

**PREPARATION OF RESVERATROL AND QUERCETIN
DERIVATIVES AND THEIR EFFECTS ON ANTIOXIDANT
AND BIOLOGICAL ACTIVITIES**

by

© **Won Young Oh**

B. Sc. (Seoul National University of Science and Technology),

M. Sc. (Seoul National University of Science and Technology)

A thesis submitted to the School of Graduate Studies
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Department of Biochemistry
Memorial University of Newfoundland

July 2018

St. John's

Newfoundland and Labrador

Canada

ABSTRACT

Resveratrol and quercetin, two representative polyphenolics found in red wine, onions, and many other sources, have been well documented for having numerous health benefits such as antioxidant, anti-inflammatory, cardioprotective, and anticancer effects. Despite their health-related beneficial properties, their widespread use in lipophilic media such as lipid-based foods and cosmetic formulations is restricted due to their hydrophilicity. Moreover, phenolic compounds, including flavonoids, generally undergo extensive metabolism after consumption, thus they have low bioavailabilities. Even if these compounds can survive from metabolism, they can hardly penetrate lipophilic cell membrane bilayers. One possible solution to this problem is to modify their structures which can be carried out simply as a first approach via esterification. Esterification with fatty acids can enhance lipophilicity that would improve their use in different lipophilic media. Moreover, in the case of esterification with polyunsaturated fatty acids (PUFA), potential health benefits may be enhanced due to the presence of both phenolics and certain healthful PUFA such as those of the omega-3 family. For this reason, fatty acids of different chain length and degree of unsaturation (C3:0-C22:6) were employed as acyl donors. The resveratrol and quercetin derivatives so prepared were evaluated for their antioxidant activities in different systems, such as the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay; the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical cation scavenging assay; a bulk oil system; β -carotene bleaching assay; a hydroxyl radical-induced DNA scission assay, and the copper-induced low-density lipoprotein (LDL) oxidation assay. Selected resveratrol derivatives were further

evaluated for their antioxidant activity and cell viabilities (A431, MCF7, HT-29, and AGS cells). All test compounds showed antioxidant activity in a system-dependent manner. The resveratrol and quercetin derivatives showed a decreased radical scavenging activity compared to their parent molecules. However, some derivatives exhibited a similar and/or a better antioxidant activity than resveratrol and quercetin themselves in different systems, such as in the bulk oil assay and copper-induced low-density lipoprotein (LDL) oxidation assays. In this work, both resveratrol and quercetin derivatives showed potential use in lipophilic systems, therefore they might serve as possible functional food ingredients as well as health promoting supplements.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Fereidoon Shahidi for accepting me as one of his students and for his endless professional guidance, constructive personal encouragements and financial support (through NSERC). I would also like to thank my supervisory committee members Dr. Philip Davis, Dr. Sherri Christian, and Dr. Robert Bertolo for their valuable suggestions. My gratitude is extended to the School of Graduate Studies at Memorial University of Newfoundland for financial support. I would like to acknowledge Ms. Linda Winsor and Dr. Celine Schneider for assistance for HPLC-MS and NMR analysis, respectively. My sincere thanks are extended to Dr. Chao-Mei Ma and Dr. Min-Hsiung Pan for their generous contribution for cell line studies. I am grateful to Dr. Priyatharini Ambigaipalan for continuous help throughout my study and my lab mates for humorous, enjoyable, and heart-warming time in the lab. I appreciate my family, mom, dad, Min Young Oh, and JuDong Yeo for dedication, encouragement and support.

TABLE OF CONTENTS

Abstract	1
Acknowledgement	III
Table of contents	IV
List of tables	XII
List of figures	XIII
List of abbreviations	XVII
Chapter 1 Introduction	1
Chapter 2 Literature review	5
2.1. Phenolics	5
2.1.1 Chemistry and classification	5
2.1.2 Bioactivities and bioavailabilities	10
2.2. Lipid oxidation and phenolics as antioxidants	15
2.2.1 Lipid oxidation	15
2.2.2 Phenolics compounds as antioxidants	20
2.3. Resveratrol	22
2.3.1 Resveratrol in foodstuffs	22

2.3.2 Resveratrol as an antioxidant	26
2.3.3 Other properties of resveratrol	30
2.3.4 Antioxidant and biological activity of modified resveratrol	33
2.4. Quercetin	35
2.4.1 Quercetin in foodstuffs	35
2.4.2 Quercetin as an antioxidant	37
2.4.3 Other properties of quercetin	43
2.4.4 Antioxidant and biological activity of modified quercetin	45
Chapter 3 Materials and methods	47
3.1 Materials	47
3.2 Methods	48
3.2.1 Lipophilisation of resveratrol and effects on antioxidant activities	48
3.2.1.1 Preparation of EPA and DHA	48
3.2.1.2 Preparation of resveratrol derivatives	49
3.2.1.3 Purification of resveratrol derivatives	50
3.2.1.4 Identification of resveratrol derivatives	50
3.2.1.5 Determination of lipophilicity	51
3.2.1.6 DPPH radical scavenging assay	51

