2008; Sajilata *et al.* 2008; Ho *et al.* 2009).

3.2 Epigallocatechin gallate (EGCG) as an antioxidant

EGCG is the most predominant catechin in tea, and 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 confer 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 α -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 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 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. 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, peroxy, 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 soluble

antioxidants such as α -tocopherol and β -carotene (Lotito and Fraga 2000). EGCG can also protect lipids against oxidation in the liver, serum and brain (Skrzydowska *et al.* 2005). In addition, EGCG may act indirectly as antioxidant through its effects on content and activity of certain enzymes. Rats orally receiving green tea extracts containing EGCG showed higher levels of antioxidant enzymes such as glutathione peroxidase and reductase, superoxide dismutase and catalase in the brain (Skrzydowska *et al.* 2005). Moreover, EGCG inhibits redox enzymes (monooxygenase, cyclooxygenase, lipoxygenase, xanthine oxidase, NADH oxidase) and therefore suppress the production of ROS *in vivo* (Halliwell and Gutteridge 1989; Korkina and Afanas'ev 1997). EGCG is also capable of protecting erythrocyte membrane-bound ATPases against oxidative stress (Saffari and Sadrzadeh 2004).

3.3 Other properties of EGCG

EGCG has been known to possess promising anticancer potential, which is thought to be attributed to its antioxidant activity, induction of phase II enzymes, inhibition against tumor necrosis factor (TNF)- α expression and release, inhibition against cell proliferation, and induction of apoptosis, among others (Pham-Huy *et al.* 2008). EGCG readily reacts with ROS and creates differential oxidative environments in normal and cancerous cells. It has been reported that EGCG treatment resulted in decreased oxidative stress in normal epithelial and salivary gland cells while the oxidative stress was increased in oral-carcinoma cells (Yamamoto *et al.* 2003). EGCG may exert prooxidant property, which is apoptosis inducing, and hence an antiproliferative effect on cancerous cells (Azam *et al.*

2004). EGCG can directly bind to target molecules involved in cancer development, such as selected protein kinases, matrix metalloproteinases and DNA methyltransferases, and thus inhibiting their action (Yang *et al.* 2006). Effects of EGCG on DNA synthesis may also contribute to its anticarcinogenic activity. EGCG has been shown to accelerate the *in vitro* degradation of 2-hydroxyamino-6-methyldipyrido [1, 2-a, 3',2'-d] imidazole, a heterocyclic amine that induces single strand DNA breaks (Arimoto-Kobayashi *et al.* 2003). EGCG exhibited strong inhibition against DNA synthesis in HTC rat hepatoma cells and DS19 mouse erythroleukemia cells (Lea *et al.* 1993). EGCG has been found to decrease telomerase activity, which plays an important role in tumorigenesis, and thus shortening the life span of cancerous cells, as observed in monoblastoid U937 leukemia cells and HT29 colon adenocarcinoma cells (Naasani *et al.* 2003). EGCG imparts a growth inhibitory effect on cancer cells and has been shown *in vitro* to inhibit cell proliferation and stimulate apoptosis of various cancer cells, including prostate, lymphoma, colon and lung cancer cell lines (Pham-Huy *et al.* 2008). EGCG may affect the molecular mechanisms involved in angiogenesis, extracellular matrix degradation, regulation of cell death, and multi-drug resistance (Demeule *et al.* 2002). EGCG is also considered to be a topical protective agent against some types of radiation (e.g. UV radiation), one of the factors accounting for skin disease, photoaging and cancer development (Elmets *et al.* 2001). A significant dose-dependent inhibitory effect of topically applied EGCG against photocarcinogenesis has been demonstrated in mice (Gensler *et al.* 1996).

EGCG has been suggested to improve lipid metabolism and play a protective role against metabolic syndrome. EGCG interferes with the micellar solubilization of cholesterol in

the digestive tract, thus lowering the intestinal absorption of cholesterol (Ikeda 2008). EGCG can reduce body fat accumulation by suppressing postprandial hypertriacylglycerolemia and delaying intestinal absorption of triacylglycerols (Ideka 2008). Visceral fat deposition can also be reduced by EGCG through inhibition of fatty acid synthase (Wang and Tian 2001). Another underlying mechanism for antiobesity effect of EGCG is through acting upon AMPc levels by enhancing the energy expenditure (Juhel *et al.* 2000). EGCG is also the major active compound in green tea that can increase insulin activity and contribute to prevention of diabetes (Anderson and Polansky 2002).

Immunomodulatory effects of EGCG have been reported. EGCG exerts a cytoprotective effect on macrophages subjected to asbestos-induced damage, and the effect has been associated with its antiradical activity against superoxide anion radical (Potapovich and Kostyuk 2003). EGCG suppressed the intracellular growth of *L. monocytogenes* in macrophages, by inhibiting its escaping from the phagosome into cytosolic space (Kohda *et al.* 2008). EGCG has been shown to possess anti-inflammatory activity, the mechanism of which includes an ability to scavenge NO and the peroxynitrite anion, to reduce the activity of NO synthase, and to down-regulate gene expression of pro-inflammatory mediators (Paquay *et al.* 2000). Other physiological properties and potential health benefits of EGCG include antiviral, such as anti-HIV (Kawai *et al.* 2003; Williamson *et al.*, 2006), and anti-carcinogenic activities (Hamilton-Miller 2001) as well as prevention of neurodegenerative diseases such as Alzheimer's disease (Weinreb *et al.* 2009) and chronic liver diseases such as liver fibrosis (Sakata *et al.* 2004; Bayer *et al.* 2004), among others.

3.4 Bioavailability and structure 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 (Zhang *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 enhance the hydrophilicity of catechins and hence facilitate their elimination through urine and bile. However, a large portion of EGCG is present in the free form in the 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 forms (Van Amelsvoort *et al.* 2001). Breakdown of EGCG by acid hydrolysis mainly occurs 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 other organs, possibly owing to poor systematic absorption of EGCG (Cabrera *et al.* 2006). Studies in rats indicated that EGCG is mainly excreted through the bile, while 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 for 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 health effect in living organisms. EGCG has a four-ring structure with 8 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 to its effectiveness in protecting lipophilic systems, such as fats and oils, lipid-based foods or cosmetic formulas and emulsions as well as 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 on 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 of these derivatives in drug delivery and bioavailability in the body over their parent phenolic compounds.

Structural modification of EGCG via esterification with aliphatic molecules such as long chain fatty acids can be a useful tool to improve its lipophilicity. Additional advantages

can be expected when long-chain omega-3 polyunsaturated fatty acids (PUFA) are selected as the acyl donor. Long-chain omega-3 PUFA originating from marine sources have been demonstrated to have cardioprotective, immune-enhancing and mental health promoting effects, among other health benefits (Shahidi and Miraliakbari 2004, 2005). Combination of EGCG and omega-3 PUFA in one molecule not only leads to enhanced lipophilicity of EGCG, but also takes advantage of the health beneficial effects of omega-3 PUFA. This thesis reports investigations about lipophilization of the water-soluble EGCG by esterification with fatty acids and evaluation of the derivatives so-produced as EGCG alternatives for their bioactivities, including antioxidant, anti-inflammatory, antiviral, and anti-cancer effects.

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CHAPTER 2

Lipophilized epigallocatechin gallate (EGCG) derivatives as novel antioxidants

Introduction

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 polyphenols that are known to render a myriad of health benefits (Rietveld and Wiseman 2003). There has been strong evidence demonstrating that green tea polyphenols (GTP) play a protective role in the risk and pathogenesis of several chronic ailments, especially cardiovascular disease and cancer (Stensvold *et al.* 1992; Hamilton-Miller 2001). The dry leaves of tea contain approximately 30% (by weight) of polyphenols, majority of which are catechins (flavan-3-ols) (Graham 1992; McKay and Blumberg 2002). Epigallocatechin gallate (EGCG) is the predominant catechin in green tea (7.36%), but less abundant (1.58%) in black tea due to the formation of polymerized catechins (Cabrera *et al.* 2006). EGCG has a four-ring structure with 8 hydroxyl groups (Figure 1-3, Chapter 1), and is therefore highly hydrophilic. It renders its functional properties or health effects mainly in aqueous environments or water compartments in body tissues. In humans, EGCG may have limited bioavailability due to its physical and chemical characteristics, which dictate its rate of absorption through the gastrointestinal tract, metabolism and elimination from the body.

EGCG is known as a powerful antioxidant, possessing the highest antioxidant potency among all tea catechins (Guo *et al.* 1996). EGCG has been reported to inhibit oxidation in various food model systems, including pork, fish, and highly unsaturated marine oils

(Wanasundara and Shahidi 1996; He and Shahidi 1997; Shahidi and Alexander 1998). It also plays a protective role against oxidative stress in biological environments. A number of researchers have reported that EGCG effectively suppresses lipid oxidation in biological systems and subcellular fractions such as microsome and low-density lipoprotein (LDL) (Kondo *et al.* 1999). EGCG retards lipid oxidation and depletion of endogenous lipid soluble antioxidants such as α -tocopherol and β -carotene in the human blood plasma (Lotito and Fraga 2000). The antioxidant potential of EGCG arises from its polyphenolic structure and may vary depending on the reaction environment (e.g. temperature, pH, lipophilicity, type of oxidants, presence of synergist/antagonist, etc.) involved.

EGCG, like many other natural antioxidants, shows good solubility in aqueous media. The hydrophilic nature of EGCG may restrict its effectiveness in protecting lipophilic systems, such as fats, oils, and lipid-based food or cosmetic formulas and emulsions as well as biological environments. EGCG can be structurally modified in order to improve its lipophilicity and hence expand its application in the food, medicinal and cosmetic industries. Structure modification of EGCG via esterification with aliphatic molecules such as long chain fatty acids can serve as a useful tool in altering its physical properties such as solubility and miscibility and hence its antioxidant activity and bioavailability. Additional advantages or synergism can be expected when long chain omega-3 polyunsaturated fatty acids (PUFA) are selected as acyl donors; long chain PUFA such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to render a broad range of health benefits, including cardioprotective, immuno-enhancing

and anti-cancer effects, among others (Shahidi and Miraliakbari 2004, 2005). In this chapter, the preparation of lipophilic derivatives of EGCG (compounds **1**, **2** and **3**) and evaluation of their antioxidant activity *in vitro* are presented.

Materials and methods

Materials. EGCG was supplied by Dr. Yu Shao of GlaxoSmithKline Consumer Healthcare (Parsippany, NJ, USA). Stearoyl chloride was purchased from Nu-chek Prep Inc. (Elysian, MN, USA). EPA was obtained from Fuso Pharmaceutical Industries, Ltd. (Osaka, Japan) and DHASCO (DHA single cell oil) from Martek Bioscience Corporation (Columbia, MD, USA). Trolox was purchased from Acros Organics (Fair Lawn, NJ, USA). Other chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). All solvents used were obtained from Fisher Scientific Ltd. (Ottawa, ON). The solvents employed were HPLC or reagent grade.

Preparation of crude EGCG derivatives. Long-chain fatty acids, namely EPA and DHA, used for acylation were prepared from EPA ethyl ester and DHA single cell oil (DHASCO, containing 40% DHA), respectively. EPA was obtained following saponification and DHA was prepared by saponification followed by a urea complexation process as described by Wanasundara and Shahidi (1999). Briefly, the KOH-catalyzed saponification was carried out in 95% ethanol at $62 \pm 2^\circ\text{C}$ under reflux. The unsaponifiable matters were removed with hexane and the pH of the aqueous phase was adjusted to 1.0 with HCl. Free fatty acids were then 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 hours at 4°C for urea-fatty acid adduct crystallization. The mixture was then filtered to obtain the non-urea complex fraction, whose pH was subsequently adjusted to 4-5 with HCl. DHA was extracted into hexane and the solvent was removed by evaporation. The purity of the fatty acids (EPA and DHA) so-obtained was confirmed by GC-MS. EPA and DHA were then converted to their corresponding acyl chlorides by reacting with thionyl chloride. Stearoyl chloride was a commercial product and used as such. Esterification of EGCG was carried out with acyl chlorides (stearoyl, eicosapentaenoyl, and docosahexaenoyl chloride) at a mole ratio of 1:1. 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. The reaction mixture upon completion of the esterification was cooled to ambient temperature and filtered. The filtrate was then washed 3 times with distilled water (60°C), and the ethyl acetate layer was collected and passed through a cone of anhydrous sodium sulfate. The dry powder of crude products containing a mixture of EGCG esters (at different degrees of substitution) was obtained by evaporating the solvent.

Purification and identification of EGCG derivatives. The composition of reaction mixture was 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 (95:5 – 100:0 from 0 to 30 min) and fractions were detected

at 280 nm. LC flow was further analyzed on-line by the MS detector system (LC-MSD-Trap-SL, Agilent) with APCI (atmospheric pressure chemical ionization) at positive mode for identification of each fraction.

In order to obtain individual EGCG derivatives for subsequent structure elucidation and bioactivity evaluation, 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 – 50:50:2, v/v/v). Fractions corresponding to each band were collected and solvents removed using a rotary evaporator. The identity of each fraction was confirmed by HPLC-MS as described above. The predominant fractions of EGCG esters with stearic acid (SA), EPA and DHA (referred to as compounds **1**, **2** and **3**, respectively) were analyzed for their specific structures and evaluated for their antioxidant activity *in vitro*.

The ^1H and ^{13}C NMR analyses were carried out for purified EGCG esters (compounds **1**, **2** and **3**) in order to identify their molecular structures, i.e. the location of fatty acid incorporation in the EGCG molecule. The ^1H and ^{13}C spectra were recorded on a Bruker Avance 500 MHz NMR spectrometer (Bruker Biospin Co. Billerica, MA, USA) operating at 500.13 and 125.77 MHz, respectively. The samples were dissolved in dimethyl sulfoxide (DMSO)- d_6 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.

Determination of lipophilicity. Lipophilicity of the identified EGCG derivatives was determined as octanol-water partition coefficient (P) by a shake flask method. Briefly, a flask containing a mixture of octanol (100 ml) and deionized water (100 ml) was shaken in a shaking waterbath at room temperature (22°C) for 24 hours. The content was then allowed to stand for 24 hours for separation into two phases. Test compounds (0.2 μ mol) were dissolved in 5 ml of the upper phase (pre-saturated octanol), and the absorbance (A_0) was read at 280 nm. A blank with no sample was prepared. Five millilitres of the bottom phase (pre-saturated water) were added afterwards, and the mixtures were vortexed for 1 min and allowed to stand for 24 hours for separation. Absorbance (A_x) of the upper phase in the vials was measured and octanol-water partition coefficient (P) calculated using the following equation: $P = \log A_x/(A_0 - A_x)$, where blank-corrected absorbance was used.

Antioxidant activity. Antioxidant activity of the lipophilic EGCG derivatives was evaluated as DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging capacity using EGCG as the reference. DPPH scavenging capacity of test compounds was determined by EPR (electron paramagnetic resonance) according to Madhujith and Shahidi (2006) with slight modifications. Trolox standards (50-300 μ M) and test compounds (25 μ M) were dissolved in ethanol, to which 2 ml of ethanolic DPPH solution (0.18mM) were added. Contents were mixed well and injected to the sample cavity of a Bruker e-scan EPR spectrometer (Bruker Biospin Co.) through capillary tubing. The spectrum was recorded after 1 min on a Bruker e-scan food analyzer (Bruker Biospin Co.). 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. Reduction of DPPH radical concentration in the presence of test compounds was monitored by change in the corresponding signal intensity. DPPH radical scavenging capacity (%) was calculated using the following equation:

$$\% \text{ scavenging} = 100 \times (1 - \text{signal intensity}_{\text{sample}} / \text{signal intensity}_{\text{control}})$$

where, control contained no test compounds or trolox. A standard curve was constructed and DPPH scavenging capacities for test compounds were expressed as trolox equivalents (TE).

Statistical Analysis and Data Interpretation. One-way analysis of variance (ANOVA) with pairwise comparisons (Tukey's HSD) was performed at a $P < 0.05$ level using Sigmastat for Windows version 2.0 (Jandel Corp., San Rafael, CA) to determine the significant differences.

Results and discussion

Synthesis of EGCG derivatives. Several strategies for improving the functional and biological properties of EGCG through structural modification have been investigated. For example, introduction of alkoxyl groups and peracylation have been shown to increase lipid membrane permeability and chemical/metabolic stability of EGCG, thereby improving bioactivities under physiological conditions (Tanaka *et al.* 1998; Lam *et al.* 2004). In this study, we intended to enhance the lipophilicity of the water-soluble EGCG while maintaining its antioxidant activity. This was achieved by partial esterification and incorporation of long chain saturated or polyunsaturated fatty acids. To the best of our

knowledge, incorporation of the health-beneficial omega-3 PUFA to EGCG, which may provide additional perspectives to food and health applications, was carried out for the first time.

The more lipophilic derivatives of EGCG were prepared via reaction with various acylating agents, namely the acyl chloride of SA, EPA and DHA. The yield of the crude products containing a series of EGCG polyesters (tetra ester and higher) was 56.9, 42.7 and 30.7% for SA, EPA and DHA esters, respectively. Long-chain PUFA showed lower rates of incorporation, possibly because of their non-linear (bent) structure that renders steric hindrance for the acylation reaction. Saturated fatty acids such as SA are known to allow facile packing and close intermolecular interactions. Esterification by chemical means, with less selectivity and specificity, has been reported to result in higher degrees of substitution (DS) in the products than those catalysed by enzymes. For example, polyesters of sucrose with fatty acids (DS 4-8) were prepared using acylating agents such as acyl chloride or aryl esters (Rizzi and Taylor 1978), while lipases were effective for the regioselective synthesis of mono- and diesters of sucrose (DS 1-2) (Ferret *et al.* 2005). The electrophilic acyl group may react with different number of hydroxyl groups located in different positions depending on the nature of the electrophile, the reaction conditions and catalysts employed (Ballesteros *et al.* 2007). A mixture of EGCG-monopalmitate regioisomers (yield lower than 23%) was prepared by a chemical method in tetrahydrofuran (THF) at 0°C using triethylamine as the catalyst (Mori *et al.* 2008). In the current study, polyesters of EGCG with high DS (≥ 4) were formed during acylation under the conditions employed. It was noticed that the composition of the reaction mixture varied depending on the ratio of the starting materials (EGCG/fatty acid), and esters with

higher DS (7-8) were produced at lower ratios of EGCG/fatty acid (<1) (data not shown). As the degree of substitution increases, the lipophilicity increases while hydrolysis by lipolytic enzymes decreases due to steric hindrance (Akoh and Swanson 1990), hence higher membrane permeability and metabolic stability of the esters. The antioxidant activity of the derivatives so prepared would presumably be preserved since hydroxyl groups were only partially acylated. 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 of SA, EPA and DHA esters, but at different proportions (Table 2-1). Chain length of the fatty acids appeared to play a role in their incorporation into the EGCG molecule. Pentaesters were produced at a highest level in the EGCG-SA crude product (36.7%) followed by EGCG-EPA (18.9%) and EGCG-DHA (5.33%), whose fatty acids were less effective in approaching the acyl acceptor and packing in the synthesized molecules.

Identification of EGCG derivatives. The crude products of EGCG esters were separated into different fractions by flash column chromatography, and the major products corresponding to tetraesters (named compounds **1**, **2** and **3** for the stearate, eicosapentaenoate and docosahexaenoate, respectively) were collected and subjected to lipophilicity and antioxidant activity assessment tests. The three compounds were identified by HPLC-MS; their mass spectra are shown in Figure 2-1.

For compound **1**, the molecular ion peak detected showed a m/z at 1524.5, representing $[M]^+$ radical ion of the EGCG-tetrestearate ($C_{94}H_{154}O_{15}$), which might result from loss of a hydrogen atom from the $[M+H]^+$ or its migration to the fragments. The presence and

Table 2-1. Composition (%) EGCG-fatty acid polyesters in crude products*.

	Tetraester	Pentaester	Hexaester
EGCG-SA	63.3 ± 1.99 ^c	36.7 ± 1.99 ^a	tr
EGCG-EPA	81.1 ± 0.59 ^b	18.9 ± 0.59 ^b	tr
EGCG-DHA	94.7 ± 0.63 ^a	5.33 ± 0.63 ^c	tr

*Reaction mixture also contained unreacted EGCG, which was not calculated as a component of crude products.

EGCG-SA, EGCG-EPA, and EGCG-DHA are ester products of EGCG with SA, EPA and DHA, respectively.

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

tr: trace.

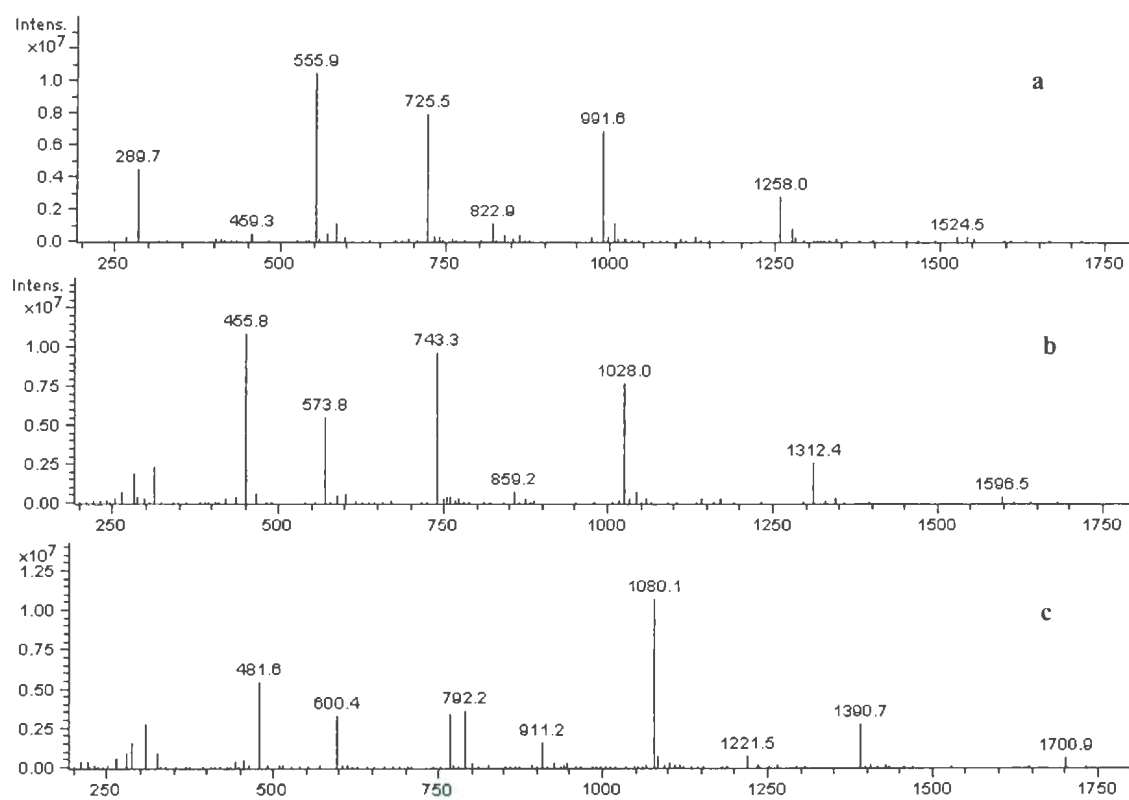


Figure 2-1. Mass spectra of EGCG derivatives (a: compound 1; b: compound 2; c: compound 3)

abundance of $[M]^+$ in relation to $[M+H]^+$ in APCI-MS have been reported to be dependant on the mobile phase in the LC as well as the H atom affinity of the analyte (Breeman 1997). The peaks at m/z 1258.0, 991.6, 725.5 and 459.3 showed one or multiple mass loss of 266.5 from the molecular ion, representing the ions of $[M-266.5]^+$, $[M-2 \times 266.5]^+$, $[M-3 \times 266.5]^+$ and $[M-4 \times 266.5]^+$, respectively. These appeared to be the fragments with 1, 2, 3 or 4 acyl groups (mass 267.5 for stearyl moiety and a hydrogen atom migrating to the fragment ions) dissociated from the molecular ion. The peaks located at m/z 822.9, 555.9 and 289.7 corresponding to $[M-2 \times 266.5-169]^+$, $[M-3 \times 266.5-169]^+$ and $[M-4 \times 266.5-169]^+$, respectively, originated from additional cleavage of the gallic acid group (mass 169). Based on the presence of the molecular ion and fragments in the mass spectrum, the identity of compound **1** was confirmed to be EGCG tetrastearate.