3.2.1.7 ABTS radical cation scavenging assay	52
3.2.2 Antioxidant activity of resveratrol ester derivatives in food and biological model systems	52
3.2.2.1 Preparation of resveratrol derivatives	52
3.2.2.2 Antioxidant activity of resveratrol derivatives in oil-in-water emulsion (β -carotene bleaching assay)	53
3.2.2.3 Antioxidant activity of resveratrol derivatives in bulk oil	54
3.2.2.4 Antioxidant activity in a meat model system (thiobarbituric acid reactive substances, TBARS)	54
3.2.2.5 Hydrogen peroxide (H_2O_2) scavenging activity	55
3.2.2.6 Inhibition of copper-induced low-density lipoprotein (LDL) oxidation	56
3.2.2.7 Inhibition of hydroxyl radical-induced DNA scission	56
3.2.3 Effect of lipophilisation of resveratrol on reactive nitrogen/oxygen species generation in murine macrophages and human cancer cell lines	57
3.2.3.1 Preparation of resveratrol esters	57
3.2.3.2 Cell culture	58
3.2.3.3 Nitrite assay	58
3.2.3.4 Determination of cytotoxicity and ROS generation on human hepatoma	

carcinoma (HepG2) cells	59
3.2.3.5 Cell viability of A431, MCF7, HT-29, and AGS	59
3.2.4 Preparation esterified quercetin derivatives and effects on antioxidant activity	60
3.2.4.1 Preparation of EPA and DHA	60
3.2.4.2 Preparation of quercetin derivatives	61
3.2.4.3 Identification of quercetin derivatives	61
3.2.4.4 Separation of quercetin and its derivatives	62
3.2.4.5 Determination of lipophilicity	62
3.2.4.6 ABTS radical cation scavenging assay	62
3.2.5 Antioxidant activity of quercetin derivatives in food and biological model systems	63
3.2.5.1 Preparation of quercetin derivatives	63
3.2.5.2 DPPH radical scavenging assay	64
3.2.5.3 Hydroxyl radical scavenging assay	64
3.2.5.4 Reducing power	64

3.2.5.5 Antioxidant activity of quercetin derivatives in oil-in-water emulsion (β -carotene bleaching assay)	65
3.2.5.6 Antioxidant activity of quercetin derivatives in bulk oil	66
3.2.5.7 Inhibition of copper-induced low-density lipoprotein (LDL) oxidation	66
3.2.5.8 Inhibition of hydroxyl radical-induced DNA scission	66
3.3 Statistical analysis	68
Results and discussions	69
Chapter 4. Lipophilisation of resveratrol and effects on antioxidant activity	70
4.1 Introduction	70
4.2 Preparation of resveratrol derivatives	71
4.3 Identification of resveratrol derivatives	72
4.4 Lipophilicity of resveratrol derivatives	80
4.5 DPPH radical scavenging assay	83
4.6 ABTS radical cation scavenging assay	85
4.7 Summary	89
Chapter 5. Antioxidant activity of resveratrol ester derivatives in food and biological model systems	90

5.1 Introduction	90
5.2 Antioxidant activity of resveratrol derivatives in oil-in-water emulsion (β -carotene bleaching assay)	93
5.3 Antioxidant activity in bulk oil	97
5.4 Antioxidant activity in a meat model system (TBARS)	103
5.5 Hydrogen peroxide (H_2O_2) scavenging activity	106
5.6 Inhibition of copper-induced low-density lipoprotein oxidation	108
5.7 Inhibition of hydroxyl radical-induced DNA scission	111
5.8 Summary	115
Chapter 6. Effect of lipophilisation of resveratrol on reactive nitrogen/oxygen species generation in murine macrophages and human cancer cell lines	116
6.1 Introduction	116
6.2 Nitric oxide (NO) production	118
6.3 Determination of cytotoxicity and ROS generation on human hepatoma carcinoma (HepG2) cells (cellular antioxidant assay)	122
6.4 Effect of resveratrol and its esters on the viability of A431, MCF7, HT-29, and AGS cells	126
6.5 Summary	129

Chapter 7. Preparation esterified quercetin derivatives and effects on antioxidant activity	131
7.1 Introduction	131
7.2 Preparation of quercetin derivatives	132
7.3 Identification of quercetin derivatives	134
7.4 ¹ H NMR assignment of quercetin derivatives	137
7.5 Lipophilicity of quercetin derivatives	140
7.6 ABTS radical cation scavenging assay	143
7.7 Summary	144
Chapter 8. Antioxidant activity of quercetin derivatives in food and biological model systems	146
8.1 Introduction	146
8.2 DPPH radical scavenging assay	149
8.3 Hydroxyl radical scavenging assay	150
8.4 Reducing power	154
8.5 Antioxidant activity of quercetin derivatives in oil-in-water emulsion (β-carotene bleaching assay)	156
8.6 Antioxidant activity of quercetin derivatives in bulk oil	159

8.7 Inhibition of copper-induced low-density lipoprotein (LDL) oxidation	164
8.8 Inhibition of hydroxyl radical-induced DNA scission	167
8.9 Summary	169
Chapter 9. Summary and recommendations	170
Publications included in this thesis	173
Permission to use copyrighted material	174
References	176

LIST OF TABLES

Table 4.1. ^1H chemical shift (δ) of resveratrol and resveratryl monodocosahexaenoate.