Similarly, compound **2** showed a molecular ion peak at m/z 1596.5 (mass of EGCG tetraeicosapentaenoate, $C_{102}H_{130}O_{15}$). The peaks at m/z 1596.5, 1312.4, 1028.0 and 743.3 had a difference in mass of 284.5, indicating the cleavage of an eicosapentaenoyl group (mass 285.5 minus a migrating H). The peak at m/z 455.8 ($[M-4 \times 285.5]^+$) might be derived from loss of all 4 eicosapentaenoyl moieties in the molecular ion (m/z 1596.5) without adding 4 H atoms. Peaks at m/z 859.2 and 573.8 indicated additional cleavage of the gallic acid group (mass 169) from the fragments m/z 1028.0 and 743.3, respectively. All of the above provides support to identification of compound **2** as EGCG tetraeicosapentaenoate.

Similar ionization and fragmentation patterns were found for MS of compound **3**. Molecular ion at m/z 1700.9 implied the presence of EGCG tetradocosaheptaenoate ($C_{110}H_{138}O_{15}$, MW 1700.3), which was further confirmed by its fragments. Peaks at m/z

1390.7 and 1080.1 correspond to the fragments with 1 and 2 docosahexaenoyl moieties (mass 311.6 minus a migrating H) cleaved off, respectively. The fragments from dissociation of 3 and 4 docosahexaenoyl groups were detected as Na-adducts at m/z 792.2 (769+23) and 481.6 (458+23), respectively. Further cleavage of the gallic acid moiety (mass 169) from the fragments m/z 769, 1080 and 1390 gave rise to peaks at m/z 600.4, 911.2 and 1221.5, respectively. Thus, it could be concluded that compound **3** was EGCG tetradocasahexaenoate.

Structure elucidation of EGCG derivatives. The purified EGCG derivatives (compounds **1**, **2** and **3**) were identified by MS as EGCG tetraesters (DS 4). However, their structures need to be determined since EGCG has 8 hydroxyl groups that are capable of acylation with fatty acids and hence the possibility of production of different regioisomers. Location of fatty acid incorporation in EGCG molecule was determined using ^1H and ^{13}C NMR by comparing the chemical shifts of the derivatives with their parent EGCG molecule. The chemical shifts detected for EGCG in both ^1H and ^{13}C NMR were very close to those reported in the literature (Valcic *et al.* 1999), while the spectra for the three derivatives displayed additional signals for corresponding alkyl groups in the expected range, i.e. δ 0.79-2.31 ppm for ^1H and δ 15.01-34.25 ppm for ^{13}C NMR (spectra not shown). Presence of alkyl protons and carbon provided evidence for incorporation of fatty acids in the EGCG molecule.

When comparing the chemical shifts of protons in the derivatives with those in EGCG, a downfield shift was observed for all proton signals of EGCG (Table 2-2), which is in accordance with the findings of Islambekov *et al.* (1976), who demonstrated that acetyl-

Table 2-2. ^1H and ^{13}C chemical shifts (δ ppm) of EGCG and its derivatives. *

C/H position	EGCG		Compound 1		Compound 2		Compound 3	
	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H
2	77.56	4.91	77.47	4.99	77.47	4.99	77.28	5.00
3	69.09	5.32	69.29	5.38	69.06	5.36	69.24	5.37
4	26.82	2.61	26.77	2.63	26.21	2.65	26.26	2.61
		2.88		2.91		2.91		2.89
5	156.71		156.68		156.77		156.58	
6	95.41	5.78	95.40	5.79	95.45	5.79	95.46	5.79
7	157.58		157.60		157.58		157.70	
8	96.60	5.88	96.67	5.89	96.67	5.90	96.76	5.90
9	157.58		157.60		157.58		157.70	
10	98.47		98.44		98.44		98.35	
1'	129.71		129.61		129.52		129.65	
2'	106.57	6.36	106.53	6.45	106.53	6.45	106.60	6.45
3'	146.47		150.72		150.72		151.05	
4'	133.45		131.97		130.25		132.73	
5'	146.47		150.72		150.72		151.05	
6'	106.57	6.36	106.53	6.45	106.53	6.45	106.60	6.45
1''	120.39		120.40		120.40		120.32	
2''	109.76	6.77	109.52	6.81	109.76	6.82	109.84	6.82
3''	146.72		151.26		151.25		151.05	
4''	139.64		137.04		137.05		137.23	
5''	146.72		151.26		151.25		151.05	
6''	109.76	6.77	106.52	6.81	109.76	6.82	109.84	6.82
COO	166.29		166.28		166.26		166.36	

* Proton and carbon in alkyl chain of incorporated fatty acids were not listed.

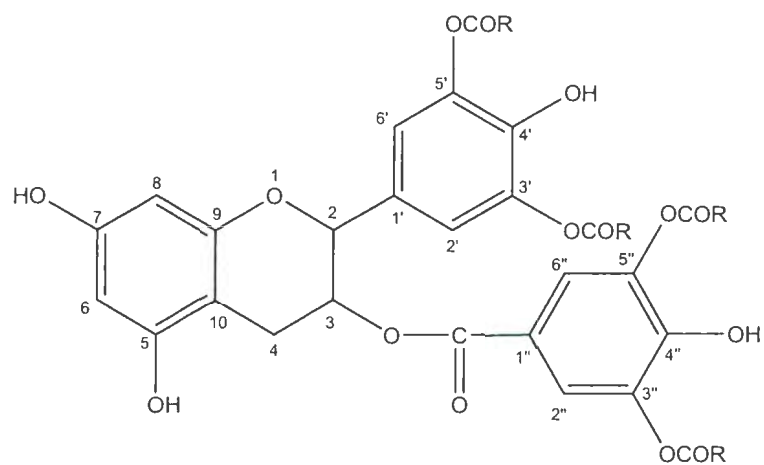
ation of catechin led to a downfield displacement of all the proton signals. Since there is no proton attached to the carbon bearing the hydroxyl substituent in the aromatic rings of EGCG, large downfield shifts ($\Delta\delta$ 1.0-1.2 ppm) normally observed for geminal protons were not detected.

Chemical shifts of protons in all three derivatives followed the same trend. H-2' and H-6' showed a downfield shift of $\Delta\delta$ 0.09 in comparison with the parent EGCG molecule, indicating the occurrence of acylation in the B ring. Downfield shift 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'' ($\Delta\delta$ 0.04-0.05 ppm), which appeared to be smaller than that 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 A ring was not the acylation site. Based on the ^1H 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 *O*-acylated carbon and an upfield shift of the neighbouring carbon. As presented in Table 2-2, a large downfield shift ($\Delta\delta$ 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 ($\Delta\delta$ 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'. The other neighbouring carbons (C-2' and C-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 ^{13}C chemical shifts. It has been demonstrated that ^{13}C chemical shifts may be determined by more complex stereochemical factors, while electronic influences dominate chemical shifts of ^1H (Ranganathan and Balaram 1984). Similarly, the positions of acylation in the D ring were assigned to C-3'' and C-5'', based on the downfield shift of C-3'' and C-5'' and upfield shift of C-4''. Absence of 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 ^1H NMR results. Thus, the structure characterization of the EGCG derivatives was conclusive based on the combined ^1H and ^{13}C NMR, and compounds **1**, **2** and **3** were identified as EGCG-3',5',3'',5''-O-tetratearate, EGCG-3',5',3'',5''-O-tetraecosapentaenoate, and EGCG-3',5',3'',5''-O-tetradocosaheptaenoate, respectively, as shown in Figure 2-2.

Lipophilicity of EGCG derivatives. EGCG is highly hydrophilic with a large polar molecular surface area, and is among those considered to be poorly absorbed in the body (Lambert *et al.* 2006). The hydrophilic nature of EGCG also poses a serious disadvantage/challenge for its use as a food preservative in stabilizing fats, oils and lipid-based food systems. Therefore, structure modification of EGCG serves as a potential means to alter its physicochemical properties and hence improving its effectiveness as an antioxidant in more diverse systems. Many water soluble bioactives have structurally been modified for improved lipophilicity and hence expanded applications in more lipo-



R: $(\text{CH}_2)_{16}\text{CH}_3$	Compound 1	(EGCG-SA)
$(\text{CH}_2)_3(\text{CH}=\text{CHCH}_2)_5\text{CH}_3$	Compound 2	(EGCG-EPA)
$(\text{CH}_2)_2(\text{CH}=\text{CHCH}_2)_6\text{CH}_3$	Compound 3	(EGCG-DHA)

Figure 2-2. Structures of EGCG derivatives.

philic environments. These include a number of antioxidant phenolics such as ferulic, chlorogenic, cinnamic, sinapic, *p*-coumaric, and caffeic acids (Sabally *et al.* 2005; Lee *et al.* 2006; Giraldo *et al.* 2007; Lue *et al.* 2005; Stevenson *et al.* 2007), which were esterified with various aliphatic alcohols, as well as ascorbic acid, tyrosol, protocatechuyl and vanillyl alcohol, esculin, rutin, naringin, genistein and daidzein (Stamatis *et al.* 1999; Aissa *et al.* 2007; Torres de Pinedo *et al.* 2007; Ardhaoui *et al.* 2004; Kontogianni *et al.* 2001; Lewis *et al.* 1998), which were converted into their corresponding fatty acid esters. In addition, sterols including cholesterol, cholestanol, and sitosterol were esterified with long chain fatty acids for better water-holding properties of the resultant esters (Shimada *et al.* 1999). Many of these studies used saturated fatty alcohols or acids as alkyl chain donors to enhance the lipophilicity of the molecules of interest. In this work, we investigated the incorporation of a saturated fatty acid as well as two long-chain omega-3 PUFA into the EGCG molecule. Long-chain omega-3 PUFA such as EPA and DHA are the major bioactive components in fish oils that are believed to render a myriad of health benefits, and are used as supplements, pharmaceutical substances, functional food ingredients and natural health products as well as cosmetics. Acylation of EGCG with these fatty acids will not only increase the lipophilicity of EGCG, but also take advantage of the health promoting effects of long-chain omega-3 PUFA. Additional perspectives may also be expected such as synergism between the two moieties, namely EGCG and the omega-3 fatty acids.

The EGCG derivatives prepared were evaluated for their lipophilicity in terms of octanol-water partition coefficient (*P*). Higher *P* values indicate higher lipophilicity of the compound. As expected, acylation with fatty acids resulted in increased lipophilicity, as

all three derivatives showed higher P than their parent molecule EGCG (Table 2-3). Compound 1 had the highest P among all, due to the presence of the highly lipophilic stearic acid side chain. Compounds 2 and 3 did not differ significantly in their lipophilicity. The lipophilicity of the test compounds was in agreement with their retention times observed in the reversed phase HPLC, which was in the order of compound 1 > 3 \geq 2 > EGCG. The enhanced lipophilicity of EGCG derivatives may lead to their improved incorporation into the lipid bilayers of cell membrane and hence better bioavailability in the body as well as greater potential in liposome-based drug delivery. Hashimoto *et al.* (1999) in a study on interaction of tea catechins with lipid bilayers reported that the incorporation rate was positively associated with the partition coefficient in octanol-water.

Antioxidant activity. EGCG and other catechins are powerful antioxidants acting as free radical scavengers, reducing agents and metal chelators. *In vitro* assays have shown the antioxidant effectiveness of EGCG in scavenging 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). Studies on antioxidant mechanism of EGCG have suggested that EGCG may have multiple reaction pathways for exerting oxidation/antioxidation effects depending on the reaction environment. The hydroxyl groups at positions 3 and 5 on the A-ring were thought to be not so important in radical scavenging of EGCG (Nanjo *et al.* 1996). However, a study on oxidation products of EGCG and H₂O₂ demonstrated that A-ring of EGCG might be a site for rendering antioxidant activity (Zhu *et al.* 2000). The trihydroxyphenyl B-ring was found to be the most active site of antioxidant action in

Table 2-3. Lipophilicity of EGCG derivatives as octanol-water partition coefficient (*P*).

Compounds	EGCG	Compound 1	Compound 2	Compound 3
<i>P</i>	0.48 ± 0.01 ^c	1.42 ± 0.02 ^a	1.10 ± 0.06 ^b	1.03 ± 0.10 ^b

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

DPPH-induced oxidation of EGCG (Zhu *et al.* 2000, 2001). Nevertheless, the gallate moiety which accounts for increased phospholipids/water partition coefficient may lead to a higher antioxidant potential *in vivo* (Caturla *et al.* 2003).

The EGCG derivatives in this study, as identified by MS and NMR, had 4 hydroxyl groups acylated with fatty acids, and the remaining hydroxyl groups on the aromatic rings of EGCG may contribute to the antioxidant property of the derivatives. However, the antioxidant efficacy of the derivatives may differ from that of EGCG, due to possible electronic and steric effects rendered by the fatty acid chains incorporated. Antioxidant activity of the modified EGCG was evaluated as scavenging capacity against DPPH radical, a stable hydrophobic radical frequently used in antioxidant assessment. Figure 2-3 shows the EPR spectra of DPPH radical as affected by the test antioxidants. The presence of test compounds significantly decreased the resonance signal intensity, indicating the scavenging effect of EGCG and its derivatives against DPPH radical. When comparing their radical scavenging capacity, compound **1** showed the highest capacity as trolox equivalents, followed by compounds **3** and **2** (Figure 2-4). All derivatives displayed a higher scavenging activity against DPPH than EGCG itself. The trend for antioxidant activity among test compounds was in accordance with that of their octanol-water coefficient.

Lipophilicity has been found to play a role in antioxidant activity of flavonoids. The lipophilic derivatives of EGCG may have greater accessibility/affinity to the lipophilic DPPH radical than the hydrophilic EGCG. Moreover, acylation may have an effect on the hydrogen atom donation capability of EGCG by altering its electron density and distribution on the aromatic rings. Maintaining and even enhancing of the antioxidant act-

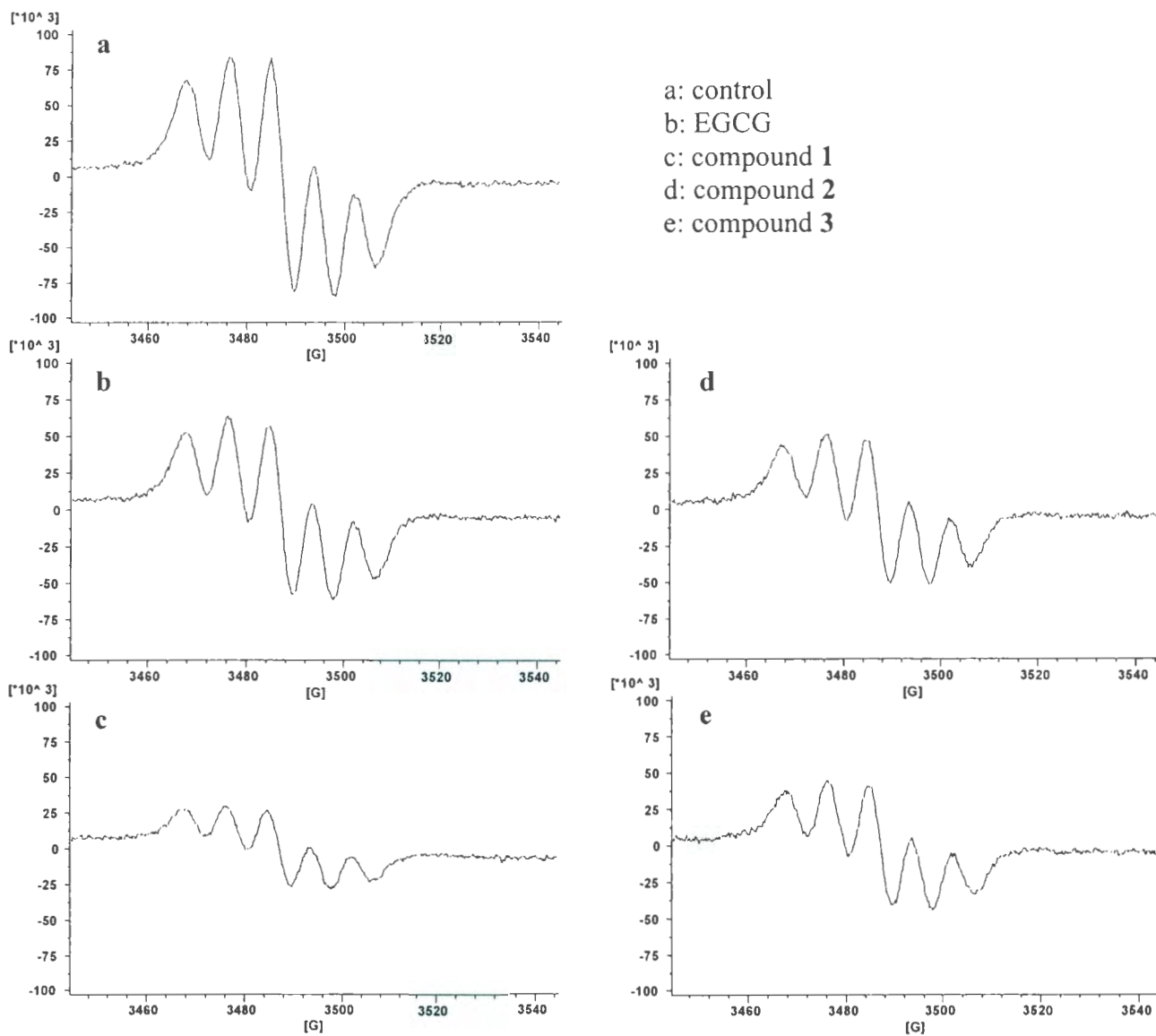


Figure 2-3. EPR spectra of DPPH scavenging of EGCG and its derivatives (compounds 1, 2 and 3 are EGCG tetraesters of SA, EPA and DHA, respectively).

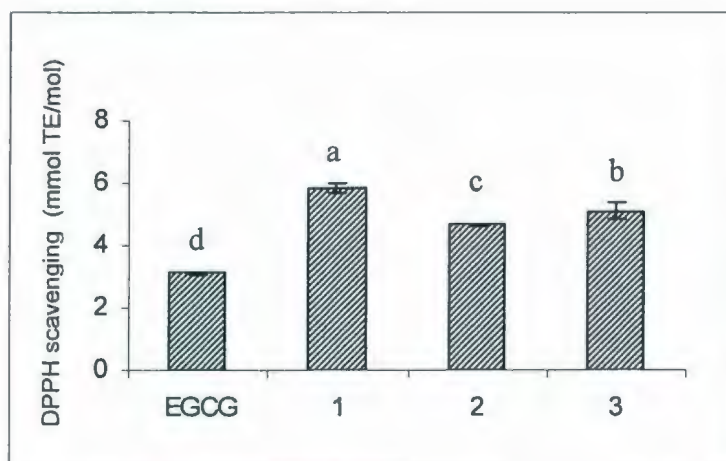


Figure 2-4. DPPH scavenging capacity in mmol trolox equivalents/mol (compounds **1**, **2** and **3** are ECGG tetraesters of SA, EPA and DHA, respectively; bars with different letters are significantly different at $P < 0.05$).

ivity for EGCG derivatives suggests that these derivatives may be used as antioxidants or EGCG alternatives in more lipophilic environments. Further investigation on the antioxidant effectiveness of EGCG derivatives in different model systems is needed for exploring their full potential for application in the food and health industries.

Conclusions

Esterification of EGCG with long-chain saturated (SA) or polyunsaturated (EPA and DHA) fatty acids yielded mainly EGCG tetraesters. The specific structures of the synthesized EGCG derivatives were confirmed by ^1H and ^{13}C NMR as EGCG-3',5',3'',5''-*O*-tetraester of the fatty acids involved. These derivatives had higher lipophilicity than EGCG itself. Antioxidant activity of all EGCG derivatives was superior to that of EGCG in terms of DPPH radical scavenging capacity, suggesting their potential use as antioxidants and EGCG alternatives in more lipophilic environments. Moreover, incorporation of long-chain omega-3 PUFA into the EGCG molecule may render additional health benefits or possible synergistic effects *in vivo*, which will be further investigated.

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CHAPTER 3

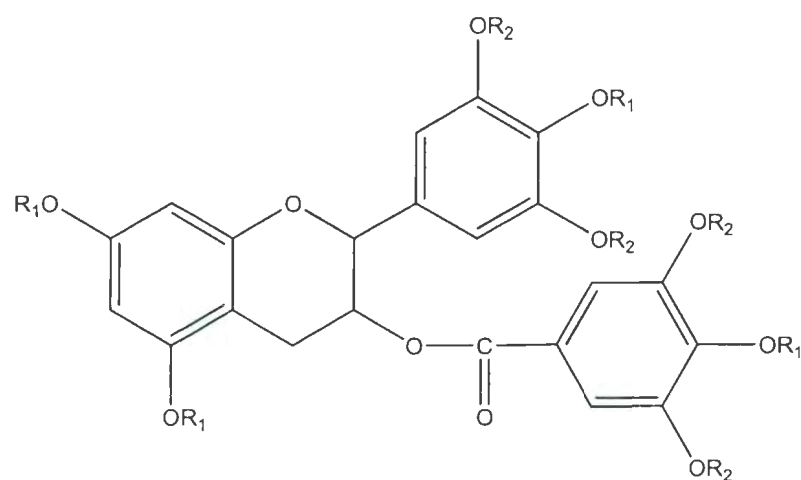
Antioxidant and antiviral activities of lipophilic epigallocatechin gallate (EGCG) derivatives *in vitro*

Introduction

Tea and tea products contain substantial amounts of bioactive flavonoids and have proven to render a multiplicity of functional and physiological effects (Cabrera *et al.* 2006). Many of the health effects related to tea consumption have been attributed to its catechin (simple or condensed) content, which is also responsible for the characteristic color, flavor and aroma of tea. Among all catechins found in tea, epigallocatechin gallate (EGCG) is the most abundant (59% of the total catechins) (McKay and Blumberg 2002) and has been extensively studied as a potential health promoting agent. EGCG has a four ring structure with 8 hydroxyl groups, and is readily dissolved in aqueous phase while sparingly soluble in hydrophobic phase. The hydrophilic nature of EGCG restricts its bioefficiency in lipophilic environments due to limited solubility and hence hindered approaching to the sites of action. Moreover, the hydrophilicity also accounts for poor cellular absorption of EGCG *in vivo* and thus low bioavailability when ingested orally. In order to take better advantage of this multifunctional compound, the water-soluble EGCG was structurally modified by esterification with different fatty acids, including stearic (SA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids (Chapter 2). The tetraesters produced showed enhanced lipophilicity and improved radical scavenging capacity compared to EGCG. Lipophilization of EGCG through esterification may be useful for improving its bioefficiency in lipophilic media or even introducing novel

bioactivities due to the fatty acid moieties incorporated, especially when the health beneficial long-chain omega-3 polyunsaturated fatty acids (PUFA) are involved. This chapter aims to investigate the lipophilized EGCG derivatives as potential alternatives of EGCG in foods and natural health products. Four EGCG-fatty acid esters (Figure 3-1), namely EGCG-3',5',3'',5''-*O*-tetrastearate (compound 1), EGCG-3',5',3'',5''-*O*-tetraeicosapentaenoate (compound 2), EGCG-3',5',3'',5''-*O*-tetradocosaheptaenoate (compound 3), and peracylated EGCG-octabutyrate (compound 4) were evaluated for their selected bioactivities (antioxidant and antiviral activities) using *in vitro* chemical assays.