Table 4.2. Lipophilicity of resveratrol and its monoesters.

Table 5.1. Conjugated dienes formation of stripped corn oil over a 6-day period in the presence of resveratrol and its derivatives.

Table 5.2. *p*-Anisidine values of stripped corn oil over a 6-day period in the presence of resveratrol and its derivatives.

Table 5.3. TBARS values of pork model system over a 7-day period in the presence of resveratrol and its derivatives (MDA eq./kg).

Table 5.4. Inhibition of hydroxyl radical-induced DNA scission by resveratrol and its derivatives.

Table 6.1. Effect of resveratrol and its DHA ester on HepG2 cell viability by resveratrol and its esters treatment

Table 7.1. Composition of quercetin (Q) and Q-C6:0 derivatives in crude products.

Table 7.2. Composition of each Q-C6:0 derivatives.

Table 7.3. ^1H chemical shift (δ) of quercetin and Q-C6:0 derivatives.

Table 7.4. Lipophilicity of quercetin and its derivatives.

LIST OF FIGURES

Figure 2.1. Formation of phenylpropanoid from phenylalanine and tyrosine by ammonia lyase.

Figure 2.2. Phenolic acids (examples of hydroxybenzoic acid derivatives (top) and hydroxycinnamic acid derivatives (bottom)).

Figure 2.3. Structures of major classes of flavonoids.

Figure 2.4. Mechanism of lipid oxidation involving initiation, propagation, and termination steps.

Figure 2.5. Formation of hydroperoxide in the autoxidation of linoleic acid (adopted from Choe and Min 2006).

Figure 2.6. Possible mechanisms of formation of secondary oxidative products.

Figure 2.7. A possible mechanism of metal chelation of phenolic compounds (flavonol).

Figure 2.8. Structures of representative stilbenoids.

Figure 2.9. Resonance structures of resveratrol radical.

Figure 2.10. Quercetin and its glycosides found in plant-based foods.

Figure 2.11. Important parts of quercetin for radical scavenging activity.

Figure 2.12. Possible sites of quercetin for cupric ion chelation.

Figure 4.1. HPLC chromatogram of RC3:0 esters with mobile phase methanol/ 5% acetonitrile in water 90:10 (I) and 60:40 (II), respectively.

Figure 4.2. Mass spectra of RC3:0 esters (a, resveratrol; b, RC3:0 monoesters; c, RC3:0 diesters).

Figure 4.3. Structures of resveratrol and its esters.

Figure 4.4. DPPH radical scavenging capacity in micromoles of trolox equivalents (TE) per micromole of resveratrol (R) or its derivatives.

Figure 4.5. ABTS radical cation scavenging assay in micromoles of trolox equivalents (TE) per micromole of resveratrol (R) or its derivatives.

Figure 5.1. Structures of resveratrol derivatives.

Figure 5.2. Antioxidant activities of resveratrol and its derivatives against β -carotene bleaching over a 105 min period (a) and inhibition against β -carotene bleaching after 105 min incubation (b) by resveratrol and its derivatives.

Figure 5.3. Hydrogen peroxide scavenging activities in micromoles of ascorbic acid equivalents (AAE) per micromoles of resveratrol and its derivatives.

Figure 5.4. Inhibition (%) of resveratrol and its derivatives against LDL oxidation induced by copper.

Figure 5.5. Inhibition (%) of hydroxyl radical induced DNA scission by (a) R, RC3:0, RC4:0, RC6:0, RC8:0, RC10:0, and RC12:0 and (b) RC14:0, RC16:0, RC18:0, RC18:1, REPA, and RDHA (B: DNA only, C: DNA + FeSO₄ + H₂O₂).

Figure 6.1. Structures of RC3:0 and RDHA monoesters.

Figure 6.2. Effect of resveratrol and its monoesters on NO production in LPS-induced RAW 264.7 macrophages.

Figure 6.3. Effect of resveratrol and RDHA on intracellular reactive oxygen species (ROS) generation.

Figure 6.4. Effect of a) resveratrol, b) RC3:0, and c) RDHA on cell viability of various human cancer cells.

Figure 7.1. HPLC chromatogram of Q-C6:0 with methanol/ 5% acetonitrile in water 90:10 as the mobile phase.

Figure 7.2. Mass spectra of quercetin (Q) and Q-C6:0 derivatives.

Figure 7.3. Structure of quercetin.

Figure 7.4. ABTS radical cation scavenging activity of quercetin (Q) and its derivatives expressed as trolox equivalents.

Figure 8.1. Structure of quercetin derivatives.

Figure 8.2. DPPH radical scavenging activity of quercetin (Q) and its derivatives expressed as trolox equivalents.

Figure 8.3. Hydroxyl radical scavenging activity of quercetin (Q) and its derivatives expressed as trolox equivalents.

Figure 8.4. DMPO-OH adduct of blank, catechin (standard, 400 μ M), and quercetin.

Figure 8.5. Reducing power of quercetin and its derivatives as ascorbic acid equivalents.

Figure 8.6. Antioxidant activities of quercetin (Q) and its derivatives against β -carotene bleaching over a 105 minutes period (a) and inhibition against β -carotene bleaching after 105 min incubation (b) by Q and its derivatives.

Figure 8.7. Conjugated dienes formation of stripped corn oil over an 8-day period in the presence of quercetin and its derivatives.

Figure 8.8. *p*-Anisidine values of stripped corn oil over an 8-day period in the presence of quercetin and its derivatives.

Figure 8.9. Inhibition (%) of quercetin and its derivatives against LDL oxidation induced by copper.

Figure 8.10. (a) DNA scission induced by hydroxyl radical and (b) their inhibition (%).

LIST OF ABBREVIATIONS

AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
ABTS	2,2'-Azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
APCI	Atmospheric pressure chemical ionization
ANOVA	Analysis of variance
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
CD	Conjugated diene
COSY	Correlation spectroscopy
COX	Cyclooxygenase
CVD	Cardiovascular disease
DCFH	Dichlorofluorescin
DCFH-DA	2',7'-Dichlorofluorescin diacetate
DHA	Docosahexaenoic acid
DHASCO	Docosahexaenoic acid single cell oil
DMEM	Dulbecco's modified eagle medium
DMPO	5,5-Dimethyl-1-pyrroline-N-oxide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl

EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
EPA	Eicosapentaenoic acid
EPR	Electron paramagnetic resonance
FA	Fatty acid
FBS	Fetal bovine serum
FRAP	Ferric reducing antioxidant power
HAT	Hydrogen atom transfer
HepG2	Human hepatoma carcinoma cells
HPLC-MS	High-performance liquid chromatography-mass spectrometry
iNOS	Inducible nitric oxide synthase
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO	Nitric oxide
PBS	Phosphate buffer solution
PG	Propyl gallate
PUFAs	Polyunsaturated fatty acids
RC3:0	Resveratrol esterified with propionic acid
RC4:0	Resveratrol esterified with butyric acid
RC6:0	Resveratrol esterified with caproic acid
RC8:0	Resveratrol esterified with caprylic acid

RC10:0	Resveratrol esterified with capric acid
RC12:0	Resveratrol esterified with lauric acid
RC14:0	Resveratrol esterified with myristic acid
RC16:0	Resveratrol esterified with palmitic acid
RC18:0	Resveratrol esterified with stearic acid
RC18:1	Resveratrol esterified with oleic acid
RDHA	Resveratrol esterified with docosahexaenoic acid
REPA	Resveratrol esterified with eicosapentaenoic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TAE	Tris-acetic acid-EDTA
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TE	Trolox equivalents
TLC	Thin layer chromatography
Q-C3:0	Quercetin esterified with propionic acid
Q-C4:0	Quercetin esterified with butyric acid
Q-C6:0	Quercetin esterified with caproic acid
Q-C8:0	Quercetin esterified with caprylic acid
Q-C10:0	Quercetin esterified with capric acid

Q-C12:0	Quercetin esterified with lauric acid
Q-C14:0	Quercetin esterified with myristic acid
Q-C16:0	Quercetin esterified with palmitic acid
Q-C18:0	Quercetin esterified with stearic acid
Q-C18:1	Quercetin esterified with oleic acid
Q-EPA	Quercetin esterified with eicosapentaenoic acid
Q-DHA	Quercetin esterified with docosahexaenoic acid
UV-DAD	UV diode array detector

CHAPTER 1

Introduction

Lipid oxidation is a major concern for the food industry, scientists and consumers alike. Attempts have been made to find the best way to minimize the risk of oxidation. In the early days, hydrogenation of unsaturated fatty acids was carried out to convert them to less unsaturated or more saturated fatty acids. The resultant products were used as shortening and margarine which were more stable than unsaturated fatty acids, thus leading to shelf life extension (Johnson, 2002). However, trans fat, a byproduct of hydrogenation, later became a significant concern due to the adverse health effects associated with the incidence of cardiovascular disease (Mozaffarian et al., 2006). Moreover, unsaturated fatty acids are known to be important in nutrition as well as biological systems (Watkins and German, 2002). For example, linoleic acid and α -linolenic acid are essential fatty acids because human body cannot synthesise them. Addition of antioxidants has proven to be the most effective means to control oxidation (Shahidi and Ambigaipalan, 2015; Laguerre et al., 2015). The most widely used synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tertiary-butylhydroquinone (TBHQ) have been questioned due to their possible carcinogenic effects (Ito et al., 1986). Hence food scientists have found alternatives, mainly phenolic compounds from natural sources as functional food ingredients. So far, numerous phenolic compounds, mostly originating from plant sources, have been identified. Phenolic compounds have been known to have a great potential to serve as antioxidants with significant health benefits such as cardioprotective, anti-

inflammatory, and anticancer effects. However, most effective phenolic compounds are hydrophilic compounds, therefore they may not be suitable for use in lipid-based systems (Laguerre et al., 2015). Besides, only a small fraction of phenolic compounds consumed may be absorbed and the rest is excreted in feces or in urine, as such or as their metabolites, hence we cannot take full advantage of their potential health benefits (Clifford, 2004). Thus, it is of interest to explore possible ways to overcome such problems, including structure modification.