EGCG is known as a powerful antioxidant protecting against free radical-mediated oxidative changes both in food and in living organisms. EGCG acts as scavengers of many reactive oxygen/nitrogen species (ROS/RNS) such as superoxide radical anion, peroxy and hydroxyl radicals, singlet oxygen, nitric oxide and peroxynitrite, among others, which are implicated in human pathogenesis including inflammation and carcinogenesis. EGCG can trap peroxy radicals and thus break the chain reaction of free radicals and terminate lipid oxidation. An electron paramagnetic resonance (EPR) study on tea catechins indicated that EGCG trapped 6 superoxide anion or hydroxyl radicals, whereas epicatechin (EC) only trapped 2 free radicals (Yang *et al.* 1994). 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 behavior, pH, solubility characteristics, bioavailability, and stability in the environment (Luczaj and Skrzydlewska 2005), which are in turn deter-



EGCG: $R_1 = R_2 = H$
 Compound 1: $R_1 = H, R_2 = CO(CH_2)_{16}CH_3$
 Compound 2: $R_1 = H, R_2 = CO(CH_2)_3(CH=CHCH_2)_5CH_3$
 Compound 3: $R_1 = H, R_2 = CO(CH_2)_2(CH=CHCH_2)_6CH_3$
 Compound 4: $R_1 = R_2 = CO(CH_2)_2CH_3$

Figure 3-1. Chemical structures of EGCG and its derivatives.

mined by the structural features of the molecule. Effect of structural modification of EGCG on its antioxidant activity is presented in this chapter.

Antiviral activities of EGCG have also been reported. EGCG has been shown to inhibit via multiple mechanisms the maturation, replication, infectivity or function of numerous viruses, including adenovirus, coronavirus, influenza virus, rotavirus, herpes simplex virus (HSV), enterovirus (EV, e.g. coxsackievirus, poliovirus, hepatitis A virus), and human immunodeficiency virus (HIV) (Weber *et al.* 2003; Clark *et al.* 1998; Friedman 2007; Ho *et al.* 2009; Nance *et al.* 2009), among others. Antiviral activities of EGCG are thought to be attributed to its ability to act as an antioxidant, to inhibit enzymes, to suppress viral RNA synthesis, to disrupt cell membranes, to bind to virulent proteins thus preventing their penetration into the receptor cells, and to trigger the host cell self-defense mechanisms (Friedman 2007). In this study, the antiviral potential of EGCG and its lipophilic derivatives was evaluated *in vitro* as inhibitory activity against NS3/4A protease, an important enzyme for the maturation of hepatitis C virus (HCV), and α -glucosidase, the enzyme essential for HIV infectivity.

Materials and methods

Materials. EGCG was supplied by Dr. Yu Shao of GlaxoSmithKline Consumer Healthcare (Parsippany, NJ, USA). Butyroyl and stearyl chlorides were purchased from Nu-chek Prep Inc. (Elysian, MN, USA). EPA and DHA were respectively prepared as described in chapter 2 from oils provided by Fuso Pharmaceutical Industries, Ltd. (Osaka, Japan) and Martek Bioscience Corporation (Columbia, MD, USA). Randomly methylated cyclodextrin (RMCD) was purchased from Cyclodextrin Technologies Inc. (High Springs,

FL, USA). Trolox was purchased from Acros Organics (Fair Lawn, NJ, USA). SensoLyte 520 HCV Protease Assay Kit was purchased from AnaSpec (San Jose, CA, USA). Other chemicals and α -glucosidase (from *Bacillus stearothermophilus*) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). All solvents used were obtained from Fisher Scientific Ltd. (Ottawa, ON). The solvents employed were of HPLC and reagent grade.

Preparation of EGCG esters. EGCG esters were prepared as previously described in Chapter 2. Briefly, EGCG was acylated with different fatty acids, namely SA, EPA and DHA, through their corresponding acyl chlorides. Peracylated EGCG butyrate was prepared with excess of butyryl chloride. The composition of the crude products containing a mixture of EGCG polyesters was determined by HPLC-MS and the predominant individual EGCG esters were isolated by flash column chromatography. The purified individual EGCG esters (compounds 1-4) were identified by HPLC-MS, ^1H NMR and ^{13}C NMR, and subjected to antioxidant and antiviral activity assessment by *in vitro* chemical assays.

ORAC (Oxygen radical absorbance capacity). The ORAC assay for EGCG and its derivatives (compounds 1-3) was carried out using a Fluostar Optima plate reader (BMG Labtech, Durham, NC) equipped with an incubator and two injector pumps. A modified method for lipophilic antioxidants was followed, in which RMCD was used as water solubility enhancer, as described by Huang *et al.* (2002). Trolox standards (6.25-50 μM) and samples (0.25 μM) were dissolved in acetone/water (1/1) containing 7% RMCD. Fluorescein and AAPH [2,2'-azobis (2-aminopropane) dihydrochloride] were used as the

probe and radical generator, respectively. Trolox standard or test compound solutions (20 μ l, 7% RMCD used as a control) were added to each well of a 96-well black microplate, followed by 200 μ l of fluorescein in phosphate buffer solution (0.11 μ M). The plate was incubated at 37°C for 15 min and the machine was programmed to inject 75 μ l of AAPH (17.2 mg/ml in buffer) 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).

Reducing power. The reducing power of EGCG and its derivatives (compounds **1-3**) was determined according to Oyaizu (1986). One millilitre of test compounds (1 mM in 95% ethanol) was mixed with 2.5 ml of phosphate buffer solution (PBS, 0.2 M, pH 6.6) and 2.5 ml of a 1% solution of potassium ferricyanide $K_3Fe(CN)_6$. The mixture was incubated at 50°C for 20 min. A portion of (2.5 ml) of trichloroacetic acid (TCA, 10%) was added to the mixture, and the content was centrifuged at 770 x g for 10 min. An aliquot (2.5 ml) of the supernatant was transferred to a tube containing 2.5 ml of distilled water and 0.5 ml of ferric chloride $FeCl_3$ (0.1%). The content was mixed well and the absorbance was recorded at 700 nm. Higher absorbance of the reaction mixture indicates greater reducing power. Ascorbic acid (1-6 mM in 95% ethanol) was used as a standard.

Metal chelation. The metal chelation capacity of EGCG and its derivatives (compounds **1-3**) was measured according to Decker and Welch (1990) with some modifications.

Ethanol solutions of test compounds (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 allowed to stand for 10 min for color development. The absorbance was then measured at 562 nm. A blank without ferrozine was used for each compound, since the antioxidant- Fe^{2+} complex gives a color that might interfere with the absorbance reading. Metal chelation capacity was calculated using the following equation:

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

where, control is in the absence of test compounds, while blank contained no ferrozine.

HCV protease inhibitory activity. Inhibitory activity of EGCG and its derivatives (compounds 1-4) against HCV protease was evaluated as an indicator for their antiviral activity. The assay was conducted following a method described by Ma *et al.* (2009) using a SensoLyte 520 HCV Protease Assay Kit. An aliquot (2 μl) of test compounds dissolved in dimethyl sulfoxide (DMSO) was added to each well of a 384-well black Assay plate, followed by addition of 8 μl of HCV NS3/4A protease solution (0.5 $\mu\text{g}/\text{ml}$). The reaction was initiated by adding 10 μl of freshly diluted substrate (100 x dilution of a DMSO stocking solution). The mixture was incubated at room temperature for 30 min, and the fluorescence intensity was measured at 485 nm for excitation and 535 nm for emission by a TECAN GENios plate reader. Inhibitory activity of test compounds was calculated as inhibition percentage according to the following equation:

$$\% \text{ inhibition} = 100 \times (\text{F}_{\text{control}} - \text{F}_{\text{sample}}) / \text{F}_{\text{control}}$$

where, F_{control} and F_{sample} stand for the fluorescence value of control without test compounds and of those with added test compounds. All fluorescence values were corrected with a substrate blank. Samples were assayed at different concentrations to plot a concentration versus inhibition percentage curve, and IC_{50} values were obtained from the curve for each sample. A known HCV protease inhibitor, embelin, was used as a reference.

α -Glucosidase inhibitory activity. The α -glucosidase inhibitory activity of EGCG and its derivatives (compounds **1-4**) was determined following the procedure described by Ma *et al.* (2008). To each well of a 96-well plate 40 μ l of 4-nitrophenyl α -D-glucopyranoside (2 mM, dissolved in 100 mM potassium phosphate buffer, pH 7.0) and 5 μ l of sample solution (in DMSO) were added. The reaction was initiated by adding 5 μ l of the enzyme solution (0.3 μ u/ml α -glucosidase from *Bacillus stearothermophilus*). The plate was incubated at 37 °C for 20 min, and the absorbance was measured before and after incubation at 405 nm with an InterMed ImmunoReader (Nippon InterMed K.K. Tokyo, Japan). The absorbance change (ΔA) was recorded and compared between control and samples. The inhibitory activity of test compounds against α -glucosidase was calculated as follow:

$$\% \text{ inhibition} = 100 \times (\Delta A_{\text{control}} - \Delta A_{\text{sample}}) / \Delta A_{\text{control}}$$

where, $\Delta A_{\text{control}}$ and ΔA_{sample} represent the absorbance change after incubation of the control (DMSO only) and samples, respectively. Acarbose was used as a reference inhibitor for α -glucosidase.

Statistical Analysis. One-way analysis of variance (ANOVA) with pairwise comparisons (Tukey's HSD) was performed at a $P < 0.05$ level using Sigmastat for Windows version 2.0 (Jandel Corp., San Rafael, CA) to determine the significant differences.

Results and discussion

Radical scavenging capacity. EGCG derivatives (compounds **1-3**) were effective in scavenging DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals, as summarized in Chapter 2. 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 than 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 life time. In order to have better understanding of their antiradical activity in biological environments, the EGCG derivatives were evaluated for their capability to scavenge peroxy 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 acetone/water as the solvent and RMCD as a solubility enhancer. The results showed that the peroxy radicals generated by AAPH were

scavenged by EGCG and its derivatives to a greater extent than by the reference antioxidant trolox, i.e. 15-85 folds more effective than trolox (Figure 3-2).

The ORAC values of the derivatives were higher than that of EGCG, indicating their greater hydrogen atom donating capability under the test conditions. The decreased number of hydroxyl groups in EGCG derivatives did not appear to negatively affect the antioxidant activity. Wright *et al.* (2001) stated that the number of hydroxyl groups in EGCG is largely irrelevant to its antioxidant activity, and it is the strategic placing of such groups that does matter. The substituents on the phenol ring play an essential role in the efficacy of phenolic antioxidant 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 antioxidant (Singh *et al.* 2005). In the EGCG derivatives, the *O*-acylation replaced the strong electron donating hydroxyl group (a combination of inductive and resonance effects) with a weaker electron donating ester group, which would presumably lead to compromised hydrogen donating capability of the molecule. However, the substitution also hindered the formation of the moderately strong hydrogen bonding of the 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 O-H bond, i.e. resistance of the 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.

Sterically, the acylation might have altered the 3D conformation of EGCG, which might facilitate hydrogen atom donation, hence increasing its radical scavenging capacity. Con-

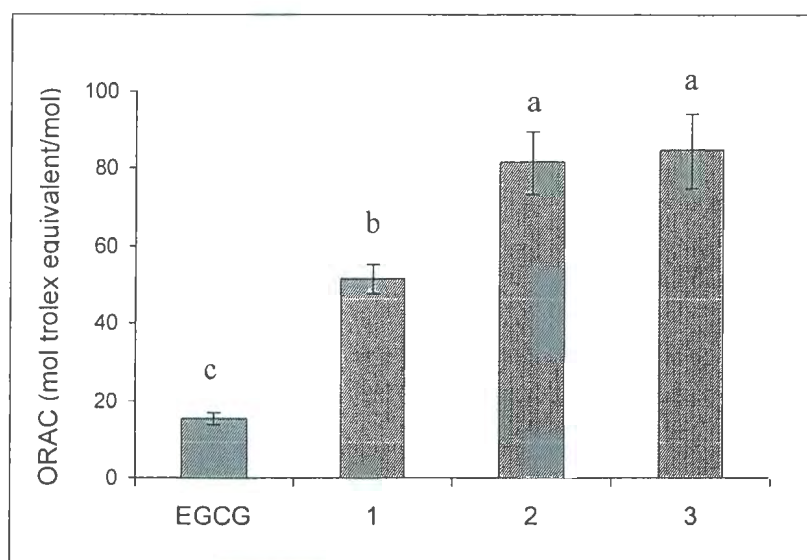


Figure 3-2. ORAC values of EGCG and its derivatives as trolox equivalents (compounds 1, 2 and 3 are EGCG tetraesters of SA, EPA and DHA, respectively).

formation characteristics are important factors affecting activity of antioxidants. Hakamata *et al.* (2006) reported that planar catechin (the catechol and chroman structure are constrained to be planar) showed a hydrogen transfer rate that was 5-fold faster than that of the native (+)-catechin. The scavenging activities of catechin against DPPH and AAPH radicals were increased by switching to the planar conformation. In this study, the acylation of hydroxyl groups on the phenyl ring might have led to planar formation or other steric changes that enhance the π -conjugation of the substituents with the phenol ring and hence the hydrogen donating capability. This, however, needs to be further investigated. In addition to the conformational attribute, the steric crowding caused by incorporation of high-molecular-weight bulky moieties might contribute to the greater hydrogen donating capability of the EGCG derivatives. Lucarini *et al.* (1996) have demonstrated that *ortho*-substituted phenols had lowered BDE due to the steric repulsion between the substituents and the hydroxyl group, especially when the substituents are bulky.

In summary, the combined electronic and steric factors discussed above might explain the overall improved radical scavenging activity of the EGCG derivatives. Among the derivatives, compounds **2** and **3** containing PUFA side chains showed higher ORAC values than compound **1** which had a saturated alkyl side chain. The superior hydrogen atom donating capacity of compounds **2** and **3** may be due to the electron-rich double bonds in the PUFA that may alter the electron distribution on the phenol rings as well as the bent structures of the PUFA chains that may help form more sterically-favored conformations for facilitating the dissociation of hydrogen atoms.

Reducing power. In addition to hydrogen atom donation, antioxidants may also 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 (Wright *et al.* 2001). The antioxidant radical formed is then stabilized by electron delocalization (resonance), as in hydrogen atom donation mechanism. Although the net result of electron transfer is the same as the hydrogen atom transfer route, the capability of an antioxidant to donate an electron or a hydrogen atom may vary depending on both intrinsic and extrinsic factors. The ability of an antioxidant to act as an electron donor, or its reducing power, is determined by the ionization potential (IP) of the compound and strongly solvent-dependant (Wright *et al.* 2001).

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 equivalents. Ascorbic acid is a known reducing agent. The results showed that both EGCG and its derivatives (compounds **1-3**) exhibited greater reducing power than ascorbic acid of 2-13 folds (Figure 3-3). EGCG had the highest reducing power among all. Acylation with long chain fatty acids led to dramatic decrease of reducing power, as found for compounds **1-3**. This is not in agreement with their free radical scavenging capacity (Figure 3-2). The poor correlation may arise from different mechanisms as well as the test environments involved in the two assays. As already mentioned, 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 phenoxyl

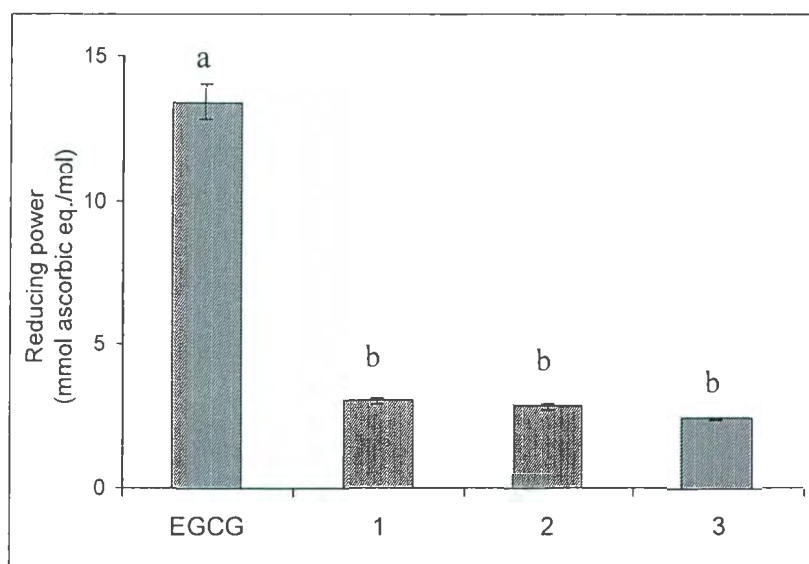


Figure 3-3. Reducing power of EGCG and its derivatives as ascorbic acid equivalents (compounds 1, 2 and 3 are EGCG tetraesters of SA, EPA and DHA, respectively).

radical cation and hence lowering the IP and enhancing the electron donating capability. Therefore, replacement of the strong electron-donating hydroxyl group with a weaker ester group results in increased IP and decreased reducing power of the antioxidant. As observed in this case, 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 BDE in a similar way, possibly because the electronic effect of the substituents on hydrogen atom donation was counteracted by other factors, such as hydrogen bonding and steric changes. Moreover, the BDE of a phenolic antioxidant seems less sensitive to substitution than the IP. For example, BDE decreases by 1 kcal/mol, while IP decreases by over 8 kcal/mol, when an aminophenol is methylated (Wright *et al.* 2001).

Solubility also affects the effectiveness of antioxidants, especially their reducing power since electron transfer mechanism is strongly solvent dependent due to solvent stabilization of charged species (Wright *et al.* 2001). EGCG derivatives with enhanced lipophilicity (data shown in chapter 2) have poor solubility in aqueous media and hence compromised activity under the hydrophilic test environment. Moreover, they might have formed micelles in 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).

Metal chelation. Metals play important roles in health. For example, iron is essential in oxygen transport, respiration, and activity of many enzymes. However, 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 peroxy 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 pro-oxidant 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, chelation capacity of EGCG and its ester derivatives for ferrous ion was evaluated. All test compounds exhibited ferrous ion chelation activity, ranging from 8 to 35% (Figure 3-4). The highest chelation activity was found for compound **2**, followed by compound **3**, indicating that the PUFA esters of EGCG were more potent metal chelators than the parent EGCG and its saturated ester. Compound **1** did not show any significant difference ($P>0.05$) from that of EGCG. Metal chelation capability has been related to geometric feature 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.

HCV protease inhibitory activity. Hepatitis C virus (HCV) infection is a serious health threat globally. More than 170 million people worldwide are chronically infected with

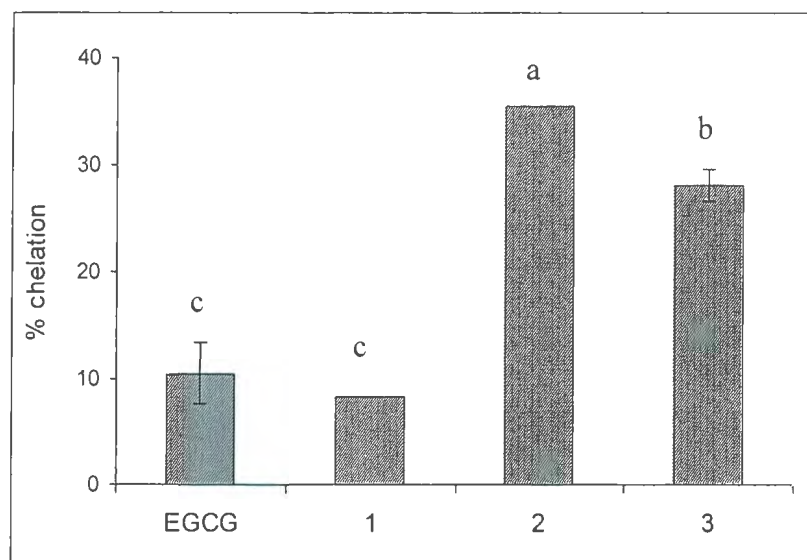


Figure 3-4. Metal chelation capacity (%) of EGCG and its derivatives (compounds **1**, **2** and **3** are EGCG tetraesters of SA, EPA and DHA, respectively).

HCV (Qiu 2009). Chronic infection with HCV is associated with liver cirrhosis that often leads to hepatic failure and hepatocellular carcinoma (HCC) (Chen and Tan 2005). Current approved therapies using interferon and pegylated interferon in combination with ribavirin can achieve only limited virologic response rates in a significant patient population (e.g. those infected with HCV genotype 1) and have been shown to have side effects (Chen and Tan 2005), necessitating development of new antiviral drugs. The NS3/4A protease, one of the most thoroughly characterized HCV enzymes has become an important target for anti-HCV treatment. The viral NS3/4A protease mediates the cleavage of the HCV polyprotein to release the functional proteins that are essential for viral propagation. Inhibitors of NS3/4A protease bind to the enzyme and inhibit activation of viral proteins, thus blocking HCV replication in infected host cells (Chen and Njoroge 2009).

The inhibitory effect of EGCG and its derivatives against HCV NS3/4A protease was examined using an *in vitro* assay and compared with embelin, a known HCV protease inhibitor. EGCG did not show any significant effect in inhibiting the protease, having a high IC_{50} value of $>200\ \mu\text{M}$ compared to that of the positive control embelin ($10.19\ \mu\text{M}$), as shown in Table 3-1. However, distinctive inhibition was observed for its ester derivatives and compounds **2** and **3** containing omega-3 PUFA displayed a protease inhibitory activity that was 1700-fold stronger than that of embelin. Although less effective than compounds **2** and **3**, compound **1** inhibited the protease to approximately 80-fold higher than embelin. The perbutyrate EGCG (compound **4**) with all 8 hydroxyl groups occupied with short chain acyl moieties was not expected to possess any antioxidant-

Table 3-1. Inhibitory effect (IC₅₀) of EGCG and its derivatives against HCV protease*.

Compounds	EGCG	1	2	3	4	Embelin
IC ₅₀ (μM)	>200	0.13	0.006	0.006	0.98	10.19
RSD%	5.5	1.8	3.4	3.0	4.4	9.0

*Compounds **1**, **2** and **3** are EGCG tetraesters of SA, EPA and DHA, respectively; compound **4** is EGCG-octabutyrate.

RSD: relative standard deviation.

ant activity due to absence of the functional OH groups. However, it exhibited a protease inhibitory activity 10-fold stronger than that of embelin. The remarkable improvement of EGCG derivatives in inhibiting HCV protease might arise from the changes in steric features and hydrophilic-lipophilic balance (HLB) of the molecules leading to the superior binding affinity to the enzyme. A study on antiviral activities of theaflavins by molecular modeling has revealed that steric and conformational effects govern the infectivity of the virus (Clark *et al.* 1998). In this study, the ester derivatives of EGCG with fatty acids, especially long chain PUFA with a bent chain structure, may be more sterically favored than EGCG in binding to the protease and thus inhibiting its function in virus replication.

α -Glucosidase inhibitory activity. α -Glucosidases are important hydrolytic enzymes in carbohydrate digestion and vital for biosynthesis of viral envelope glycoproteins. Glycosylation of the viral envelope glycoproteins is essential for infectivity of HIV, and inhibition of α -glucosidase provides a promising strategy for developing novel anti-HIV drugs. Moreover, inhibition of α -glucosidase may also have a therapeutic effect against type II diabetes by interfering with digestion of carbohydrates and delaying glucose absorption.

Catechin and its planar analogues have been investigated for α -glucosidase inhibitory activity (Hakamata *et al.* 2006). Only a weak inhibitory effect ($IC_{50} > 500 \mu M$) was found for (+)-catechin, while the lipophilic planar analogues (alkyl chains incorporated in a heterocycle ring) exhibited strong inhibition ($IC_{50} = 0.7-47.5 \mu M$) against α -glucosidase.