This study aimed to expand the potential application of phenolic compounds in food and lipophilic biological systems. To that end, two phenolic compounds, resveratrol and quercetin, were selected and esterified with 12 different fatty acids varying in chain length as well as in the degree of unsaturation. The central hypothesis of this study was that the lipophilised resveratrol and quercetin would have increased antioxidant activity in food and biological systems compared to the parent compounds. Because the lipophilisation would increase affinity between the phenolic compounds and lipophilic systems tested such as a bulk oil system and LDL oxidation inhibition assay, therefore lipophilisation would lead to enhanced antioxidant activity of resveratrol and quercetin.

The general objective of this study was to investigate the effect of lipophilisation of resveratrol and quercetin on antioxidant activity and their application in a wide range of food and biological systems.

The specific objectives of this study are summarised below.

- 1) To prepare the lipophilised resveratrol and quercetin derivatives via esterification;

- 2) To identify the structure of lipophilised resveratrol and quercetin, derivatives;
- 3) To evaluate the effect of lipophilised resveratrol and quercetin on antioxidant properties;
- 4) To determine the effect of fatty acid chain length and degree of unsaturation of lipophilised resveratrol and quercetin on antioxidant properties, and
- 5) To explore the additive, synergistic, or antagonistic effect arising from 12 different fatty acid esters of resveratrol and quercetin.

This thesis consists of nine chapters.

Chapters 1 and 2 describe the introduction and the related literature review. Chapter 3 presents material and methods, whereas chapters 4-8 provide results and discussions. The titles of chapters 4-8 are summarised below.

Chapter 4. Lipophilisation of resveratrol and effects on antioxidant activity

Chapter 5. Antioxidant activity of resveratrol ester derivatives in food and biological model systems

Chapter 6. Effect of lipophilisation of resveratrol on reactive nitrogen/oxygen species generation in murine macrophages and human cancer cell lines

Chapter 7. Preparation of esterified quercetin derivatives and effects on antioxidant activity

Chapter 8. Antioxidant activity of quercetin derivatives in food and biological model systems

Finally, Chapter 9 presents summary and recommendations.

CHAPTER 2

Literature review

2.1 Phenolics

2.1.1 Chemistry and classification

Phenolic compounds consist of one (phenol) or more (polyphenol) hydroxyl groups attached to one or more aromatic hydrocarbon rings. They are widely distributed in plants and are present in several thousand different forms (Shahidi, 2002a). They can also incorporate other groups such as methoxy groups, fatty acids, and carbohydrates. They are important in the plant for growth regulation, wound healing, and for protection from sunlight, pathogens, and external predators (Shahidi and Yeo, 2016). They are also responsible for the colour and taste of foods.

Phenolic compounds in plants are derived from phenylalanine and to a lesser extent from tyrosine. They are synthesized by phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL) to phenylpropanoids (C6-C3), which are trans-cinnamic acid and *p*-coumaric acid, respectively. (Figure 2.1; Shahidi, 2002b). These can be modified and form different types of phenolic compounds. Each phenolic compound has a different structure, however they can be classified, based on their basic carbon skeletons, into simple phenols (C6), phenolic acids (C6-C1, C6-C3), stilbenes (C6-C2-C6), and flavonoids (C6-C3-C6) (Pereira et al., 2009). Phenolic acids and flavonoids are the predominant phenolics found in food sources (Robbins, 2003).

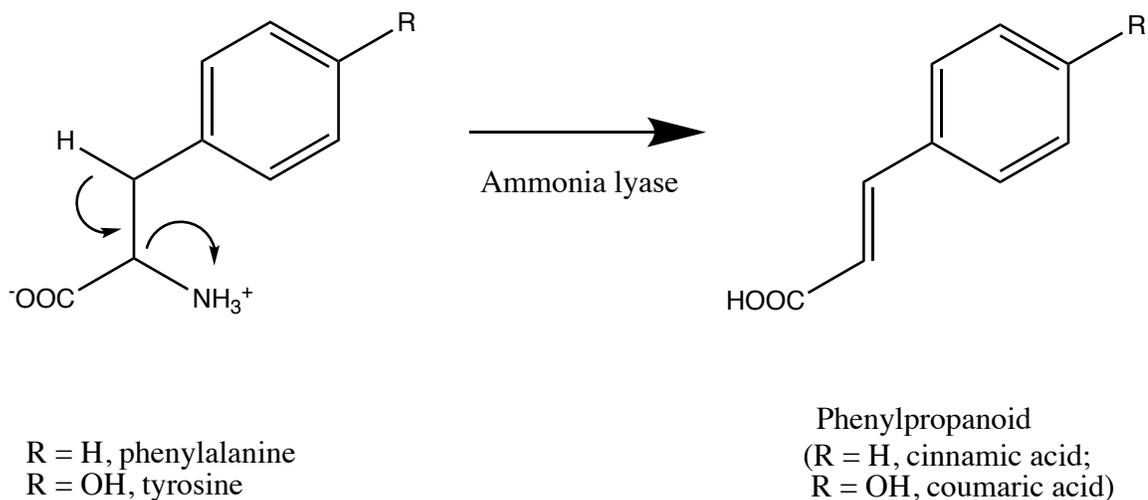
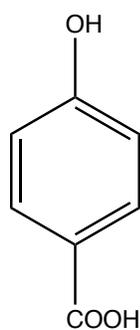


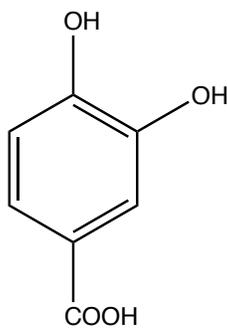
Figure 2.1. Formation of phenylpropanoids from phenylalanine and tyrosine by ammonia lyase.

Phenolic acids can be divided into hydroxybenzoic acid derivatives such as *p*-hydroxybenzoic acid, protocatechuic acid, and gallic acid, as well as hydroxycinnamic acid derivatives such as coumaric acid, ferulic acid, and sinapic acid; these are varied by the location and number of hydroxyl groups attached to the aromatic ring (Figure 2.2).

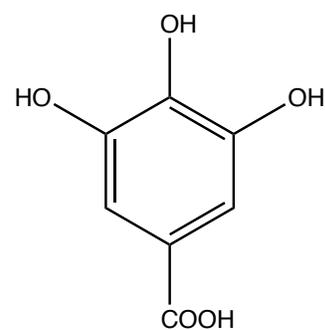
The production of stilbenes also starts from a phenylpropanoid pathway and subsequently undergoes condensation reaction of a *p*-coumaroyl-CoA and three malonyl-CoA (Vermerris and Nicholson, 2008). Phenolic compounds such as resveratrol and piceid belong to the class of stilbene derivatives (Shahidi and Ambigaipalan, 2015).



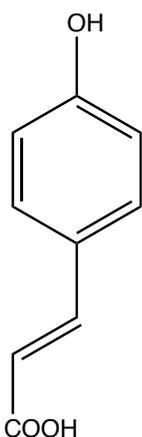
p-Hydrobenzoic acid



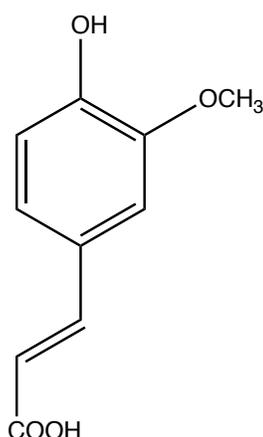
Protocatechuic acid



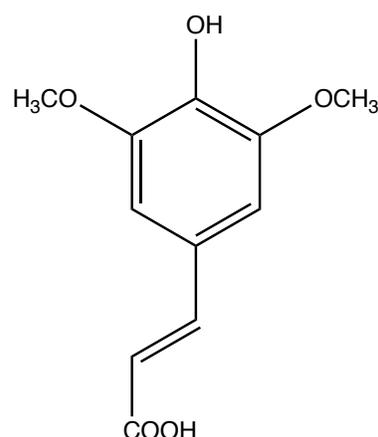
Gallic acid



p-Coumaric acid



Ferulic acid

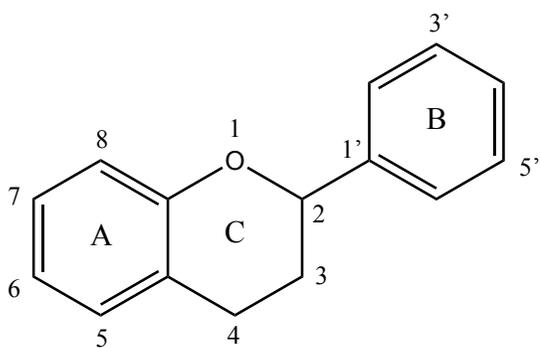


Sinapic acid

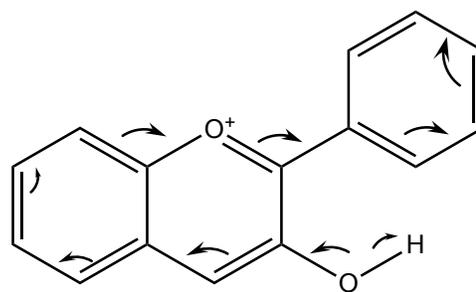
Figure 2.2. Phenolic acids (examples of hydroxybenzoic acid derivatives [top] and hydroxycinnamic acid derivatives [bottom]).

Flavonoids consist of two benzene rings (A and B) linked by a 3-carbon heterocyclic pyrene ring (C) (Figure 2.3; Kumar and Pandey, 2013). More than 6500 flavonoid compounds have been identified (Harborne and Williams, 2000), which can be further categorized into flavones, flavan-3-ols, flavonols, anthocyanidins, and isoflavones