Another study on chlorogenic acid derivatives suggested that hydrophobic interactions were involved in α -glucosidase binding and inhibiting activity of these compounds (Ma *et al.* 2008). In this study, the ester derivatives of EGCG were determined for their α -glucosidase inhibitory activity and compared with the parent EGCG molecule and a positive control acarbose, a known α -glucosidase inhibitor used to reduce postprandial hyperglycemia. Table 3-2 presents the IC_{50} values of all test compounds, among which acarbose was found to be the most potent inhibitor of α -glucosidase. EGCG showed the highest IC_{50} value ($>200 \mu M$) among all. The derivatives with enhanced lipophilicity had significantly improved effectiveness in inhibiting α -glucosidase. Compound **1** had the lowest IC_{50} value i.e. highest potency as α -glucosidase inhibitor among all derivatives, followed by compound **4**. The less lipophilic derivatives, compounds **2** and **3**, were less effective. The results suggest that the binding affinity of EGCG and its derivatives to α -glucosidase may be dependent on a combined factor of hydrophobic interaction and steric features, since compound **1** showed a higher inhibitory activity than compound **4** while having lower hydrophobicity (according to the retention times in HPLC, data not shown).

Conclusions

Ester derivatives of EGCG with enhanced lipophilicities exhibited higher antioxidant activities than the parent EGCG in scavenging peroxy radicals and chelation pro-oxidant metal ions, possibly due to a combined electronic and steric effects. However, reducing power of the derivatives was lower than EGCG, which may be explained by their poor solubility in the aqueous test medium or formation of micelle. The derivatives also show-

Table 3-2. Inhibitory effect (IC₅₀) of EGCG and its derivatives against α -glucosidase*.

Compounds	EGCG	1	2	3	4	Acarbose
IC ₅₀ (μ M)	>200	2.62	15.66	15.87	6.87	0.15
RSD%	8.0	1.9	7.0	2.7	4.7	5.3

*Compounds **1**, **2** and **3** are EGCG tetraesters of SA, EPA and DHA, respectively; compound **4** is EGCG-octabutyrate.

RSD: relative standard deviation.

ed excellent antiviral activities in inhibiting HCV protease and α -glucosidase, which were not significant for EGCG. The steric features and lipophilicity of the derivatives may be responsible for the increased binding affinity to the enzymes, leading to greater inhibition. These results suggest that lipophilic EGCG derivatives may be used as potential functional ingredients of food, cosmetics, drugs and natural health products for health promoting purposes. However, these findings are based on *in vitro* chemical assays, and more research on their bioactivities in real food and biological systems is needed for better understanding and utilization of these derivatives as functional compounds.

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CHAPTER 4

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

Introduction

Lipid oxidation is considered a major cause of quality deterioration affecting both the nutritional/sensory quality and safety of foods, especially lipid-based products, and has thus been a challenge for manufacturers and food scientists alike. Among the many strategies employed for controlling lipid oxidation, addition of antioxidants is the most effective, convenient and economical approach (Wanasundara and Shahidi 2005). Antioxidants in foods, naturally occurring as food components, produced from process-induced chemical changes, or added as preservatives, can minimize rancidity, maintain nutritional quality, retard the formation of toxic oxidation products, and increase shelf-life of the food (Jadhav *et al.* 1996). Antioxidants are also used in the 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 as or as synthetic chemicals. Polyphenols are the most abundant antioxidants in nature. The antioxidant activity of polyphenols arises from their unique structures, which render a superior hydrogen donating capability over many other compounds. Tea is a rich source of polyphenols, particularly flavonoids. The flavonoids, mainly catechins (flavan-3-ols), contribute to the characteristic bitter and astringent taste of tea, and are among the major active components responsible for the functional properties of tea and its extracts (Cabrera *et al.* 2006).

Epigallocatechin gallate (EGCG), the predominant catechin in tea, has been known as a powerful antioxidant. *In vitro* assays have shown the antioxidant effectiveness of EGCG in radical scavenging, reduction and metal chelation (Zhu *et al.* 2001; Sun and Ho 2001; Xu *et al.* 2004; Chan *et al.* 2007). 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). 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).

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 food or cosmetic formulas and emulsions as well as biological environments. In order to expand the application of EGCG as an antioxidant or other functional ingredient in more diverse systems, ester derivatives of EGCG with improved lipophilicity were synthesized (Chapter 2) and their antioxidant activity confirmed by various *in vitro* assays (Chapter 3). In this chapter, the antioxidant efficacy of the lipophilic EGCG derivatives (compounds 1-3, Figure 2-2 in Chapter 2) in selected food model systems is presented as an indicator of their potential use in the food industry.

Materials and methods

Materials. EGCG was supplied by Dr. Yu Shao of GlaxoSmithKline Consumer Healthcare (Parsippany, NJ, USA). Stearoyl chloride and linolenic acid were purchased from Nu-chek Prep Inc. (Elysian, MN, USA). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were prepared as described in chapter 2 from oils provided by Fuso Pharmaceutical Industries, Ltd. (Osaka, Japan) and Martek Bioscience Corporation (Columbia, MD, USA), respectively. Stripped corn oil, BHA, β -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). All solvents used were of HPLC grade quality and obtained from Fisher Scientific Ltd. (Ottawa, ON). Fresh lean ground pork was obtained from a local supermarket.

Preparation and characterization of lipophilic EGCG derivatives. EGCG was acylated with stearic acid (SA), EPA or DHA by reacting with their correspondent acyl chlorides as described in Chapter 2. The composition of the crude products containing a mixture of EGCG polyesters was determined by HPLC-MS and the predominant individual esters were isolated by flash column chromatography. The predominant ester products of EGCG with SA, EPA and DHA (referred to as compounds **1**, **2** and **3**, respectively), whose structures were identified by HPLC-MS, ^1H NMR and ^{13}C NMR, were analyzed for their antioxidant potential in different food model systems.

Antioxidant activity in bulk oil. Antioxidant activity of EGCG derivatives in bulk oil was measured as protection against lipid oxidation in a stripped corn oil model system by

a well-established Rancimat method. EGCG or its derivatives dissolved in ethanol (1 $\mu\text{mol/ml}$, 1 ml) 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 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 compounds involved. Antioxidant activity was interpreted as protection factor (PF), which was calculated as follow.

$$\text{PF} = \text{IP}_{\text{sample}} / \text{IP}_{\text{control}}$$

where, $\text{IP}_{\text{sample}}$ and $\text{IP}_{\text{control}}$ represent induction periods for the oil with and without test compounds, respectively.

Antioxidant activity in oil-in-water emulsion. Antioxidant activity of EGCG derivatives in an oil-in-water emulsion was 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 nitrogen stream, 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 test compounds (1 mM in ethanol) or ethanol as control. A mixture of blank (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 are 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 for kinetic curve of β -carotene bleaching. Antioxidant activity of test compounds in protecting β -carotene/linoleic acid oxidation was calculated using the following equation.

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

where, A_0 and A_t are corrected absorbance values for test samples measured at zero time and after incubation, respectively; while A_0° and A_t° are corrected absorbance values for control at zero time and after incubation, respectively.

Antioxidant activity in muscle food. A pork model system was employed for assessing antioxidant effectiveness of EGCG derivatives. The meat model systems were prepared as described by Shahidi and Alexander (1998). Fresh ground pork (40g) was mixed with deionized water (10 g) in a Mason jar. Samples and reference antioxidant compound (BHA) dissolved in ethanol were added to meat at a level of 80 μ mol/kg (14, 37, 122, 128, and 136 ppm for BHA, EGCG, compounds **1**, **2**, and **3**, respectively). A control without

any antioxidant was also prepared. The content was 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. The meat samples were taken on day 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 solution at difference 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 TABRS in the cooked meat model system, 2 g of meat were mixed with 5 ml of trichloroacetic acid (TCA, 10% w/v) in a centrifuge tube, followed by addition of 5 ml of TBA reagent. The mixture was centrifuged at 3000xg for 10 min and the supernatant was filtered. The filtrate was heated in a boiling water bath for 45 min and 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.

Statistical Analysis. One-way analysis of variance (ANOVA) with pairwise comparisons (Tukey's HSD) was performed at a $P < 0.05$ level using Sigmastat for Windows version 2.0 (Jandel Corp., San Rafael, CA) to determine the significant differences.

Results and discussion

Antioxidant activity in bulk oil. It is well known that 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 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 hence its antioxidant activity. Antioxidant activity 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 structure modification on the antioxidant activity of EGCG, the lipophilized derivatives were evaluated for their antioxidant efficacy in different food model systems, namely a bulk oil, an emulsion and a muscle food model system.

A 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). Volatile flavor and odor components such as organic acids were formed and detected in the Rancimat method as an indicator of oxidation. The calculated PF of EGCG and its derivatives as affected by their molar concentrations (converted from ppm) is shown in Figure 4-1. EGCG showed a dose-dependent antioxidant activity in the oil (0.3-0.9 $\mu\text{mol/g}$ of oil) while antioxidant efficiency of its ester derivatives decreased with increasing concentration (0.2-0.4 $\mu\text{mol/g}$ of oil). Compound 1 had the highest antioxidant activity among all derivatives tested, although the activity was higher at lower concentrations.

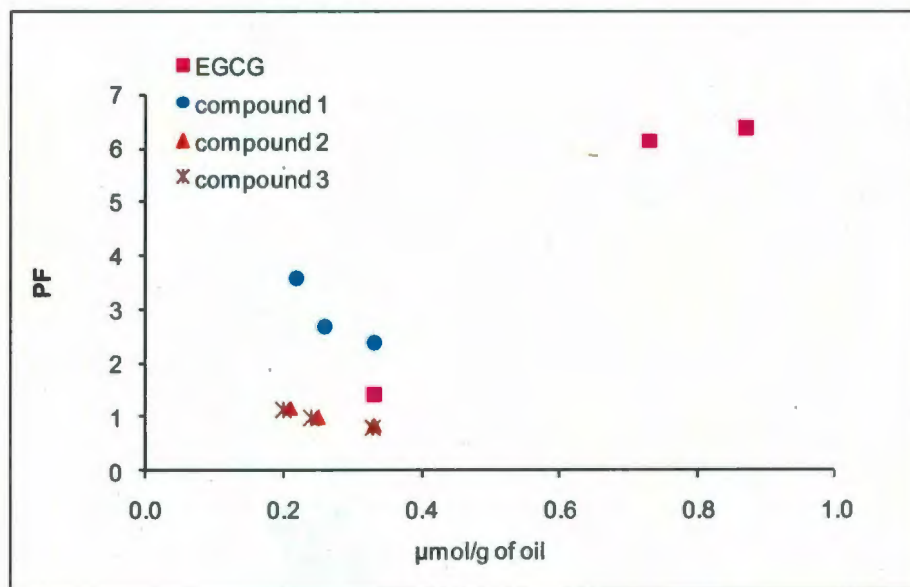


Figure 4-1. PF (protection factor) of EGCG and its derivatives in stripped corn oil at different concentrations (compounds 1, 2 and 3 are EGCG tetraesters of SA, EPA and DHA, respectively).

Compounds **2** and **3** acted as antioxidants ($PF > 1$) at the lowest concentration, and had no effect on oxidative stability of the oil ($PF = 1$) when the concentration increased, while exerted a slightly pro-oxidative effect at the highest concentration ($PF < 1$). The results suggest that these derivatives achieve their maximum efficacy in oil at relatively low concentrations compared to that of EGCG, and that higher dose leads to loss of antioxidant power or even prooxidative action. EGCG appears to require higher concentrations for exhibiting greater antioxidant efficacy, possibly due to its poor solubility in the oil. However, a high PF of 6.37 was observed for EGCG at a concentration of $0.87 \mu\text{mol/g}$ of oil, which may be explained by the interfacial phenomenon of hydrophilic antioxidants in bulk oil. The partially soluble hydrophilic antioxidants tend to be oriented in the air-oil interface where surface oxidation occurs, and therefore protects 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). However, the hydrophilic antioxidant EGCG and its lipophilic derivatives at the same molar concentration ($0.33 \mu\text{mol/g}$ of oil) showed a different trend in their antioxidant activity in the stripped corn oil. Compound **1** was more effective than EGCG in inhibiting oxidation. It is hypothesized that at this concentration the effect of solubility on antioxidant efficiency dominates over the effect of interfacial phenomenon and hence compound **1** with higher lipophilicity exerted greater antioxidant efficiency than EGCG. It is possible that the polar paradox theory only applies when the antioxidant under investigation reaches a critical concentration so that

interfacial phenomenon dominates over the solubility issue. More research is needed for confirmation of this hypothesis. With respect to compounds **2** and **3**, which had slightly pro-oxidative activity, the long-chain PUFA side chains in the molecules might have compromised their oxidation inhibitory effect at this concentration by contributing to 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 and may serve as potential candidates for possible application in bulk oil systems. Although EGCG at higher concentrations protected the oil from oxidation, better than its derivatives, the poor solubility remains a bottleneck since it would exist as a suspension in the oil and the appearance of oil products is one of the important parameters for quality judgment. Concentration of EGCG-PUFA esters added to oil should be closely monitored, since they may have compromised efficacy as antioxidants or may even exert a pro-oxidant activity at higher concentrations.

Antioxidant activity in oil-in-water emulsion. Antioxidants are found to behave in different manners when used in different media. Hence, their efficacy in bulk oil may not necessarily reflect that in oil/water emulsions. Moreover, it has been recognized that high surface-to-volume ratio emulsions are the natural conditions, while 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 oil/water emulsions for a more comprehensive assessment of antioxidant activity. In this work, the antioxidant activity of EGCG and its lipophilic derivatives in

oil-in-water emulsion was determined using a β -carotene/linoleic acid emulsion model system. Their antioxidant activity was detected by monitoring the bleaching of β -carotene as affected by test compounds. The decoloration 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 the emulsified aqueous system.

The results indicate that EGCG and its derivatives inhibited bleaching of β -carotene by 7.90 – 32.1% over the 105 min incubation period (Table 4-1). Lower rates of absorbance decay were observed for emulsions containing test compounds compared to the control, as shown in Figure 4-2. When comparing the antioxidant activity of EGCG and its derivatives, compound **1** was the most effective in retaining β -carotene in the first 45 min, followed by compound **3** and compound **2**, while EGCG had the lowest effectiveness. The antioxidant activity of test compounds was well correlated with their hydrophobicity, which is in the order of compound **1** > **3** \geq **2** > EGCG (measured as octanol-water partition coefficient). Similar correlation was found for naturally occurring tea catechins, which demonstrated antioxidant activity in a β -carotene/linoleic acid model system following the order of ECG > EGCG \approx EC > EGC, reflecting the retention times of these catechins in reverse-phase HPLC (Amarowicz and Shahidi 1995). These findings lend further support to the antioxidant “polar paradox” theory, which also states that nonpolar antioxidants are more effective than polar ones in emulsions, indicating an opposite trend from that in bulk oil. In the oil-in-water emulsion systems, hydrophilic antioxidants such as EGCG tend to move to the water phase and hence providing less protection to the oil.

Table 4-1. Inhibitory effect of EGCG and its derivatives against β -carotene bleaching and TBARS formation in cooked pork.

Inhibition (%)	EGCG	Compound 1	Compound 2	Compound 3	BHA
<i>β-carotene bleaching</i> ¹					
	7.90 \pm 0.64 ^c	16.8 \pm 4.19 ^b	28.0 \pm 0.17 ^a	32.1 \pm 4.48 ^a	-
<i>TBARS formation in cooked pork</i> ²					
	71.9 \pm 1.56 ^d	86.3 \pm 2.10 ^a	80.5 \pm 0.80 ^{bc}	78.4 \pm 1.62 ^c	83.3 \pm 2.34 ^{ab}

Values (mean \pm SD of three replicates) in the same row with different letters were significantly different at $P < 0.05$. Compounds 1, 2 and 3 are EGCG tetraesters of SA, EPA and DHA, respectively

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

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

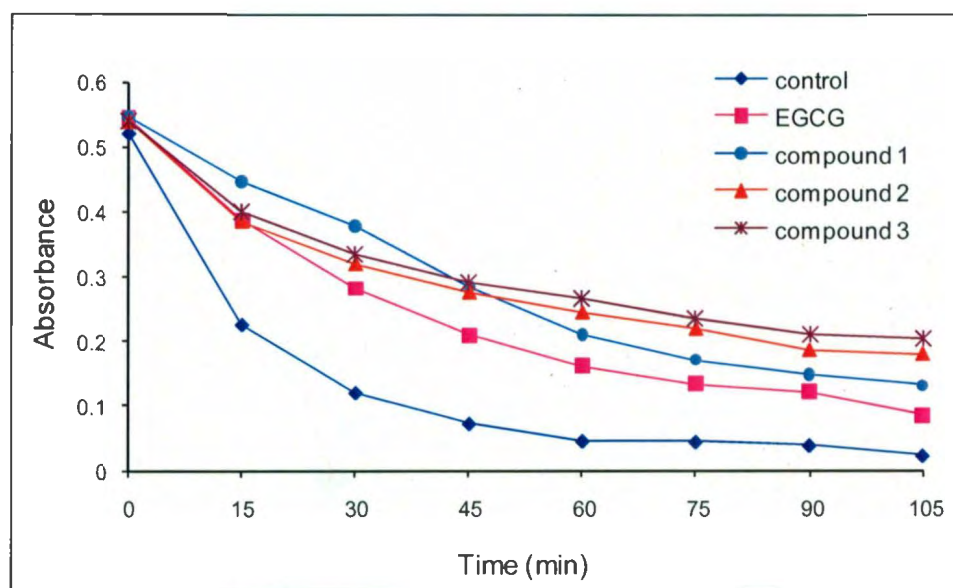


Figure 4-2. β -Carotene bleaching as affected by EGCG and its derivatives (compounds 1, 2 and 3 are EGCG tetraesters of SA, EPA and DHA, respectively).

The lipophilic derivatives of EGCG, in contrast, are more soluble in the oil phase, or oriented in the oil-water interface due to the presence of both hydrophobic aliphatic side chains and hydrophilic hydroxyl groups in their molecules, thus showing higher antioxidant activity than their hydrophilic counterpart EGCG. However, the test compounds demonstrated different antioxidant kinetics in the last 60 min of the assay. Compound 1 exhibited a lower inhibitory effect against β -carotene bleaching than compounds 2 and 3 after 45 min, possibly due to their depletion or the fact that compound 1 plays a better protective role in the early as compared to the later stages of oxidation. All derivatives possessed a superior antioxidant effectiveness than their parent EGCG molecule at all time intervals (0-105 min), thus suggesting their greater potential as antioxidants in emulsion systems of most foods, cosmetics and biological environments.

Antioxidant activity in 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). These foods generally require 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 effectiveness of antioxidants in thermally processed whole or modified tissue foods, where heating causes

rapid oxidation of lipid and meat flavor deterioration as well as development of “warmed-over flavor” (Rubin and Shahidi 1988).

Antioxidant activity of EGCG and other tea catechins has been evaluated in muscle food model systems. EGCG inhibited lipid oxidation in cooked fish (mackerel) meat with a higher efficacy than that of the other catechins and TBHQ (*tert*-butylhydroquinone), a commonly used synthetic antioxidant (He and Shahidi 1997). In a cooked pork model system, EGCG showed antioxidant activity higher than other catechins and typical food antioxidants BHT (butylated hydroxytoluene) and α -tocopherol (Shahidi and Alexander 1998).

In this work, the antioxidant activity of EGCG and its derivatives was determined in a cooked pork model system 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). During cold storage (4°C), all meat samples showed increasing concentrations of TBARS with time as a result of lipid oxidation (Figure 4-3). The groups with added EGCG and its derivatives had significantly lower TBARS values than the control over the entire storage period. The antioxidants added to fresh meat exerted inhibitory effects against oxidation during cooking of the meat prior to storage, which explains the higher TBARS value in control at day 0. From day 0-5, test compounds did not differ significantly in their antioxidant activity. However, varied antioxidant potencies were observed for EGCG and its derivatives during day 5-14. The inhibition of TBARS formation by test compounds and BHA in cooked meat after a 14-day storage period is given in Table 4-1. Compound 1 was most effective in inhibiting the production of TBARS followed by compounds 2 and 3, while all lipophilic derivatives

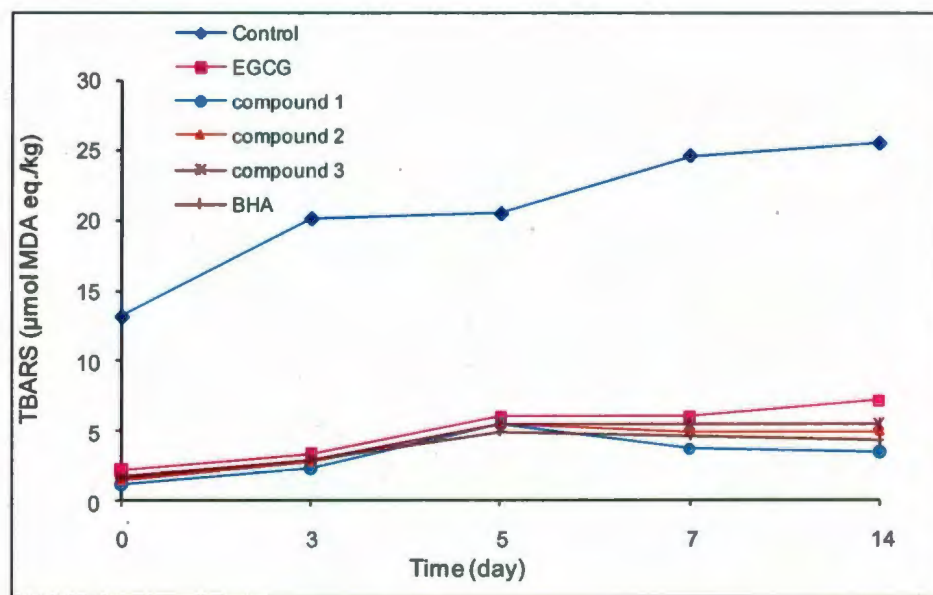


Figure 4-3. TBARS in cooked pork as affected by EGCG and its derivatives.

exhibited greater antioxidant efficacy than their parent EGCG molecule. This trend is in accordance with that obtained from β -carotene bleaching assay (first 45 min), which correlated well with the lipophilicity of the compounds. Lipophilicity of antioxidants appeared to be positively associated with their activity in cooked muscle food, as in oil-in-water emulsions. Two of the derivatives (compounds **1** and **2**) 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 for better oxidative stability and longer shelf life of the products.

Conclusions

The lipophilic ester derivatives of EGCG exhibited great antioxidant potential in inhibiting lipid oxidation in bulk oil, emulsion and muscle food model systems. The antioxidant activity of EGCG derivatives was similar or superior to that of the parent EGCG molecule, suggesting their potential for possible use in food formulations.

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CHAPTER 5

Lipophilized epigallocatechin gallate (EGCG) derivatives and their antioxidant potential in biological systems

Introduction

Oxidative changes in biological systems mediated by reactive oxygen species (ROS) render a destructive cellular effect *in vivo* and has been implicated in the pathogenesis of numerous chronic diseases and health conditions. Lipids in living organisms undergo oxidation reaction during normal aerobic metabolism (Beckman and Ames 1998). Approximately 2-5% oxygen consumed by a cell is converted to oxygen radicals, the important intermediates in oxidation chain reaction (Floyd and Hensley 2002). Polyunsaturated fatty acids (PUFA) in triacylglycerols (as well as mono- and diacylglycerols) and membrane phospholipids are generally the major reactants affected by such reactions. However, the substrates may be extended, in principle, to all cellular and extracellular components, including cholesterol, proteins, enzymes, lipoproteins and DNA, causing damage to cells and tissues. Antioxidant defense systems by endogenous and exogenous antioxidant substances provide protection against oxidative damage *in vivo*. Antioxidant strategy has been employed in preventing/treating oxidative stress-associated diseases, including cardiovascular disease, cancers, hypertension, diabetes, inflammation, and other autoimmune disorders.