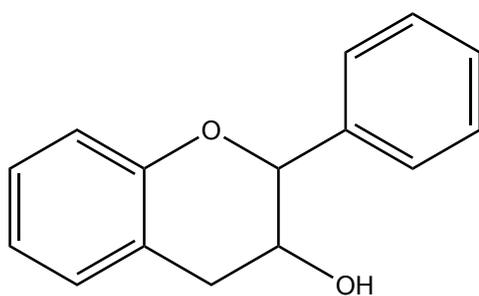
(Shahidi and Ambigaipalan, 2015). All phenolic acids and flavonoids may also be found as their glycosides. Most flavonoids are 3-*O*-glycosides (monoglycosides) and 3,7-di-*O*-glycosides (diglycosides) (Shahidi and Naczki, 2004). Flavan-3-ols consist of a C6-C3-C6 basic carbon skeleton with a hydroxyl group attached to the C-3 position of the C ring. Flavan-3-ols include catechin, epicatechin, epicatechin gallate, and epigallocatechin gallate, and these are found in green tea. Flavones have a 2,3-double bond and a 4-keto group in the C ring. Flavones are mainly found in fruit skins (Kumar and Pandey, 2013). Isoflavones, which can be found in soybeans, have a similar structure to flavones, however the B ring of isoflavones is linked via carbon 3 of the C ring. Flavonols such as quercetin and kaempferol have an additional hydroxyl group linked to carbon 3 of the C ring of flavone. They are widely distributed in vegetables and fruits such as berries, onions, lettuce, and broccoli (Shahidi and Ambigaipalan, 2015). Meanwhile, anthocyanins, which can be found in flowers and fruits, play an important role in plant colour that can change with pH, being red in acidic solutions, blue to violet in neutral solutions, and green to yellow in alkaline solutions (Michaelis et al., 1936). Anthocyanins are abundant in blueberries, blackberries, cherries, pomegranates and eggplants (Shahidi and Naczki, 2004).



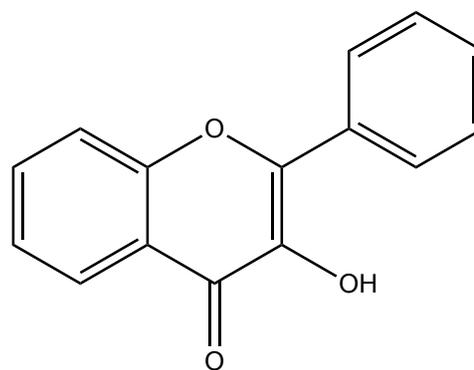
Basic flavonoid structure



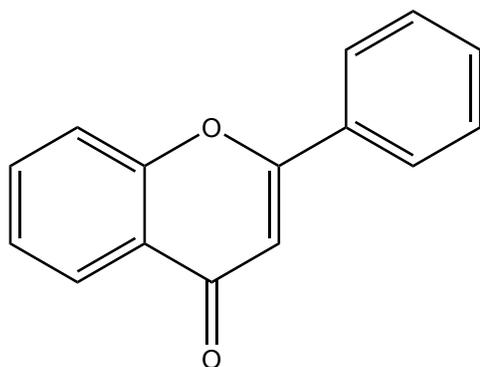
Anthocyanidin



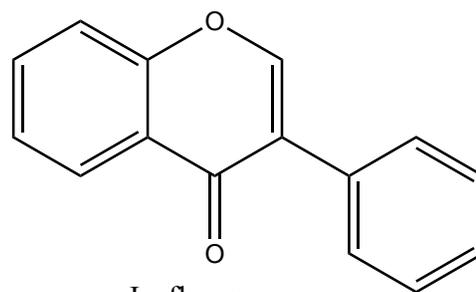
Flavan-3-ol



Flavonol



Flavone



Isoflavone

Figure 2.3. Structures of major classes of flavonoids.

2.1.2 Bioactivities and bioavailabilities

It is of interest to the general public to learn about healthy intake of food in order to keep themselves in good health. There are notable volumes of epidemiological evidence that consumption of fruits and vegetables can decrease the risk of chronic diseases such as cancer, hypertension, and heart disease, therefore fruits and vegetables are generally considered as healthy foods (Van Duyn and Pivonka, 2000). Healthy food consumption is closely related to phenolic intake because food from plant sources such as fruits, vegetables, herbs, seeds, and cereals are important sources of phenolics. Phenolic compounds have been shown to have numerous anti-pathological activities such as antioxidant, anti-inflammatory, and antiulcer effects (Bravo, 1998), among others. Phenolic compounds display strong antioxidant activity, which prevents oxidation of biomolecules such as low density lipoprotein (LDL) cholesterol, DNA, and proteins, thus suppressing the development of cardiovascular disease and cancer. For example, catechins, abundant in green tea, can delay propagating lipid peroxy radicals, DNA oxidation, and LDL cholesterol oxidation *in vitro* (Salah et al., 1995; Zhong and Shahidi, 2012). Phenolics from olive oils could protect against reactive oxygen species (ROS) but also suppress xanthine oxidase activity, which can produce superoxide *in vitro* (Owen et al., 2000). Phenolic compounds also exert anti-inflammatory activity. Epigallocatechin gallate (EGCG) and its derivatives showed anti-inflammatory activity via inhibiting pro-inflammatory mediators such as nitric oxide and prostaglandin (PGE₂) *in vitro* (Zhong et al., 2012). This is exemplified for resveratrol, which also showed anti-inflammatory activity by reducing the production of nitric oxide and TNF- α (Bi et al., 2005). Phenolic