Epigallocatechin gallate (EGCG), the most predominant polyphenol in tea, has been shown to possess antioxidant activity, among other bioactivities, and has been proposed as a potential health-promoting agent. 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). 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. For this purpose, the water-soluble EGCG was structurally modified by esterification with long-chain saturated or polyunsaturated fatty acids, and three ester derivatives (compounds 1-3, Figure 2-2) with enhanced lipophilicity were produced (Chapter 2). These partially substituted EGCG esters with retained/improved/novel bioactivities may serve as potential lipophilic alternatives to EGCG for diverse areas of application and in different media. The antioxidant activities of the lipophilic EGCG derivatives in different chemical assays and food model systems as well as their antiviral activities were previously studied, and found to be similar or superior to that of the original EGCG (Chapters 3 and 4). Their affinity to lipid membranes and effectiveness as antioxidants to protect against oxidation in biological systems are presented in this chapter.

Materials and methods

Materials. EGCG was supplied by Dr. Yu Shao of GlaxoSmithKline Consumer Healthcare (Parsippany, NJ, USA). Stearoyl chloride were purchased from Nu-chek Prep Inc. (Elysian, MN, USA). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were prepared as described in Chapter 2 from oils provided by Fuso Pharmaceutical

Industries, Ltd. (Osaka, Japan) and Martek Bioscience Corporation (Columbia, MD, USA), respectively. Egg yolk phosphatidylcholine (PC), human LDL and supercoiled plasmid pBR322 DNA were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON), and SYBR safe gel stain from Invitrogen Corporation (Carlsbad, CA, USA). Other chemicals were purchased from Sigma-Aldrich Canada Ltd. All solvents used were obtained from Fisher Scientific Ltd. (Ottawa, ON). The solvents employed were of reagent or HPLC grade.

Preparation of lipophilic EGCG derivatives. Ester derivatives of EGCG were prepared as described in Chapter 2. Briefly, EGCG was allowed to react with acyl chlorides of stearic acid (SA), EPA and DHA. The crude product containing a mixture of EGCG polyesters (at different degrees of substitution) was then fractionated by flash column chromatography. The predominant fraction was identified by HPLC-MS, ^1H NMR and ^{13}C NMR and subjected to subsequent antioxidant analysis in selected biological systems.

Incorporation into liposomes. The incorporation of EGCG and its derivatives into liposomes through lipid bilayers was measured in order to estimate their affinities for cell membranes and cellular uptake. The experiment was carried out using phosphatidylcholine (PC) liposome multilamellar vesicles (MLV) as model membranes, according to Hashimoto *et al.* (1999) with modifications. PC (25 mg) was dissolved in a small amount of chloroform in a round bottom flask, followed by evaporation of the solvent using a rotary evaporator to obtain a thin layer film on the inner surface of the flask. Traces of solvent were removed by nitrogen flush. An aqueous glucose solution

(300mM, 5 ml) was added to the flask, and the content was thoroughly vortexed and sonicated in an ultrosonicator, followed by four freeze-thaw cycles. The liposomal solution was diluted with 45 ml of phosphate buffer saline (PBS, 50mM, pH7.4) and transferred into a centrifuge tube. The content was centrifuged at 13000 g for 5 min to remove the untrapped glucose. The sediment was re-suspended in 25 ml of PBS to obtain a final liposomal MLV. An aliquot (0.9 ml) of the above MLV was transferred to an Eppendorf tube containing 0.1 ml of test compounds (500 μ M in ethanol) or ethanol blank. The content was mixed by vortexing and incubated for 20 min at 37°C. After incubation the mixture was centrifuged at 13000 g for 5 min, and the sediment was washed with 1 ml of 10% ethanol (in PBS), followed by a second centrifugation. The supernatants were combined and their absorbance read at 280 nm. A series of standard solutions in 10% ethanol at different concentrations was prepared for each test compound and their absorbance measured at 280 nm to construct a standard curve (absorbance vs. concentration). The un-incorporated test compounds in the supernatant were quantified using their corresponding standard curves. Percentage incorporation of each compound into the liposomes was calculated according to the following equation:

$$\% \text{ incorporation} =$$

$$100 \times (1 - \text{amount un-incorporated} / \text{amount added}) = 100 \times (1 - C \times 2 / 50)$$

where, C is the concentration of test compounds read from the standard curve.

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 (10mM, 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 (500 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 (0.125mg/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 CuSO₄ (20µM, previously incubated at 37°C). A blank containing only sample without LDL or CuSO₄ was prepared for each test compound. After incubation of the reaction mixture at 37°C for 22 hours, 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:

$$\% \text{ inhibition} = 100 \times (\text{Abs.}_{\text{control}} - \text{Abs.}_{\text{sample}}) / (\text{Abs.}_{\text{control}} - \text{Abs.}_{\text{native LDL}})$$

where, control had LDL and CuSO₄ without antioxidants, and native LDL contained LDL only. All absorbances for samples were blank-corrected.

Inhibition against DNA scission. Antioxidant activity of EGCG and its derivatives against DNA scission was determined as described by Hiramoto *et al.* (1996) with some modifications. DNA strand breaking was induced by hydroxyl and peroxy radicals and monitored by gel-electrophoresis. For hydroxyl radical-induced DNA oxidation, 2 µl of test compounds dissolved in ethanol were added into an Eppendorf tube and the solvent was evaporated under a stream of nitrogen. To the tube 2 µl of distilled water were added,

followed by thorough vortexing for 1 min. The following reagents were then added to the tube in the order stated: 2 μ l of PBS (pH7.4), 2 μ l of supercoiled pBR322 DNA, 2 μ l of H_2O_2 and 2 μ l of Fe_2SO_4 . The mixture (10 μ l) containing 1 μ M test compounds, 0.1 M PBS, 10 μ g/ml DNA, 0.2 mM H_2O_2 and 0.1 mM Fe_2SO_4 (final concentration/assay) was incubated at 37°C for 1 hour. For peroxyl radical-induced DNA oxidation, a higher concentration of samples were used, and H_2O_2 and Fe_2SO_4 were replaced with 4 μ l of AAPH [2,2'-azobis (2-aminopropane) dihydrochloride]. The reaction mixture contained 10 μ M test compounds, 0.1 M PBS, 10 μ g/ml DNA and 9 mM AAPH. A blank (DNA only) and a control (DNA + free radicals) were also prepared for both assays.

After incubation, 1 μ l of loading dye (0.25% bromophenol blue/0.25% xylene cyanol/50% glycerol) was added, and the whole 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 the images were photographed by a GelDoc apparatus equipped with a Sony digital camera. The images were analyzed using AlphaEase stand-alone software (Alpha Innotech Co., San Leandro, CA, USA) and the intensity or density of the bands (obtained from 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:

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

where, $\text{DNA}_{\text{sample}}$ and $\text{DNA}_{\text{blank}}$ are normalized concentrations of native supercoiled DNA in total DNA for sample groups and blank, respectively.

Inhibition against UV-induced liposome oxidation. Antioxidant activity of EGCG and its derivatives in inhibiting UV-induced liposome oxidation was measured. To prepare liposome MLV, 10 mg of egg yolk PC were dissolved in a small amount of chloroform in a round bottom flask and the solvent was removed using a rotary evaporator followed by nitrogen flush. The thin film on the inner surface of the flask was suspended in 10 ml of PBS (10mM, pH7.4, 150mM NaCl) by vortexing and sonication, and the emulsion was then frozen and thawed four times. The liposomal MLV obtained were diluted with 90 ml of PBS, and an aliquot (750 μl) was transferred into an Eppendorf tube containing 10 μl of the test compounds dissolved in ethanol and 240 μl of PBS. The content was mixed well and incubated at 37°C for 15 min. After incubation, the mixture was exposed to UV irradiation (254 nm) for 45 min. The oxidation was detected by measuring conjugated dienes (CD) spectrophotometrically at 234 nm against a blank without liposome (test compounds + PBS). Conjugated diene values were calculated as follows using blank-corrected absorbances:

$$\text{CD (mol/g of PC)} = \text{Abs.}/\epsilon\text{LC} ,$$

where ϵ is the absorptivity constant ($29500 \text{ M}^{-1}\text{cm}^{-1}$), L is the length of the cuvette (1 cm) and C is the final concentration of PC (g/L). Antioxidant activity was then obtained as percentage inhibition of conjugated diene formation according to the following equation.

$$\% \text{ inhibition} = 100 \times (\text{CD}_{\text{control}} - \text{CD}_{\text{sample}}) / \text{CD}_{\text{control}}$$

where control had only liposome and PBS without test compounds.

Statistical Analysis. One-way analysis of variance (ANOVA) with pairwise comparisons (Tukey's HSD) was performed at a $P < 0.05$ level using Sigmastat for Windows version 2.0 (Jandel Corp., San Rafael, CA) to determine the significant differences.

Results and discussion

Incorporation into liposomes lipid bilayers. The biological activities of tea catechins and other plant polyphenols have been examined by various *in vitro* assays for prediction of their ability to prevent diseases and promote health. However, their activities found in *in vitro* experiments may not necessarily reflect their protective effectiveness in living organisms, mostly due to their low cellular absorption. The structure–activity relationships have revealed that the dose-dependency of polyphenol bioactives *in vivo* is largely governed by the amount incorporated into the cells. Since no transporter specific to plant polyphenols has been found in mammalian cells, it is supposed that most polyphenols are incorporated into the cells by passive transport and the amount incorporated is related to their affinity for the cell membranes (Nakayama *et al.* 2006).

In this study, the cell membrane affinity and cellular uptake of EGCG and its ester derivatives were estimated using liposome MLV as model membranes. Separation of liposomes by centrifugation was facilitated by trapping a dense solution of glucose, as proposed by Hashimoto *et al.* (1999). The amount of test compounds incorporated into the core and/or lipid bilayers of the liposomes was quantified by subtracting the amount un-incorporated from the total amount added. The rates of incorporation into the liposome MLV ranged from 65.2 to 91.2% for EGCG and its derivatives (Table 5-1). The highest incorporation was found for compound 1 (91.2%), followed by compound 3 (80.9%) and

Table 5-1. Incorporation (%) of EGCG and its derivatives into liposomes.

	EGCG	Compound 1	Compound 2	Compound 3
% incorporation	65.2 ± 0.41 ^d	91.2 ± 1.89 ^a	71.8 ± 0.33 ^c	80.9 ± 1.43 ^b

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

Compounds 1, 2 and 3 are EGCG tetraesters of SA, EPA and DHA, respectively.

2 (71.8%); while EGCG (65.2%) showed the poorest liposome incorporation. The trend for incorporation of test compounds was well correlated with that of their lipophilicity, which was in the order of compound $1 > 3 \geq 2 > \text{EGCG}$ (measured as octanol-water partition coefficient, data shown in Chapter 2).

Studies on interaction of tea catechins with liposome have revealed that the affinity of catechins for lipid membrane bilayers was governed by their lipophilicity and stereochemical structure (Hashimoto *et al.* 1999; Kajiya *et al.* 2002). The authors demonstrated that the amount of tea catechins incorporated into liposome lipid bilayers followed the same order of their partition coefficients in an octanol-PBS system, i.e. epicatechin gallate (ECG) with the highest lipophilicity had the highest incorporation rate among all. It was also suggested that conformational features affect the membrane affinity of catechins (e.g. EGCG > gallocatechin gallate). The ester derivatives of EGCG with enhanced lipophilicity in this study exhibited significantly ($P < 0.05$) improved affinity to membrane phospholipids compared with EGCG. However, the effect of steric changes caused by acylation on membrane affinity remains to be unraveled. The results suggest that the lipophilic derivatives of EGCG with greater cell membrane incorporation may have better cellular absorption *in vivo* than the parent EGCG, leading to improved bioavailability and bioefficiency when ingested orally. Moreover, the liposome-water partition coefficient is an important parameter for evaluation of drug delivery in pharmaceuticals. Lipophilic EGCG derivatives may be superior to EGCG in terms of compatibility in the liposome drug delivery systems. Further research investigating the specific incorporation location of these derivatives in the liposome MLV and their

interaction with the lipid bilayers may help shed light on the absorption and antioxidant mechanisms of action of EGCG derivatives at cellular levels.

Inhibition against LDL-cholesterol oxidation. Catechins from tea have been shown to render cardioprotective effects, mostly through their interaction with LDL-cholesterol, including reducing LDL uptake by macrophages, inhibiting LDL oxidation, and decreasing the susceptibility of LDL to aggregation (Scalbert *et al.* 2005). 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). 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 given in Table 5-2. After 22 hours 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 free radicals generated. Among them, compounds **1** and **2** were significantly more effective than the parent EGCG molecule in inhibiting the formation of conjugated dienes. Compound **3** had a slightly higher effect than EGCG although statistically insignificant

Table 5-2. Inhibition (%) of EGCG and its derivatives against copper-induced LDL-cholesterol oxidation.

	EGCG	Compound 1	Compound 2	Compound 3
% inhibition	6.53 ± 0.92 ^c	16.33 ± 1.07 ^a	11.80 ± 2.26 ^{ab}	8.18 ± 2.90 ^{bc}

Values (mean ± SD of three replicates) with different letters were significantly different at P < 0.05. Compounds 1, 2 and 3 are EGCG tetraesters of SA, EPA and DHA, respectively.

($P > 0.05$). The improved inhibitory activity of the derivatives against LDL oxidation may be due to their greater lipophilicity, hence greater affinity to the phospholipids which are the major components of the surface region of LDL. Moreover, introduction of hydrophobic fatty acid side chains into the water soluble EGCG may improve its amphiphilic nature, thus allowing the derivatives to exert their antioxidant activities within the LDL particle and/or on the surface or extraparticle environment of LDL. In addition to lipophilicity, the steric features could influence the interaction between the antioxidants and LDL particles, which may be responsible for the lower efficacy of compound **3**. It is also possible that the highly unsaturated EPA and DHA side chains in compounds **2** and **3**, respectively, contributed to conjugated dienes formation, slightly counteracting its inhibition against oxidation as compared to compound **1** with saturated side chains. The overall results suggest that the lipophilic derivatives of EGCG were superior or similar to their parent EGCG in inhibiting LDL-cholesterol oxidation and may be used as potential anti-atherogenic agents in cardiovascular diseases risk reduction.

Inhibition against DNA scission. Oxidative stress in cells caused by ROS such as hydroxyl and peroxy radicals also leads to DNA damage, which is implicated in mutagenesis and carcinogenesis, among other pathological processes. Oxidative damage to the 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 as well as 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

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 was evaluated. As shown in Figures 5-1 and 5-2 (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 5-3, 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

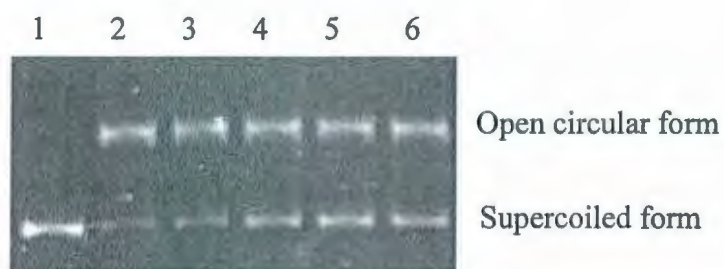


Figure 5-1. Hydroxyl radical induced DNA scission (lane 1: DNA only; lane 2: DNA + FeSO_4 + H_2O_2 ; lane 3: DNA + FeSO_4 + H_2O_2 + EGCG; lane 4: DNA + FeSO_4 + H_2O_2 + compound 1; lane 5: DNA + FeSO_4 + H_2O_2 + compound 2; lane 6: DNA + FeSO_4 + H_2O_2 + compound 3; Compounds 1, 2 and 3 are EGCG tetraesters of SA, EPA and DHA, respectively).

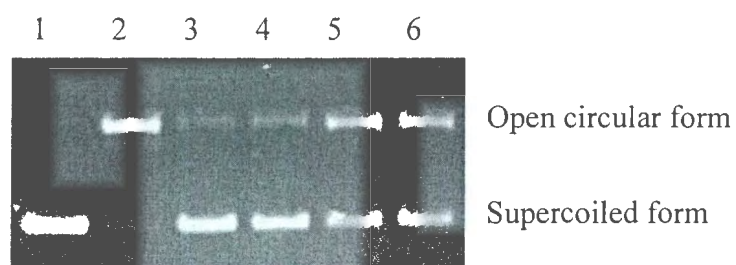


Figure 5-2. Peroxyl radical induced DNA scission (lane 1: DNA only; lane 2: DNA + AAPH; lane 3: DNA + AAPH + EGCG; lane 4: DNA + AAPH + compound **1**; lane 5: DNA + AAPH + compound **2**; lane 6: DNA + AAPH + compound **3**; Compounds **1**, **2** and **3** are EGCG tetraesters of SA, EPA and DHA, respectively).

Table 5-3. DNA retention (%) in hydroxyl and peroxy radical induced oxidative scission.

DNA retention (%)	Hydroxyl radical induced	Peroxy radical induced
Control	30.15	23.53
EGCG	35.37 ± 0.77 ^c	90.02 ± 0.18 ^a
Compound 1	48.19 ± 0.56 ^b	82.84 ± 0.49 ^b
Compound 2	53.37 ± 1.15 ^a	50.75 ± 1.02 ^d
Compound 3	47.44 ± 0.91 ^b	56.25 ± 0.89 ^c

Values (mean ± SD of three replicates) in the same row with different letters were significantly different at $P < 0.05$. Compounds **1**, **2** and **3** are EGCG tetraesters of SA, EPA and DHA, respectively.

* Control contained DNA and free radicals only.

DNA to various extents. The ester derivatives of EGCG were more effective than EGCG in protecting against DNA scission, with the EPA ester (compound 2) being the most potent antioxidant among all. This may be due to 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 greater antioxidant efficacy of compound 2 may largely be attributed to its higher metal chelation capacity (results shown in Chapter 3). 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 chapter 3) may reduce Fe^{3+} 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. EGCG at certain concentrations, where its stimulating effect dominates over scavenging effect on hydroxyl radical, have been reported to accelerate oxidative damage of DNA (Hiramoto *et al.* 1996). This may partially explain the low antioxidant efficacy of EGCG compared to that of its ester derivatives.

Incubation of supercoiled DNA with peroxy radical (generated from AAPH) also resulted in single strand breakage of the DNA (Figure 5-2). Addition of EGCG and its derivatives significantly improved the oxidative stability and retention of native DNA, which ranged from 50.75 to 90.02% (Table 5-3). However, the test compounds showed a nearly opposite trend of antioxidant efficacy to that in hydroxyl radical induced DNA scission. The ester derivatives were less effective in inhibiting DNA scission than their parent EGCG molecule. The EPA ester (compound 2), while being the most potent

antioxidant against hydroxyl radical induced DNA scission, was the least effective among all in inhibiting peroxy radical induced DNA damage. The degree of DNA oxidation varied depending on the nature of the catalysts/prooxidants 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. The ester derivatives, although less potent than EGCG at the concentrations tested, showed excellent protection (50.75-82.84% DNA retention) against peroxy radical induced DNA scission. This and their superior antioxidant activities against hydroxyl radical induced DNA scission suggest that lipophilic derivatives of EGCG may be good candidates as antimutagenic agents.

Inhibition against UV-induced liposome oxidation. UV radiation, as an oxidizing agent, is generally considered harmful to biological organisms by initiating photooxidation and causing oxidative damage in cells and tissues. Repeated exposure to UV radiation may overwhelm the body's natural antioxidant defense mechanisms, leading to oxidative stress and development of related pathogenesis including carcinogenesis. The effect of UV radiation on multicellular organisms is localized on the skin as the outermost tissue, which is constantly exposed to solar UV radiation. The cutaneous damage induced has been linked to increased risk of numerous skin disorders, including phototoxicity, photoallergy, photosenescence, photoaging and photocarcinogenesis (Guercio-Hauer *et al.* 1994). Antioxidants such as tea catechins topically or orally administered are known to provide protection against UV-induced oxidation (Bonina *et al.* 1996; Cabrielska *et al.* 2005). The *in vitro* and *in vivo* animal and human studies have reported that EGCG acts

as a photoprotective agent against UV-induced oxidative stress (Katiyar 2003; Morley *et al.* 2005).

UV radiation can induce lipid oxidation in biological membranes, causing structural and functional changes that eventually lead to oxidation of cellular components of the skin and other cells. Hence, many studies on the effects of UV radiation and antioxidant protection have focused on lipid oxidation in cell membranes and related models thereof (liposomes). In this study, the ability of EGCG and its derivatives to inhibit UV-induced liposome oxidation was investigated.

As shown in Table 5-4, all test compounds exhibited inhibitory effects against lipid oxidation in the liposomes, ranging from 3.47% for compound **2** to 47.6% for compound **1**. The antioxidant activities of EGCG and its derivatives may be attributed to their ability for scavenging free radicals (e.g. superoxide anion, hydroxyl, peroxy radicals, etc.), quenching singlet oxygen and absorbing UV radiation, among others. Their antioxidant efficacy increased in the order of compound **2** < EGCG < **3** < **1**, which did not correlate well with their incorporation rates into liposomes (Table 5-1). Compounds **1** and **3** exhibited significantly higher antioxidant activity than EGCG, as expected, based on their greater liposome affinity. However, compound **2** had greater liposome incorporation than EGCG but lower antioxidant effectiveness. The observed variations may be caused by the existing differences in their interaction with the liposome lipid bilayers, i.e. their location and effects on membrane structure. Polyphenols are incorporated into liposomes differently, depending on the lipophilicity, stereochemical structure and concentration of the molecules. Some polyphenols are absorbed on the membrane surface, while others may permeate deeper into the hydrophobic region of the lipid bilayers, as observed for

Table 5-4. Inhibition (%) of EGCG and its derivatives against UV-induced liposome oxidation.

	EGCG	Compound 1	Compound 2	Compound 3
% inhibition	9.53 ± 1.61 ^c	47.6 ± 0.96 ^a	3.47 ± 0.53 ^d	16.2 ± 3.53 ^b

Values (mean ± SD of three replicates) with different letters were significantly different at P < 0.05. Compounds 1, 2 and 3 are EGCG tetraesters of SA, EPA and DHA, respectively.

different catechins (Hashimoto *et al.* 1999; Kubota *et al.* 2007). Antioxidants at different locations of the liposomes may render different efficacies in inhibiting oxidation. It has been suggested that antioxidants absorbed on the membrane surface or those (interface antioxidants) located in the polar region of the bilayer near the hydrophobic-hydrophilic border are effective in protecting the membranes against oxidation (Gabrielska *et al.* 2006). It is possible that compound **2** was distributed in a lower proportion on the liposome surface compared to other compounds, or it was preferentially located in the hydrophobic core of the lipid bilayers, hence being less effective as an antioxidant. Moreover, it is worth noting that some polyphenols including EGCG at higher concentrations may enter into the lipid bilayer and broaden or even perturb the membrane, leading to compromised protection or even prooxidant effect (Kajiya *et al.* 2002; Kubota *et al.* 2007), which may also account for lower antioxidant efficacy of compound **2**. Further investigation is required to better understand the mechanism(s) of the interaction between the EGCG derivatives and membrane lipid bilayers.

Conclusions

Ester derivatives of EGCG with improved lipophilicity were readily incorporated into the model membrane (liposome MLV) and exhibited better or comparable antioxidant activities to that of the parent EGCG molecule in biological model systems. The derivatives had higher liposome incorporation than EGCG, suggesting their higher cellular absorption *in vivo* and greater potential in liposome drug delivery. LDL-cholesterol oxidation was inhibited by EGCG derivatives to a greater extent than by EGCG, which indicates their greater potency as anti-atherogenic and cardioprotective

agents. The derivatives were also able to inhibit hydroxyl and peroxy radical-induced DNA scission, and may be used as potential inhibitors for mutagenesis and carcinogenesis. The derivatives exhibited inhibitory effect on UV-induced oxidation of liposome, which supports their potential use in skin care and cosmetic products.

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CHAPTER 6

Anti-inflammatory activity of lipophilic epigallocatechin gallate (EGCG) derivatives in LPS-stimulated murine macrophages

Introduction

Inflammation is a normal physiological response of the immune system to counteract pathological states such as irritation/infection caused by chemicals, microbial pathogens and/or wounding. However, unbalanced or prolonged inflammation leads to progressive tissue damage and has been implicated in the development of many chronic diseases such as cancer and neurodegenerative disorders as well as diabetes and cardiovascular disease. Inflammation is characterized by recruitment of a wide range of immune cells (e.g. neutrophils, macrophages and monocytes, etc.) to the inflamed sites and the release of various pro-inflammatory cytokines and reactive oxygen/nitrogen species (ROS/RNS). Oxidative stress through continuous overproduction of ROS/RNS by activated phagocytes constitutes the major tissue-destructive force *in vivo*, and in turn promotes inflammation by stimulating production of inflammatory mediators and cytokines (Shahidi and Zhong 2009). Evidence has shown that ROS/RNS are involved in the activation of a variety of kinases and transcription factors, whose regulation is dependent on the redox changes. The transcription factors nuclear factor-kappa B (NF- κ B) and activator protein (AP)-1, for example, are redox-sensitive and become activated under oxidative/nitrosative stress. Once activated, NF- κ B and AP-1 translocate from the cytoplasm to the nucleus, leading to the up-regulation of numerous inflammatory genes, such as those coding for inducible nitric oxide synthase (iNOS) and cyclooxygenase

(COX)-2, among others (Kamata and Hirata 1999). Hence, ROS/RNS and pro-inflammatory cytokines work in a synergistic manner through a ROS/RNS-cytokine-transcription factor regulatory loop, thereby augmenting the inflammatory response and tissue damage (Fiocchi, 1998). The production of NO and prostaglandins by iNOS and COX-2, respectively, are considered to be the most prominent molecular mechanisms in the inflammatory processes (Moncada 1999; Turini and DuBois 2002), and are also involved in the multistage carcinogenesis, especially the promotion stage (Pan and Ho 2008). Excessive and prolonged NO generation caused by overexpression of iNOS has also been implicated in inflammatory tumorigenesis; while COX-2-mediated prostaglandin production stimulates cell proliferation, invasion and angiogenesis in cancer development (Mann *et al.* 2005).

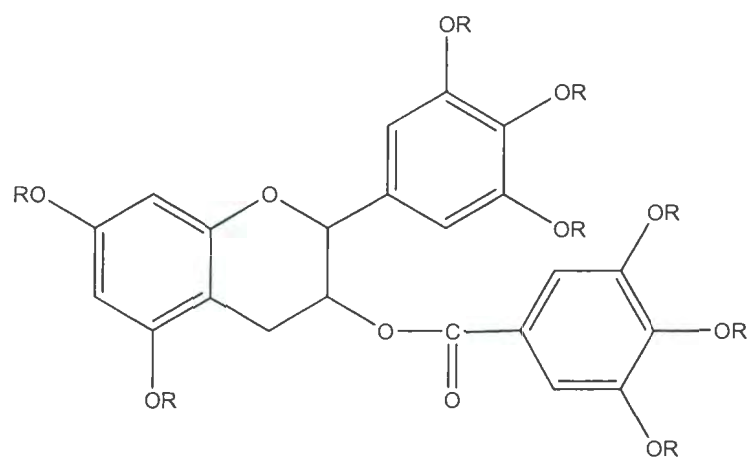
Anti-inflammatory agents can block the ROS/RNS and cytokine-involved inflammatory cascade. Compared to steroidal or non-steroidal chemical drugs for treating inflammation, naturally derived substances are readily available at lower costs while having limited side effects and intolerance. Polyphenols found abundantly in plant foods have been studied for their anti-inflammatory activities in suppressing the synthesis (gene expression) and action (enzyme activity) of many pro-inflammatory mediators. Tea catechins, especially epigallocatechin gallate (EGCG), have been reported to exhibit anti-inflammatory activity through their ability to scavenge NO, peroxynitrite (ONOO⁻) and other ROS/RNS, to inhibit the translocation of NF- κ B and AP-1 from cytoplasm to nucleus, and to inhibit the activity of iNOS and COX-2 (Paquay *et al.*, 2000; Nagai *et al.*, 2002; Tedeschi *et al.*, 2004). EGCG is found abundantly in green tea as a major polyphenol with a variety of health benefits. However, the molecule is hydrophilic with poor solubility in lipid systems,

which, at least partially, accounts for its low absorption by the cells and limited efficacy in the cellular environment. Moreover, cell line studies have suggested that EGCG and other tea catechins are subject to active efflux by multidrug resistance-related proteins, leading to restricted bioefficiency *in vivo* (Hong *et al.* 2003). In such cases, prodrug strategies may be employed. Ester-based prodrugs are known to provide improved bioavailability and reduced toxicity by occlusion of polar side chains, increasing hydrophobicity and making the hydroxyl groups unavailable for phase II biotransformation or oxidative degradation (Lambert *et al.* 2006).

In this work, EGCG was structurally modified by esterification with different fatty acids to produce lipophilic ester derivatives with enhanced cellular absorption and bioefficiency *in vivo*. EGCG was esterified with docosapentaenoic acid (DPA) and the crude product (EGCG-DPA ester mixture containing mainly tetra- and pentaesters, Figure 6-1) was evaluated for its anti-inflammatory activities in terms of inhibition against NO and prostaglandin E₂ (PGE₂) production as well as iNOS and COX-2 gene expression in murine macrophages. Three isolated EGCG ester compounds (**1**, tetrastearate; **2**, tetraeicosapentaenoate; **3**, tetradocosahexaenoate) were also examined for their effect on NO production and iNOS and COX-2 expression, in order to compare the anti-inflammatory efficacy of the esters with that of the original EGCG on a molar basis.

Materials and methods

Materials. EGCG was supplied by Dr. Yu Shao of GlaxoSmithKline Consumer Healthcare (Parsippany, NJ, USA). Stearoyl chloride was purchased from Nu-chek Prep Inc. (Elysian, MN, USA). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)



EGCG: R=H

EGCG-DPA: R=CO(CH₂)₅(CH=CHCH₂)₅CH₃ at some positions, and =H at others

Figure 6-1. Chemical structures of EGCG and EGCG-DPA esters.

were prepared as described in Chapter 2 from oils provided by Fuso Pharmaceutical Industries, Ltd. (Osaka, Japan) and Martek Bioscience Corporation (Columbia, MD, USA), respectively. DPA was produced via a proprietary procedure from seal blubber oil. RAW 264.7 cells, derived from murine macrophages, were purchased from the American Type Culture Collection (Rockville, MD, USA); cell culture medium from GIBCO (Grand Island, NY, USA); ACE TM EIA kit from Cayman Chemical Co. (Ann Arbor, MI, USA); bicinchoninic acid assay (BCA) kit from Pierce (Rockford, IL, USA); PVDF membranes from Millipore Corporation (Bedford, MA, USA); iNOS and COX-2 monoclonal antibodies, anti-mouse and anti-rabbit IgG antibodies from BD Transduction Laboratories (Lexington, KY, USA); anti- β -actin monoclonal antibodies from Oncogene Science Inc. (Uniondale, NJ, USA); ECL western blotting detection agent from Amersham Corporation (Arlington Heights, IL, USA); Trizol Reagent from Invitrogen Life Technologies (Carlsbad, CA, USA); and PCR Beads from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Other chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). All solvents used were obtained from Fisher Scientific Ltd. (Ottawa, ON). The solvents employed were of reagent or HPLC grade.

Preparation of EGCG ester derivatives. Ester derivatives of EGCG were prepared as previously described in Chapter 2. Briefly, EGCG was reacted with acyl chloride of DPA, stearic acid (SA), EPA or DHA. The crude product containing a mixture of EGCG-DPA polyesters (mainly tetra- and pentaesters) was used as such without further purification for subsequent anti-inflammation analyses. The SA, EPA and DHA esters were fractionated by flash column chromatography. The predominant fraction was identified

by HPLC-MS, ^1H NMR and ^{13}C NMR. These isolated pure compounds were evaluated on a molar basis for their effect on NO production and iNOS and COX-2 expression.

Cell culture. RAW 264.7 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 media (without phenol red) supplemented with 10% endotoxin-free, heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. When the cells reached a density of $2\text{--}3 \times 10^6$ cells/ml, they were activated by incubation in a medium containing *E. coli* LPS (100 ng/ml). Various concentrations of test compounds dissolved in dimethyl sulfoxide (DMSO) were added together with LPS. Cells were treated with 0.05% DMSO as vehicle control.

Nitrite assay. The LPS-induced NO production by the macrophages was determined as the nitrite concentration in the culture medium according to the Griess reaction (Kim *et al.* 1995). The culture medium of control and treated cells was collected and centrifuged. An aliquot (100 μL) of the supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). Absorbance of the mixture was read at 550 nm with an ELISA plate reader (Dynatech MR-7000, Dynatech Laboratories, Chantilly, VA, USA).

Determination of PGE₂

The level of PGE₂ released into the culture medium was quantified by a specific enzyme immunoassay (EIA) method using a ACE TM EIA kit according to the manufacturer's instructions.

Western Blotting. The stimulated murine macrophage cell line RAW 264.7 cells were washed with phosphate buffer solution (PBS) and lysed in an ice cold radioimmunoprecipitation assay (RIPA) buffer (Tris-HCl, pH 7.2, 25 mM; 0.1% sodium dodecyl sulfate (SDS); 1 mM ethylenediaminetetraacetic acid (EDTA); 1% Triton X-100; 1% sodium deoxycholate; 0.15 M NaCl) containing 1 mM phenylmethyl sulfonyl fluoride (PMSF), 10 µg/mL aprotinin, 1 mM sodium orthovanadate, and 5 µg/mL leupeptin. Protein concentrations were determined using the bicinchoninic acid assay (BCA) kit. The samples (50 µg of protein) were mixed with 5-fold sample buffer containing 0.3 M Tris-HCl (pH 6.8), 25% 2-mercaptoethanol, 12% SDS, 25 mM EDTA, 20% glycerol, and 0.1% bromophenol blue. The mixtures were boiled at 100 °C for 5 min, pre-run on a stacking gel, and then resolved by 12% SDS-polyacrylamide minigels at a constant current of 20 mA. For western blot, proteins on the gel were electro-transferred onto a 45 µm immobile polyvinylidene fluoride (PVDF) membrane with a transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol, as described by Pan *et al.* (2000). Membrane blocking was then carried out at room temperature for 1 h in blocking solution (20 mM Tris-HCl pH 7.4, 125 mM NaCl, 0.2% Tween 20, 1% bovine serum albumin, and 0.1% sodium azide). The membrane was incubated with the primary antibody of iNOS and COX-2 at 4°C overnight. The membrane was subsequently probed with anti-mouse or anti-rabbit IgG antibody conjugated to horseradish peroxidase and visualized using enhanced chemiluminescence. The densities of the bands were quantitated with a computer densitometer (AlphaImager 2200 System, Alpha Innotech Corp., San Leandro, CA, USA). All the membranes were stripped and reprobed for β-actin as loading control.

Reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was isolated from mouse macrophage RAW264.7 cell using Trizol Reagent according to the manufacturer's instructions. Changes in the steady-state concentration of mRNA in iNOS, COX-2, and β -actin (control) were assessed by RT-PCR. Total RNA (2 μ g) was converted to cDNA in a series of standard 10 μ l reverse transcription reactions. DNA amplification was carried out in "Ready To Go" PCR Beads. The initial conditions were 95 °C for 5 min. Amplification 30 cycles of iNOS were 95 °C for 40 s, 65 °C for 60 s, and 72 °C for 2 min, followed by a 10 min extension at 72 °C. The thermal cycle conditions of COX-2 were initiated at 95 °C for 60 min, then 30 cycles of amplification (94 °C for 45 s, 55 °C for 60 s, and 72 °C for 2 min) were performed followed by 10 min extension at 72 °C. The PCR products were separated by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. Amplification of β -actin served as a control for sample loading and integrity. PCR was performed on the cDNA using the following sense and antisense primer: iNOS, forward primer 5'-CCCTTCCGAAGTTTC-TGGCAGCAGC-3' (2944-2968), reverse primer 5'-GGCTGTCAGAGCCTCG-TGGCTTTGG-3' (3419-3443); COX-2, forward primer 5'-GGAGAGACTATCAAGATAGTGATC-3' (1094-1117), reverse primer 5'-ATGGTCAGTAGACTTTTACAGCTC-3' (1931-1954); β -actin, forward primer 5'- AAGAGAGGCATCCTCACCCCT-3', reverse primer 5'-TACATGGCTGGGGTGTTGAA-3'. Confirmation of the correct amplicons was obtained by direct DNA sequencing of the PCR products.

Results and discussion

Inhibition of LPS-induced nitrite production by EGCG-DPA esters. Nitric oxide (NO) is an important oxidative and inflammatory mediator produced by NOS under physiological and pathophysiological conditions. It is a lipid-soluble free radical with a considerably long life and capable of diffusing several cell diameters from its synthesis site (Kruidenier and Verspaget, 2002). NO itself at nanomolar concentrations is not particularly harmful and in some occasions may even exert beneficial effects (Kruidenier and Verspaget, 2002). However, it is the precursor of a more damaging RNS peroxynitrite anion (ONOO^-), a stable and reactive oxidizing/nitrating agent that can damage a broad array of biomolecules in cells. Excessive and uncontrolled production of NO in activated immune cells during inflammation contributes to the major destructive force in tissue injury. NO production by immune cells has been used as an indicator of the presence and extent of inflammation as well as the effectiveness of anti-inflammatory agents.

In this work, the ability of EGCG-DPA esters to inhibit NO production in murine RAW 264.7 macrophages was evaluated and compared with that of their acyl donor DPA. The macrophages were activated by LPS, and NO production was measured as nitrite concentration in the culture medium. LPS is a cell wall component of gram-negative bacteria that is known to induce the activation of monocytes and macrophages and production of pro-inflammatory mediators and cytokines (Nicholas *et al.* 2007). As shown in Figure 6-2, LPS treatment resulted in a sharp increase in the nitrite level in the macrophages. Both DPA and EGCG-DPA were effective in alleviating the LPS-induced NO overproduction. Their inhibitory effect did not differ significantly within the concentration range of 2.5-25 $\mu\text{g/ml}$, whereas a dramatic drop (~50%) in nitrite level was

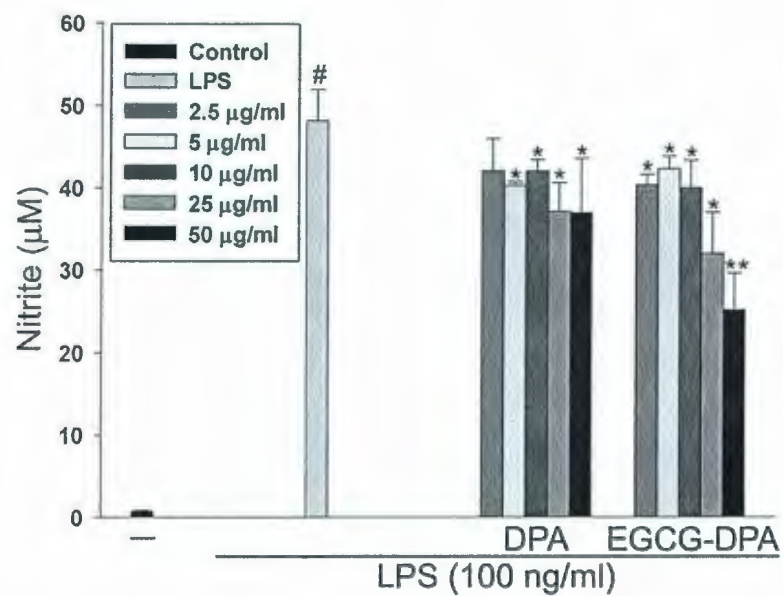


Figure 6-2. Effect of DPA and EGCG-DPA on NO production in LPS-stimulated RAW264.7 macrophages (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ compared with the LPS treatment only; #, $P<0.001$ compared with the control).

observed in macrophages treated with 50 µg/ml of EGCG-DPA but not for the DPA counterpart. This suggests that EGCG-DPA esters at certain concentrations may act as efficient anti-inflammatory agents by inhibiting production of NO.

Direct and indirect mechanisms have been reported for NO inhibition by EGCG and other tea catechins (Paquay *et al.* 2000). Similar mechanisms may be involved for EGCG ester derivatives with possible additional contribution from the fatty acid side chain. EGCG has been demonstrated to inhibit NO production by suppressing gene expression of iNOS, an enzyme catalyzing production of NO and an important target for anti-inflammatory drug search or design. EGCG-DPA may render a similar suppressing effect on iNOS biosynthesis. This hypothesis is supported by the effect of EGCG-DPA esters on iNOS gene expression, which was also investigated in this study (results in Figure 6-4). Moreover, EGCG-DPA esters may inhibit the activity of iNOS at the enzyme level through the polyphenol-protein binding, as observed for many polyphenols including EGCG. In addition to the inhibition of iNOS, the EGCG moiety in the ester may render a direct scavenging effect on NO, and the lipophilic DPA moiety may contribute to a higher affinity of the molecule to the lipid soluble free radical.

Inhibition of LPS-induced PGE₂ production by EGCG-DPA esters. Prostaglandins are a group of arachidonic acid (AA)-derived eicosanoids biosynthesized through a cyclooxygenase pathway. They are lipid mediators that coordinate a wide variety of physiologic and pathologic processes (Fitzpatrick and Soberman 2001). Prostaglandins under physiologic conditions play important roles in the cytoprotection of gastric mucosa, hemostasis, and renal homodynamic. However, their stimulated biosynthesis is implicated

in pathogenesis of various diseases including inflammation and cancer (Patrignani *et al.* 2005). Enhanced prostaglandin production is observed in immune cells, due to the relatively high levels of AA as the precursor in the immune cell membrane phospholipids as well as the induction of COX-2 in response to cellular stimulation. PGE₂ is the best characterized prostaglandin and a major target for anti-inflammation and anti-cancer research.

As shown in Figure 6-3, PGE₂ production in LPS-stimulated macrophages was inhibited by EGCG-DPA esters and DPA, especially at high concentrations (25 and 50 µg/ml). The inhibitory effect was found to be stronger by EGCG-DPA esters than by the fatty acid DPA. DPA as an omega-3 fatty acid may act as a competitive inhibitor against the omega-6 fatty acid AA for the COX enzymes. It has been well documented that incorporation of increased amounts of omega-3 fatty acids such as EPA and DHA into cell membranes results in decreased production of arachidonic acid-derived eicosanoid mediators such as PGE₂ (Clifton 2009). Meanwhile, EGCG is able to inhibit both the gene expression and activity of COX-2, the enzyme that catalyses the conversion of AA into prostaglandins (Kundu *et al.* 2003). The EGCG-DPA esters with improved lipophilicity and cellular absorption may take advantage of both the DPA and EGCG moieties as well as possible synergistic effects between them. It is worth noting that both NO and PGE₂ inhibitory effects of the test compounds were determined on a weight basis, and an even more distinctive effect may be expected for the ester derivatives than for DPA at equimolar levels, due to the much higher molecular weight of the esters.

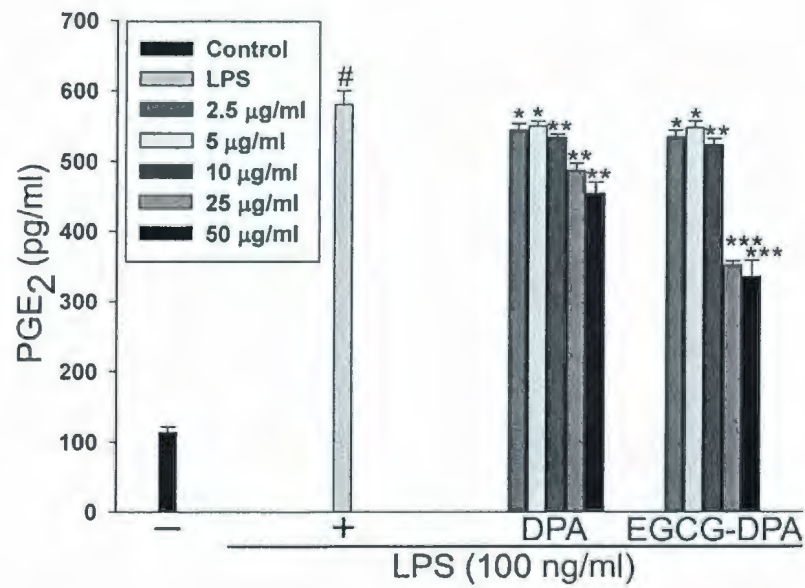


Figure 6-3. Effect of DPA and EGCG-DPA on PGE₂ production in LPS-stimulated RAW264.7 macrophages (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with the LPS treatment only; #, $P < 0.001$ compared with the control).

Inhibition iNOS and COX-2 induction by EGCG-DPA esters. In order to further confirm that NO and PGE₂ production was inhibited by the test compounds through, at least partially, down-regulation of iNOS and COX-2, the protein levels of the two inflammatory mediators were determined. NOS comprise a family of enzymes that catalyse the production of NO *in vivo*, and are commonly known in two forms: cNOS that is constitutively present in several types of cells (e.g. neurons and endothelial cells), and iNOS whose expression is induced in response to pro-inflammatory cytokines and bacterial LPS (Pan *et al.* 2009). In addition to its pro-inflammatory role, iNOS has also been proposed to be a major factor involved in pathological vessel dilation and tumorigenesis (Biesalski 2007). COX-2 is an inducible enzyme catalyzing the conversion of AA to prostaglandins. Bioactive lipids produced by COX-2 such as PGE₂, have been identified to be potent inflammatory mediators that can also promote tumor growth and metastasis (Mann *et al.* 2005). Suppression of induction and activities of iNOS and COX-2 is an important approach to preventing inflammation and carcinogenesis.

The effect of EGCG-DPA esters on induction of iNOS and COX-2 protein was evaluated in LPS-stimulated murine RAW 264.7 macrophages using β -actin as the control protein. According to the results from western blotting (Figure 6-4), LPS remarkably increased the protein level of both iNOS and COX-2, while EGCG-DPA esters and DPA were able to attenuate the increase caused by LPS to various extents. Both the esters and DPA showed a dose-dependent relationship in suppressing the production of iNOS and COX-2, and the inhibitory effect was greater for the EGCG-DPA esters than for DPA. The iNOS concentrations in macrophages treated with EGCG-DPA were reduced by >50% compared to those treated with LPS alone, and were even lower than the negative control,

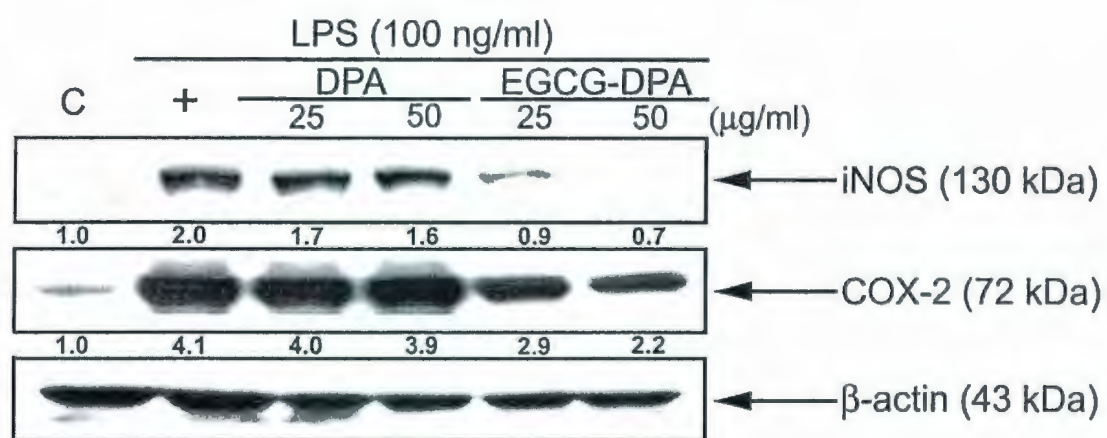


Figure 6-4. Effect of DPA and EGCG-DPA on expression of iNOS and COX-2 proteins in LPS-stimulated RAW264.7 macrophages (C: negative control without LPS; + : positive control with LPS).

i.e. in the absence of LPS stimulator. COX-2 induction was also inhibited to a large extent by EGCG-DPA esters, while only a slight inhibition was observed for DPA. These results correlated well with their inhibition against NO and PGE₂ production, suggesting that suppression of iNOS and COX-2 induction plays a major role in the anti-inflammatory mechanism of EGCG-DPA esters.

The inhibition of iNOS and COX-2 induction by EGCG-DPA esters may be due to the down-regulation of their gene expression at transcriptional level, as reported for EGCG and other tea catechins (Lin and Lin, 1997; Kim *et al.* 2007). Gene expression of iNOS and COX-2 is promoted by the transcription factor NF- κ B and AP-1, which are redox-sensitive and become activated under oxidative stress. This may explain why many antioxidants also possess anti-inflammatory activity. The EGCG-DPA esters with antioxidant potential (as reported for SA, EPA and DHA esters in chapters 2-5) may alleviate oxidative stress and inhibit activation and/or translocation of NF- κ B and AP-1, thus down-regulating the expression of the iNOS and COX-2 genes.

Inhibition of iNOS and COX-2 mRNA expression by EGCG-DPA esters. The decrease in the concentration of iNOS and COX-2 in EGCG-DPA treated macrophages could be due to inhibited synthesis or promoted degradation of the proteins. The mRNA expression of the two inflammatory mediators was monitored in order to test the involvement of transcriptional events during their biosynthesis. Changes in the steady-state concentration of mRNA for iNOS and COX-2 were assessed by RT-PCR using β -actin as the control gene.

As shown in Figure 6-5, mRNA expression of iNOS and COX-2 was markedly enhanced by LPS, while EGCG-DPA esters effectively inhibited the LPS-induced up-regulation of the their mRNA. The level of iNOS mRNA was reduced by >50% in EGCG-DPA treated macrophages, and a greater inhibition was achieved at the lower concentration (25 µg/ml). The mRNA expression for COX-2 was also inhibited, albeit to a lesser extent (<50%), by EGCG-DPA esters. The effect of DPA on mRNA expression, however, was inconsistent between the two inflammatory mediators. DPA-treated macrophages showed a mild decrease in iNOS mRNA level, while the mRNA for COX-2 was slightly up-regulated by DPA at 50 µg/ml.

The results from RT-PCR on the iNOS and COX-2 mRNA expression as affected by EGCG-DPA suggest involvement of a pretranslational or transcriptional mechanism in the inhibition of iNOS and COX-2 protein production. As discussed earlier, the down-regulation effect of EGCG-DPA esters on the two inflammatory mediators may arise from their EGCG moiety, which has been demonstrated to inhibit the activation of transcription factors NF-κB and AP-1 through various mechanisms (Surh 2006). The DPA, according to Figure 6-5, exerted an insignificant effect on transcription of the iNOS and COX-2 genes. However, its incorporation into the EGCG molecule may lead to improved cell membrane affinity and enhanced cellular absorption of the esters into the macrophages, and hence improved bioefficiency. The DPA moiety of the esters after absorption may then contribute to the anti-inflammatory activity through other mechanisms.

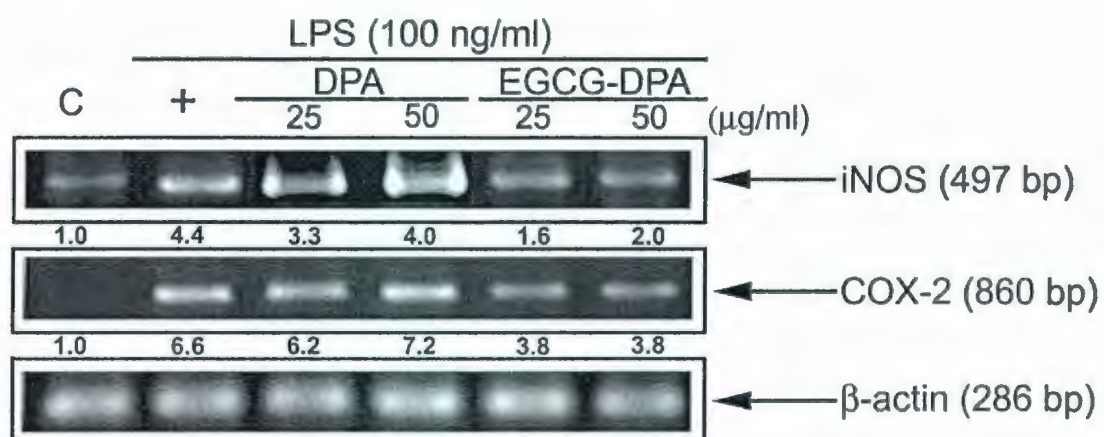


Figure 6-5. Effect of DPA and EGCG-DPA on expression of iNOS and COX-2 mRNA in LPS-stimulated RAW264.7 macrophages (C: negative control without LPS; + : positive control with LPS).

Anti-inflammatory activity of EGCG and its individual ester derivatives. In addition to the DPA ester mixture, three isolated EGCG esters (SA, EPA and DHA tetraesters, structure shown in Figure 2-2 of Chapter 2) were also investigated on a molar basis for their inhibitory effect against NO production and iNOS and COX-2 expression.

All test compounds (including EGCG and its esters) inhibited NO production in LPS-stimulated macrophages in a dose dependent manner. The IC_{50} value (concentration to achieve 50% inhibition) obtained for each compound (Figure 6-6) indicated the highest IC_{50} (103.3 μ M) for EGCG, and hence the lowest efficiency in inhibiting NO production. The ester derivatives showed greater inhibitory effects than the EGCG molecule itself, with low IC_{50} ranging from 29.8 to 40.7 μ M. The derivatives were estimated to have higher cellular absorption than EGCG due to their higher lipophilicity and cell membrane affinity (Chapter 5), suggesting larger amounts of the compounds incorporated into the macrophage cells that are available to render their anti-inflammatory effect. Among the derivatives, compounds **2** and **3** with polyunsaturated side chains had slightly lower IC_{50} than compound **1** with saturated side chain. The steric characteristics of the derivatives, especially those of compounds **2** and **3** with bending fatty acid side chains, may favor iNOS binding and account for greater inhibition of the enzyme activity than that exerted by EGCG.

The EGCG derivatives exhibited down-regulation effects on iNOS and COX-2 biosynthesis. All test compounds inhibited the LPS-induced iNOS expression in the macrophages in a dose-dependent manner (Figure 6-7). Compound **3** was the most potent inhibitor, followed by EGCG. Compounds **1** and **2** were less effective, except for compound **2** at a higher concentration (40 μ M). This trend did not correlate with that of

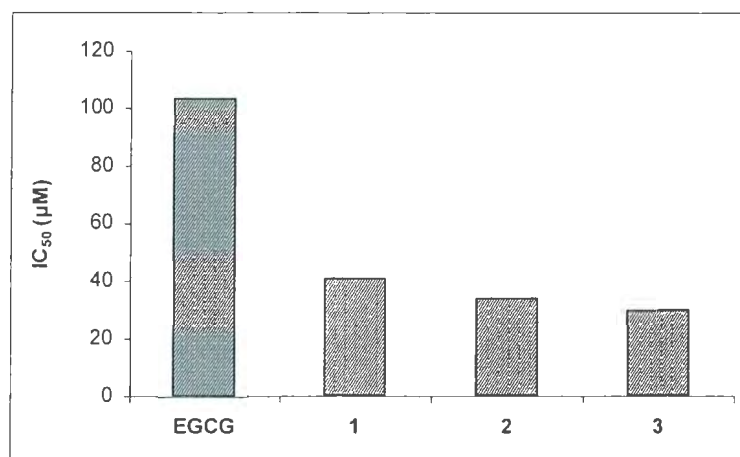


Figure 6-6. Inhibitory effect of EGCG and its derivatives on nitrite production in LPS-stimulated RAW264.7 macrophages (compounds **1**, **2** and **3** are EGCG tetraesters of SA, EPA and DHA, respectively).

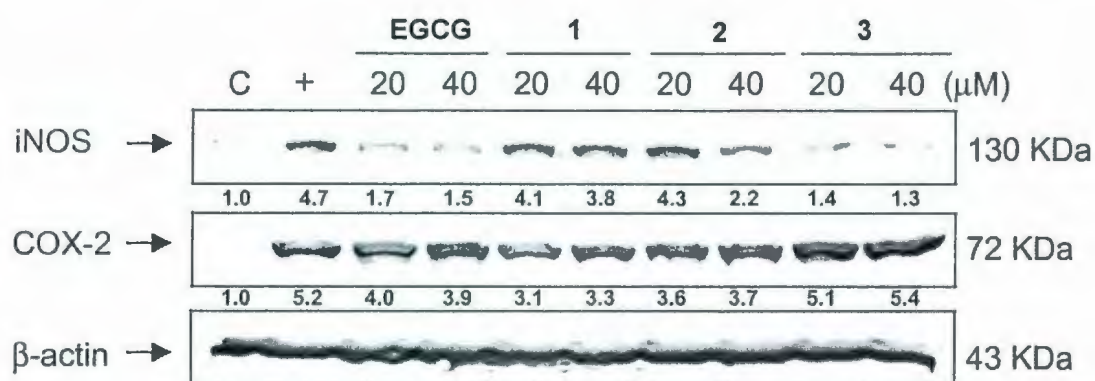


Figure 6-7. Effect of EGCG and its derivatives on expression of iNOS and COX-2 proteins in LPS-stimulated RAW264.7 macrophages (Compounds 1, 2 and 3 are EGCG tetraesters of SA, EPA and DHA, respectively; C: negative control without LPS; +: positive control with LPS).

their NO inhibition capability, where the derivatives (compounds **1-3**) showed a higher potency than EGCG. The variation may be attributed to the contribution of other possible mechanisms to NO inhibition, such as inhibition of the iNOS activity and NO radical scavenging, as discussed earlier.

Macrophages treated with EGCG and its derivatives showed altered protein levels of COX-2 compared to those treated with LPS alone (Figure 6-7). All test compounds inhibited COX-2 induction at 20 μ M, while compound **3** at 40 μ M resulted in slightly increased level of COX-2. The inhibitory effect of compounds tested decreased in the order of Compound **1** > **2** > EGCG > **3**, which is opposite to that observed for iNOS inhibition. The difference in their inhibitory trend against iNOS and COX-2 expression may be due to other mechanisms involved in addition to blocking of the NF- κ B and AP-1 signalling pathways. EGCG and its derivatives may also suppress the production of the two inflammatory mediators by breaking down the protein and mRNA of iNOS and COX-2 to various degrees, which requires further investigation.

Conclusions

Ester derivatives of EGCG with improved lipophilicity exhibited anti-inflammatory activities in LPS-stimulated murine macrophages, possibly through enhanced cellular absorption and contribution from both EGCG and the fatty acid moieties. The EGCG esters showed inhibition against LPS-induced production of NO and PGE₂ in the macrophages. The inhibitory effect is largely related to their ability to down-regulate the inflammatory mediators (iNOS and COX-2) biosynthesis at transcriptional level, while other mechanisms may also be operative, such as direct inhibition of the enzyme activity

and scavenging of NO by EGCG, and substrate competition of the omega-3 fatty acids with arachidonic acid, the precursor for pro-inflammatory prostaglandins. These findings suggest that the EGCG ester derivatives may serve as lipophilic alternatives to EGCG in preventing/treating inflammation-mediated diseases.

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CHAPTER 7

Protective effects of epigallocatechin gallate (EGCG) derivatives on azoxymethane-induced colonic tumorigenesis in mice

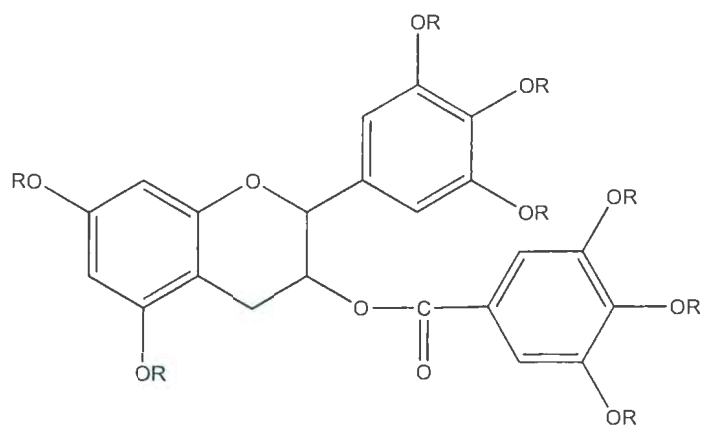
Introduction

Colorectal cancer (CRC) is the second most often diagnosed cancer and a significant cause of mortality in the western countries (Stevens *et al.* 2007). Both hereditary and environmental factors (through ingestion of genotoxins and carcinogens in alimentation) contribute to the development of CRC (Yuan *et al.* 2008). The pathogenesis of CRC involves sequential and multistep progression of epithelial cells initiated to a cancerous state with defined precancerous intermediaries (Raju 2008). Colonic aberrant crypt foci (ACF) are identified as intermediate precancerous lesions and precursors of adenoma and cancer, and may serve as predictive biomarkers of CRC. ACF are both morphological and genetic abnormalities in single crypts during the development of colonic mucosal preneoplasia with potential for progression, and are often used to identify modulators of colon carcinogenesis, including carcinogens and anticarcinogens (Stevens *et al.* 2007). In the rat model, the ACF was enhanced by cancer promoters such as azoxymethane (AOM) and suppressed by chemopreventive agents (Yakayama *et al.* 1998). The ACF were observed in rat colon a few weeks after treatment with a colon-specific carcinogen and grew larger with time with more marked nuclear dysplasia (McLellan *et al.* 1991). It has been widely accepted that the incidence of ACF in rodents correlates strongly with the final tumor outcome. In humans, significant correlation has also been established between

the number of ACF, the size of the foci, the presence of dysplastic foci and the number of adenomas (Yakayama *et al.* 1998).

Epigallocatechin gallate (EGCG), the major bioactive polyphenolic compound in tea, has been known to possess anticancer potential, which is thought to be attributed to its antioxidant activity, induction of phase II enzymes, inhibition against cell proliferation, and induction of apoptosis, among others (Pham-Huy *et al.* 2008). EGCG and other tea polyphenols have been shown to inhibit tumorigenesis in different animal models, including those with induced cancers of the skin, lung, oral cavity, esophagus, stomach, small intestine, colon, bladder, liver pancrease, prostate and mammary glands (Yang *et al.* 2008). Inhibitory effects of tea and tea polyphenols on intestinal tumorigenesis have been reported in mice (Yin *et al.* 1994; Suganuma *et al.* 2001). Xiao *et al.* (2008) demonstrated that dietary tea polyphenols (containing 65% EGCG) significantly and dose-dependently inhibited the colonic ACF formation, especially the high-grade dysplastic ACF in AOM-treated F344 rats. The inhibition was proposed to be associated with the capability of EGCG and other polyphenols to promote apoptosis, prevent aberrant nuclear β -catenin accumulation, decrease cyclin D1 level and maintain RXR α expression.

In this work, the EGCG molecule was structurally modified by esterification with selected fatty acids to yield ester derivatives in order to take advantage of the ester-based prodrugs, including improved lipophilicity and bioavailability *in vivo* as well as additional benefits or synergism arising from the fatty acid moiety, in this case, docosahexaenoic acid (DHA) and butyric acid. A EGCG-DHA mixture (54.7% tetraester and 3.1% pentaester and 42.2% unreacted EGCG) and perbutyrate EGCG (EGCG octabutryrate) (Figure 7-1) were evaluated for their effect on colon tumorigenesis in AOM-treated mice



EGCG-octabutyrate: $R = \text{CO}(\text{CH}_2)_2\text{CH}_3$

EGCG-DHA: $R = \text{CO}(\text{CH}_2)_2(\text{CH}=\text{CHCH}_2)_6\text{CH}_3$ at some positions, and $=\text{H}$ at others

Figure 7-1. Chemical structures of EGCG and its derivatives.

by monitoring the ACF formation and expression of two tumor-promoting enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2).

Materials and methods

Materials. EGCG was supplied by Dr. Yu Shao of GlaxoSmithKline Consumer Healthcare (Parsippany, NJ, USA). Butyroyl chloride was purchased from Nu-chek Prep Inc. (Elysian, MN, USA). DHA was prepared as described in Chapter 2 from DHASCO (Martek Bioscience Corporation, Columbia, MD, USA). Polyvinylidene fluoride (PVDF) membranes were purchased from Millipore Corp. (Bedford, MA, USA); iNOS and COX-2 monoclonal antibodies, anti-mouse and anti-rabbit IgG antibodies from BD Transduction Laboratories (Lexington, KY, USA); ECL western blotting detection agent from Amersham Corp. (Arlington Heights, IL, USA). Other chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). All solvents used were obtained from Fisher Scientific Ltd. (Ottawa, ON). The solvents employed were of reagent or HPLC grade.

Preparation of EGCG esters. Ester derivatives of EGCG were prepared as described in Chapter 2. Briefly, EGCG was acylated with DHA through its acyl chloride, and the composition of the crude products containing a mixture of EGCG polyesters was determined by HPLC-MS. EGCG octabutyrate was prepared by reacting EGCG with an excess of butyroyl chloride and its identity was confirmed with HPLC-MS. The EGCG-DHA crude mixture (54.7% tetraester, 3.1% pentaester and 42.2% unreacted EGCG

according to HPLC) and EGCG octabutyrate were evaluated for their protective effect against AOM-induced colon tumorigenesis in mice.

Animals. Male ICR (Institute of Cancer Research) mice at 5 weeks of age were purchased from the BioLASCO Experimental Animal Center (Taipei, Taiwan). After 1 week of acclimation, the animals were randomly distributed into the control and experimental groups (12 mice in each group, Figure 7-2). Groups 1 and 2 were given a basal diet, whereas groups 3 through 6 were given a diet supplemented with different concentrations (0.25 and 0.50%) of EGCG derivatives. Mice in groups 2 through 6 were injected with AOM (5 mg/kg body weight) twice a week for two weeks. All mice were housed in a controlled atmosphere ($25 \pm 1^{\circ}\text{C}$, 50 % relative humidity, and 12-hour light/12-hour dark cycle) with free access to food and water at all times. Food containers were replenished with fresh diet every day. At the termination of the study (after 5 weeks), the animals were sacrificed with CO_2 asphyxiation and dissected, and the weights of their whole body and selected tissues, including the liver, kidney and spleen, were recorded.

Identification of colonic ACF. The formalin-fixed colonic tissues were cut into proximal (1-2 cm from the cecum), middle (3-4 cm) and distal (1-2 cm from anus) segments and stained in a 0.2 % methylene blue solution for 10 min. Aberrant crypts (AC) were identified under a microscope ($\times 40$) as large thick crypts that are more darkly stained than normal crypts. The stained AC in each focus were counted and the location (distance from anus), number and size (number of AC) of ACF were recorded.

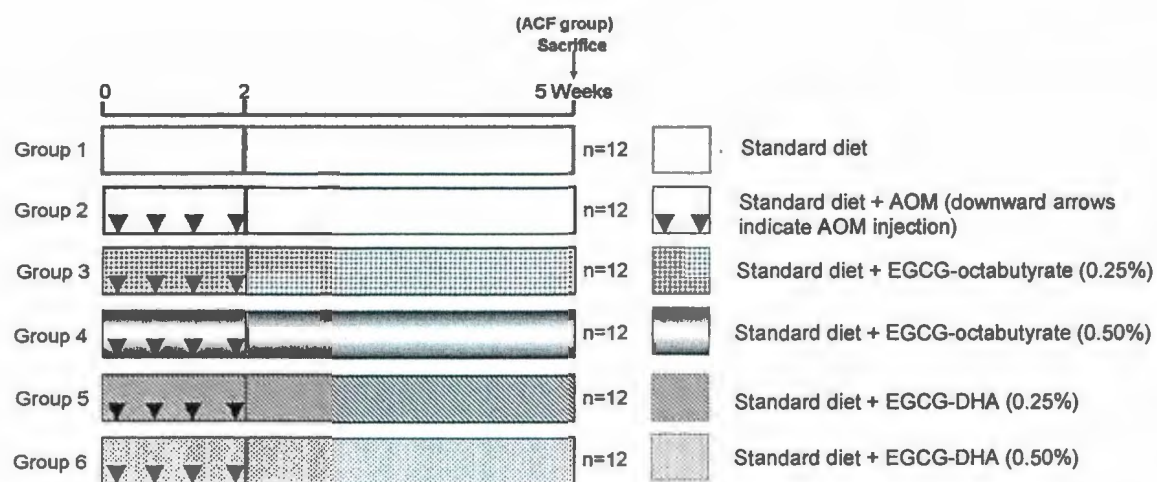


Figure 7-2. Experimental treatment of mice with AOM and EGCG derivatives.

Western blot analysis for iNOS and COX-2 expression. For protein analyses, total scraped colon mucosa was homogenized on ice for 15 s with a Polytron tissue homogenizer and lysed in 0.5 mL ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM NaF, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethane-sulfonyl fluoride, 1% NP-40, and 10 mg/mL leupeptin) on ice for 30 min, followed by centrifugation at 10,000g for 30 min at 4 °C. The samples (50 µg of protein) were mixed with 5× sample buffer containing 0.3 M Tris-HCl (pH 6.8), 25% 2-mercaptoethanol, 12% sodium dodecyl sulfate (SDS), 25 mM EDTA, 20% glycerol, and 0.1% bromophenol blue. The mixtures were boiled at 100 °C for 5 min and were subjected to gel electrophoresis with 12% SDS-polyacrylamide minigels at a constant current of 20 mA. For Western blot analysis, proteins on the gel were electrotransferred onto the 45 micron immobile membrane (PVDF) with transfer buffer containing 25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membranes were blocked with blocking solution (20 mM Tris-HCl pH 7.4, 0.2% Tween 20, 1% bovine serum albumin, and 0.1% sodium azide). The membrane was further incubated with respective specific antibodies, at appropriate dilution (1: 1000) using blocking solution with the primary antibody of iNOS, COX-2 monoclonal antibodies overnight at 4°C. The membranes were subsequently probed with anti-mouse or anti-rabbit IgG antibody conjugated to horseradish peroxidase and visualized using enhanced chemiluminescence. The densities of the bands were quantified with a computer densitometer (AlphaImager TM 2200 System, Alpha Innotech Corporation, San Leandro, CA, USA). All the membranes were stripped and reprobed for β-actin as loading control.

Results and discussion

General observations. Figure 7-3 shows the body and selected tissue weights of the mice at the termination of the experiment, including the vehicle (negative control) and AOM-treated (positive control) mice as well as those administered with different concentrations of EGCG esters. No significant variations were observed for the body and tissue weight among the groups. Injection of the colon-specific carcinogen AOM did not result in abnormal weight gain or loss of the mice in a short term (5 weeks). The tissue weight of liver, kidney and spleen did not seem to be affected by AOM injection or EGCG ester administration. Similar results have been reported in AOM and green tea polyphenols-treated rats, which did not show any significant difference in their body and spleen weights (Xiao *et al.* 2008). However, Shimizu *et al.* (2008) demonstrated that AOM treatment resulted in reduced weight gain in mice with obese and diabetic mellitus. The authors also stated that this may be due to greater susceptibility of the diabetic mice to AOM toxicity.

Inhibition of AOM-induced ACF in mouse colon. After 5 weeks of dietary treatment with EGCG derivatives, all mice were killed and the entire colon harvested for analysis of ACF, the reliable intermediate biomarkers for colon carcinogenesis. Colonic ACF were identified after methylene blue staining and their location, number and size recorded. Figure 7-4 shows the morphology of the colon crypts as affected by treatment with AOM and EGCG derivatives. Compared to normal crypts, AC were more darkly stained with thicker epithelial linings and increased pericryptal space and often had irregular lumens, e.g. oval or slit-shaped lumens. Distribution of ACF in the colon was not even. ACF,

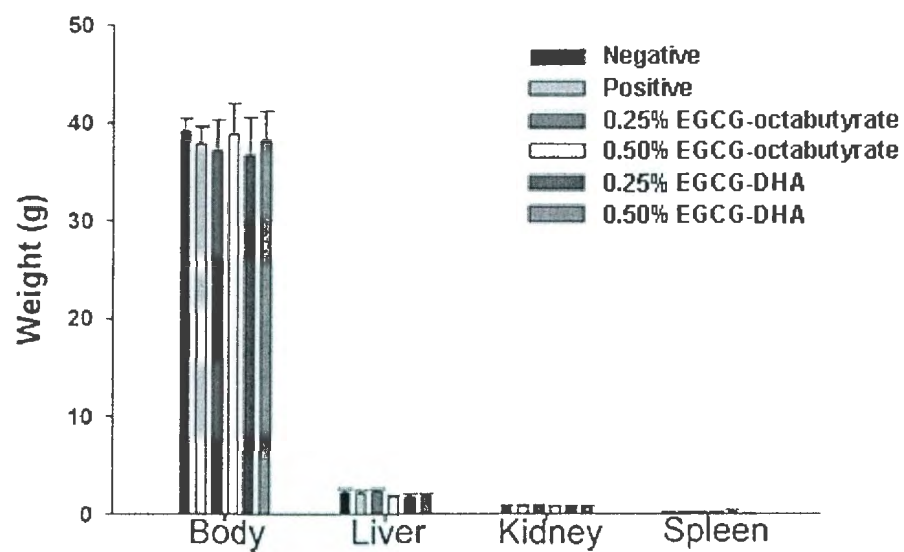


Figure 7-3. Whole body and selected tissue weights of AOM-treated mice.

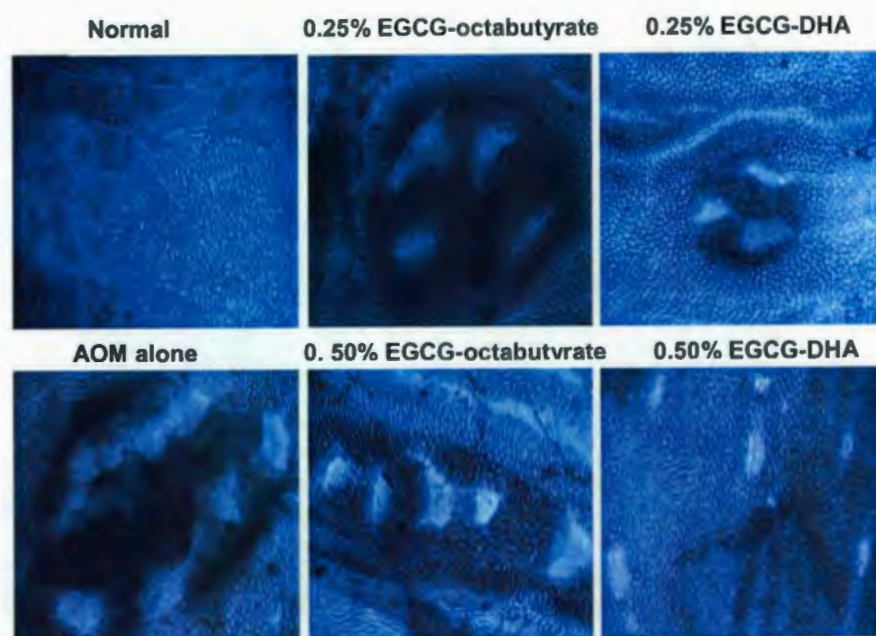


Figure 7-4. Morphological observation of normal and aberrant crypts in the mice colon as affected by EGCG derivatives.

especially those larger than 7 AC/focus, occurred mostly in the distal and middle colon, while less detected in the proximal colon (Table 7-1). The prevalent localization of ACF in the distal and middle colon has been reported by other researchers, who suggested that ACF are not random lesions of the rodent colon treated with genotoxic colon carcinogens (Geng *et al.* 1998; Rodrigues *et al.* 2002). Among the different treatment groups, the mice receiving 0.25% EGCG derivatives had smaller number of large ACF (≥ 7 AC/focus) in their distal colon. ACF containing more than seven AC were specifically investigated as they are believed to be more significant in identifying colonic carcinogenesis (Magnuson *et al.* 1993; Davies *et al.* 1998).

The formation of ACF in the entire colon was stimulated by AOM injection, while dietary supplementation with EGCG derivatives inhibited the AOM-induced ACF formation to different extents (Figure 7-4). The inhibition was characterized by a decrease in total number of AC per mouse and number of AC per focus (Table 7-2). However, the total number of ACF per mouse was not affected significantly ($P>0.05$) by EGCG derivatives administration. Thus, treatment of EGCG esters appears to have a greater effect in reducing the size than the number of ACF in the mice colon. The esters were able to inhibit the formation of large ACF, i.e. foci with more than 7 AC/focus. EGCG-DHA esters in the diet remarkably decreased the number of ACF larger than 7 AC/focus, while EGCG-octabutyrate did not show any significant effect (Figure 7-5). Both the DHA esters and octabutyrate significantly decreased the number of ACF larger than 10 AC/focus. The EGCG derivatives were effective in inhibiting the intermediate precancerous lesions in the colon, thus exerting an anti-cancer activity in the sequential progression of colon tumorigenesis. Tumors, defined as lesions with a crypt multiplicity of ≥ 32 AC/lesion,

Table 7-1. ACF development (number of foci) in the colon of AOM-treated mice.

Treatment	Proximal		Middle		Distal	
	<7 AC	≥7 AC	<7 AC	≥7 AC	<7 AC	≥7 AC
Positive control	9.0 ± 2.6	5.0 ± 1.0	6.3 ± 2.3	11.5 ± 6.7	4.3 ± 3.0	19.3 ± 1.5
0.25% EGCG-octabutyrate	7.0 ± 2.8	3.0 ± 2.8	8.3 ± 6.8	15.0 ± 8.5	3.0 ± 1.4	14.0 ± 1.4*
0.50% EGCG-octabutyrate	11.3 ± 3.5	10.3 ± 3.0	7.3 ± 3.1	14.7 ± 8.5	14.3 ± 4.9	13.7 ± 8.0
0.25% EGCG-DHA	10.0 ± 6.9	6.0 ± 2.0	9.0 ± 5.0	9.0 ± 5.3	17.3 ± 5.9	4.3 ± 1.2*
0.50% EGCG-DHA	8.3 ± 3.8	4.7 ± 2.1	9.3 ± 7.4	9.3 ± 2.1	15.7 ± 8.6	13.0 ± 6.6

* Numbers are significantly different from the positive control (AOM only) at P<0.05.

Table 7-2. ACF formation (number and size) in the colon as affected by AOM and EGCG derivatives treatment.

Treatment	Total number of ACF/mouse	Total number of AC/mouse	Number of AC/focus
Positive control	61.0 ± 7.9	511.7 ± 31.8	8.4 ± 0.6
0.25% EGCG-octabutyrate	52.0 ± 24.0	488.5 ± 16.8	8.0 ± 5.3
0.50% EGCG-octabutyrate	72.3 ± 25.4	461.0 ± 24.2	10.2 ± 5.1
0.25% EGCG-DHA	57.0 ± 23.3	398.5 ± 11.2*	5.2 ± 6.1*
0.50% EGCG-DHA	59.7 ± 8.5	366.0 ± 7.4*	6.6 ± 1.1*

* Numbers are significantly different from the positive control (AOM alone) at P<0.05.

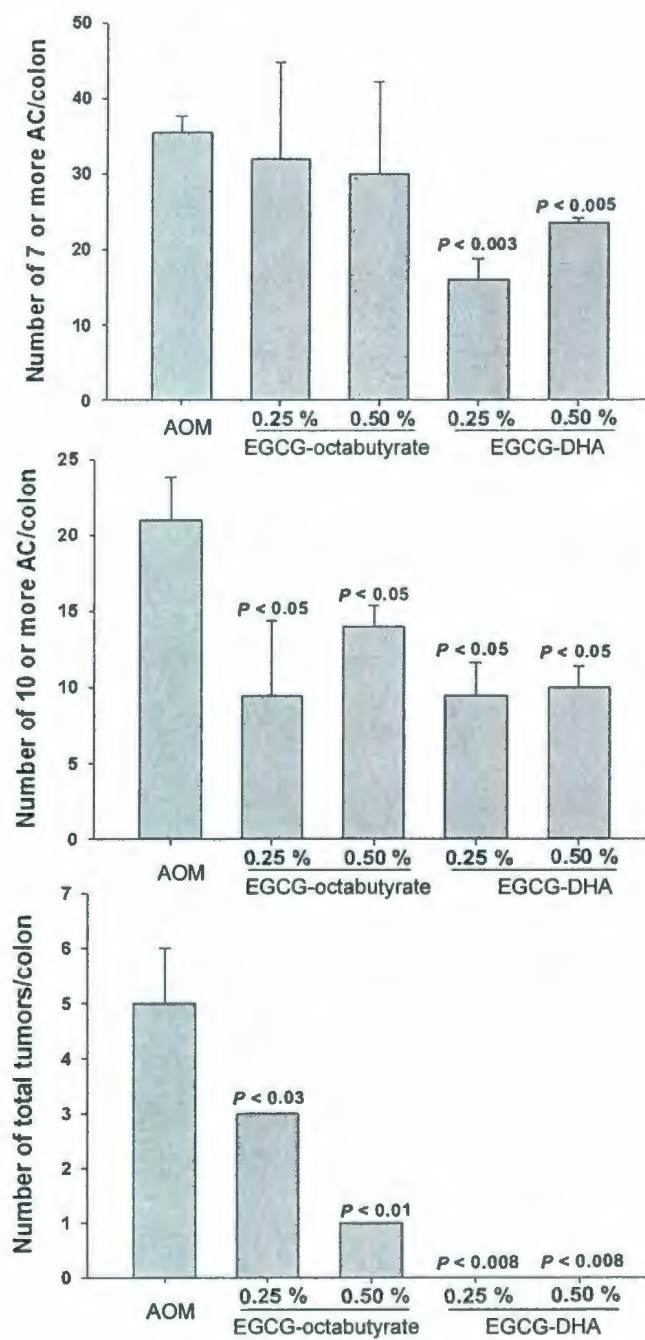


Figure 7-5. Number of ACF and tumor in the mice colon.

were detected in the colon of the mice treated with AOM except for the groups supplemented with EGCG-DHA esters, which exhibited a 100% inhibition against tumor formation (Figure 7-5). The octabutyrate, although less potent than the DHA esters, also significantly ($P < 0.03$) decreased the number of tumors in the mouse colon in a dose-dependent manner.

ACF formation is known as a putative indicator of colon tumorigenesis and efficacy of anticarcinogenic agents. ACF were first reported in a rodent model by Bird (1987), and are now recognized as the earliest identifiable lesions on the pathway to CRC in humans. Formation of ACF has been related to a number of neoplastic alterations of the colon epithelial cells, such as increased proliferation, nuclear and cytoplasmic β -catenin expression, K-RAS and APC mutations as well as microsatellite instability (Augenlicht *et al.* 1996; Takayama *et al.* 2001). More recently, it has been suggested that ACF containing β -catenin accumulated crypts with dysplasia in nature are more relevant and informative preneoplastic lesions for chemical carcinogen-induced colon cancer (Femia *et al.* 2007).

The colon is considered a promising site for chemoprevention with polyphenols, as it is exposed to considerable levels of polyphenols from dietary sources that have low systemic bioavailability (Yang *et al.* 2008). EGCG from green tea, for example, has only limited systemic bioavailability after oral ingestion, and even the absorbed EGCG is excreted mostly into the intestine through the bile (Yang *et al.* 2008). EGCG has been reported to inhibit the formation of colonic ACF and the growth and metastasis of cancer cells, mostly via inhibiting cell proliferation and/or inducing apoptosis in colon cancer (Carter *et al.* 2007). The exact mechanism of EGCG in the inhibition of colon

tumorigenesis is not yet fully unravelled, but is suggested to involve its roles in preventing aberrant nuclear β -catenin accumulation, decreasing cyclin D1 level, maintaining RXR α expression as well as activating Nrf2-UGTs (nuclear factor E2-related factor 2-uridine 5'-diphosphate glucuronosyltransferase, a phase II metabolism) signalling pathway, among others (Xiao *et al.* 2008; Yuan *et al.* 2008). Esterification of EGCG with fatty acids employed in this study might have led to improved lipophilicity and membrane affinity (data shown for EGCG-DHA tetraester in Chapters 2 and 5), and presumably enhanced absorption through the intestine. The absorbed compounds with less free hydroxyl groups might lead to decreased conjugation (glucuronidation, sulfation and methylation, etc.) during metabolism and reduced rate of urinary excretion, in other words, increased deposition in the colon through the bile. In addition, the fatty acid moiety in the derivatives may also contribute to the anti-carcinogenic effect of these compounds, possibly in a synergistic manner with EGCG. Both DHA and butyrate have been reported to induce apoptosis in colorectal tumor cells (Johnson 2002). DHA not only inhibited AOM-induced ACF formation, but also reduced the incidence of spontaneous ACF in rats without carcinogen treatment (Takahashi *et al.* 1994). The mechanisms of the protective effect of the EGCG ester derivatives needs to be further clarified in future investigations.

Inhibition against expression of pro-tumorigenic inflammatory mediators. There has been a causal relationship between tumorigenesis (including cellular transformation, promotion, proliferation and metastasis steps) and chronic inflammation, characterized by increased expression of many pro-inflammatory mediators. Some pro-inflammatory

mediators such as iNOS and COX-2 are known to act as promoters of carcinogenesis, whose expressions in the colonic mucosa are altered during CRC development (Cheng and Lai 2003). iNOS belongs to the family of enzymes that catalyse the production of nitric oxide (NO) *in vivo*. It is induced in response to inflammation and the excessive and prolonged iNOS-mediated NO production has been linked to promotion of tumorigenesis. COX-2 is an inducible enzyme catalyzing the conversion of arachidonic acid to prostaglandins. Recent studies have suggested that increased levels of prostaglandins and COX-2 activity may play important roles in many epithelial cancers including colon carcinoma (Dubois *et al.* 1998). Therefore, suppression of gene expression of iNOS and COX-2 may serve as an important approach to inhibiting CRC development and a useful indicator for assessing chemopreventive efficacy of novel anticarcinogens. Inhibitory effects of EGCG on iNOS and COX-2 expression have been reported (Lin and Lin 1997; Kundu *et al.* 2003). EGCG and other tea polyphenols are believed to down-regulate iNOS and COX-2 expression at the transcriptional level through inhibiting the activation and/or translocation of the transcription factor NF (nuclear factor)- κ B and AP (activator protein)-1 (Surh 2006).

The ester derivatives of EGCG used in this study with the improved lipophilicity were evaluated for their efficacy in inhibiting colon mucosal expression of the tumor-promoting enzymes iNOS and COX-2. As shown in Figure 7-6, AOM treatment (positive control) had a stimulating effect on the protein expression of both iNOS and COX-2 in the mice colon. Oral administration of EGCG derivatives was able to alleviate the carcinogen-induced overexpression of the two enzymes. EGCG-DHA esters significantly suppressed the expression of iNOS and COX-2 in a dose-dependent manner. Mice treated

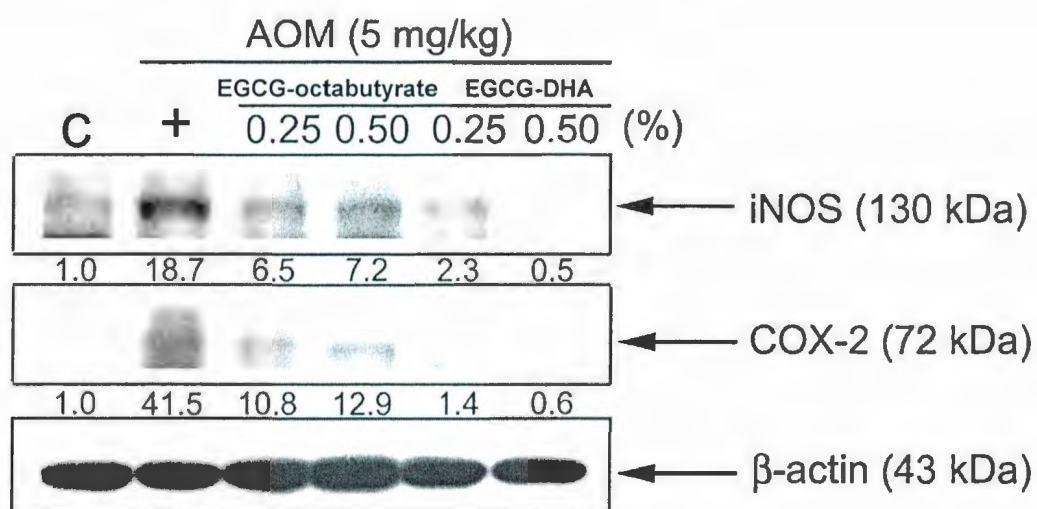


Figure 7-6. Mucosal expression of iNOS and COX-2 in the mice colon (C: negative control; +: positive control, AOM only).

with 0.5% of EGCG-DHA showed even lower levels of iNOS and COX-2 proteins in their colon mucosa compared to those fed a standard diet without AOM injection (negative control). EGCG-octabutyrate, although less effective than the DHA esters, also exhibited a remarkable inhibitory effect against AOM-induced expression of the two tumor-promoting enzymes, and the effect was found to be greater at a lower concentration (0.25%). The excellent suppressing activity of the EGCG derivatives on the pro-tumorigenic enzymes may be attributed to both EGCG and the fatty acids, as compartments in the ester molecules or in their free form after hydrolysis, as well as possible synergism between the two moieties. DHA and other long-chain omega 3 PUFA have been demonstrated to possess anti-inflammatory effects (Bouwens *et al.* 2009). Moreover, a synergistic effect has been reported for DHA and the polyphenol curcumin in suppressing the pro-inflammatory and tumor-promoting enzymes iNOS and COX-2 (Saw *et al.* 2010). The short chain butyric acid, resulting from colonic fermentation of dietary fiber, was found to act as an efficient anti-inflammatory agent and be responsible for the protective role of dietary fiber against colon cancer (Salimath *et al.* 1999).

Conclusions

Esterification of the green tea polyphenol EGCG with butyric acid and DHA provides a novel approach to search for anti-cancer agents. The ester derivatives with improved lipophilicity may have altered metabolism and elimination in the body from that of the parent EGCG, and therefore varied bioactivity *in vivo*. In mice treated with a colon-specific carcinogen (AOM), oral administration of the EGCG derivatives significantly inhibited the colon tumorigenesis. Formation of ACF was significantly inhibited by the

derivatives, observed as the decrease in the total number of AC and size of the foci. Supplementation of the EGCG derivatives, especially the DHA esters, showed a striking effect in reducing the total number of colon tumors in mice. The expression of the tumor-promoting enzymes iNOS and COX-2 was also suppressed to great extents by the derivatives. The findings suggest that EGCG ester derivatives with chemopreventive potential may serve as anticarcinogenic agents in preventing and/or treating colon cancers.

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CHAPTER 8

Summary and recommendations 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 and degrees of unsaturation, including stearic (SA, C18:0), eicosapentaenoic (EPA, C20:5) and docosahexaenoic acids (DHA, C22:6). 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, ^1H and ^{13}C NMR as EGCG-3',5',3'',5''-*O*-tetrastearate (compound 1), EGCG-3',5',3'',5''-*O*-tetraeicosapentaenoate (compound 2), and EGCG-3',5',3'',5''-*O*-tetradocosahexaenoate (compound 3). These ester derivatives showed enhanced lipophilicity and liposome incorporation (indicator of cellular uptake), 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, antiviral activity in inhibiting different virulent enzymes, anti-inflammatory activity in lipopolysaccharides (LPS)-stimulated murine macrophages, and anticancer activity in a carcinogen-induced mouse colon cancer model. In addition to the three isolated tetraesters (compounds 1-3), a crude product of EGCG-DPA (docosapentaenoic acid, C22:5) ester mixture and perbutyrate EGCG (EGCG-octabutyrate) were also prepared and subjected to selected bioactivity tests.

The EGCG derivatives (compounds **1-3**) exhibited excellent antioxidant activity in various *in vitro* chemical assays, including the oxygen radical absorbance capacity (ORAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, reducing power, and metal ion chelation capability. The antioxidant activity of EGCG and its derivatives varied depending on the system environment involved and EGCG derivatives had generally similar or superior antioxidant activity when compared to the original EGCG. In order to evaluate the potential of EGCG derivatives as food preservatives, their antioxidant activity in several food model systems were investigated. EGCG derivatives effectively inhibited lipid oxidation in a bulk oil (stripped corn oil), an emulsion (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 derivatives to a greater extent than by EGCG, suggesting their anti-atherogenic effect and hence cardioprotective potency. The derivatives also inhibited hydroxyl and peroxy radical-induced DNA scission, the free radical-mediated oxidative damage involved in mutagenesis and carcinogenesis. The derivatives were able to render protective effects against UV-induced oxidation in a liposome model. Thus, it is suggested that the lipophilic derivatives of EGCG be considered for use as potential skin care agents.

The antiviral activity of EGCG derivatives, including the tetraesters (compounds **1-3**) and the perbutyrate (EGCG-octabutyrates), was studied in order to explore the potential role of

these compounds in protecting against virus-initiated infections or diseases. The derivatives showed great potential in inhibiting NS3/4A protease, an important enzyme required for the maturation of hepatitis C virus (HCV), and α -glucosidase, the enzyme essential for HIV infectivity, while the effect was not observed for the parent compound EGCG. It was also noted that the HCV protease inhibitory activities of the EGCG-PUFA (polyunsaturated fatty acids) esters (compounds 2 and 3) were about 1700-fold greater than that of the positive control embelin.

The anti-inflammatory activity of EGCG derivatives, including the tetraesters (compounds 1-3) and the EGCG-DPA ester mixture, was evaluated in LPS-stimulated murine macrophages. The derivatives were effective in inhibiting LPS-induced nitric oxide (NO) and prostaglandin E2 (PGE2) production and the effectiveness was attributed to their ability to down-regulate the gene expression of inducible NO synthase (iNOS) and cyclooxygenase (COX)-2 at the transcriptional level.

The anti-cancer activity of selected EGCG derivatives (EGCG-octabutyrate and crude EGCG-DHA mixture) was evaluated using a mouse model of colon carcinogenesis induced by azoxymethane (AOM). Oral administration of the EGCG derivatives significantly inhibited the AOM-induced colon tumorigenesis, observed as decreased number of aberrant crypts (AC) and total number of tumors as well as suppressed iNOS and COX-2 expression in the mice colon. The DHA esters of EGCG, in particular, were able to totally eliminate tumorigenesis in the limited number of animals used. These findings suggest that EGCG ester derivatives possess chemopreventive potential against colon cancer.

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 may be suggested. However, further investigations need to be carried out on 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 such as stearidonic and α -linolenic acids as well as selected omega-6 fatty acids can also be considered for their potential health benefits.