compounds also exert protective effects against coronary heart disease (CHD). Tresserra-Rimbau et al. (2014) studied the association between polyphenol intake and the incidence of CVD in the Prevención con Dieta Mediterránea (PREDIMED) study. They divided 7447 participants into three groups: Mediterranean diet supplemented with extra virgin olive oil (EVOO), Mediterranean diet supplemented with nuts or the control diet (low-fat diet). They reported that greater polyphenol intake leads to decreased risk of CVD. Hertog et al. (1993) studied the effects of flavonoid intake (25.9 mg/day) by 805 males (65-84 years old) for mortality from CHD and suggested that regular consumption of flavonoids might have protective effects on CHD. In addition, phenolic compounds are known to have anti-diabetic activity. Phenolics from Pakhanbhed, a traditional remedy for diabetes used by Nepalese, showed anti-diabetic potential by inhibiting rat intestinal α -glucosidase as well as porcine pancreatic α -amylase (Bhandari et al., 2008). Besides, phenolics from muscadine (grapevine) exhibited anti-diabetic activities by inhibiting α -glucosidase and pancreatic lipase (You et al., 2012). Phenolic compounds also show antimicrobial activity. For example, Rauha et al. (2000) reported that quercetin and naringenin effectively inhibited the growth of *Staphylococcus aureus*, which can cause food poisoning. According to Puupponen-Pimiä et al. (2001), phenolics from cloudberry, raspberry, and strawberry were able to inhibit the growth of *Salmonella enterica*.

Although phenolics generally act as antioxidants, they can also behave as pro-oxidants which might be useful for their anticancer activity via apoptotic DNA fragmentation (Galati and O'Brien, 2004; Dai and Mumper, 2010). The action mechanism of anticancer drugs and γ -radiation treatment is highly related to apoptotic DNA fragmentation activity

via ROS (Hadi et al., 2000). This mechanism of action is similar to when phenolic compounds are binding and cleaving DNA then generating ROS in the presence of transition metal ions (Hadi et al., 2000). Juan et al. (2008) studied the anticancer activity of resveratrol in the colorectal carcinoma cell line (HP-29). They observed both anti-proliferation activity and activation of caspase-3 in a dose-dependent manner as well as membrane permeability and DNA fragmentation. ROS level was also elevated. Phenolics such as secoiridoids from extra virgin olive oil showed inhibitory effects on proliferation in human breast cancer cells *in vitro* (García-Villalba et al., 2010). Phenolic extract from strawberries inhibited the growth of different human cancer cell lines such as oral (CAL-27, KB), colon (HT-29, HCT-116), and prostate (LNCaP, DU145) (Zhang et al. 2008). Traditional Chinese medicinal herbs used for cancer treatment contain phenolic compounds from 0.22 to 50.3 g of gallic acid equivalents/100 g dry weight (Cai et al., 2004).

Despite the potential health benefits of phenolic compounds, it is extremely difficult to utilize them due to their low bioavailability and fast metabolism. Moreover, only limited information is available on the bioavailability of phenolics due to the variety, varying chemical structures and occurrence in specific plants (Bravo, 1998). Daily intake of phenolic compounds is varied from less than 100 mg to more than 2 g, depending on the food intake pattern (Clifford, 2004). However, generally only up to 10 % of the phenolic compounds consumed can be absorbed and the remaining 90 % goes to the colon where they are fermented by the gut microflora (Clifford, 2004). The absorption and metabolism of phenolics depend on their chemical structure, including degree of glycosylation, conjugation with other phenolic compounds, degree of polymerisation, and solubility

characteristics (Bravo, 1998; Kumar and Pandey, 2013). For example, flavonoids can either be absorbed in the intestine or go to the colon, whereas most of the flavonoid glycosides need to be hydrolysed to the aglycone form and then absorbed via passive diffusion (Kumar and Pandey, 2013; Manna et al., 2000). Absorption of phenolic compounds can also be affected by their affinity to the cell membrane. Murota et al. (2002) studied the cellular uptake of isoflavonoids and flavonoids by human intestinal Caco-2 cells. The highest affinity to the cell membrane was by flavonoid aglycones, whereas genistin and daidzin showed the least affinity to cell membranes. They suggested that this might be due to their lipophilicity, but earlier it was believed that mammals lacked β -glycosidases and hence absorption of phenolic glycosides in the intestine did not occur (Bravo, 1998). However, some phenolic glycosides may be absorbed in the intestine via glucose transporter (SGLT1) (Hollman et al., 1999) and/or hydrolysed by lactase-phlorizin hydrolase (β -glycosidase) on the brush border of the mammalian small intestine (Day et al., 2000). The phenolic glycosides, which did not absorb in the small intestine, are transported to the colon where they are hydrolysed by bacteria. The absorption in the colon can occur, however it is very limited compared to that in the small intestine (Kumar and Pandey, 2013). After absorption, phenolic compounds are metabolised via glucuronidation, sulphation, or methylation and this mostly occurs in the liver. Other organs such as the kidney and the intestine can also perform the conjugation of phenolic compounds (Bravo, 1998). The metabolites can either be secreted in the bile/urine or re-enter the intestine via the enterohepatic cycle (van Duynhoven et al., 2011; Chiou et al., 2014). Subsequently, phenolic compounds enter systemic circulation, however only small amounts (5-10%) can enter the plasma in the unchanged form

(Clifford, 2004). Manach et al. (2005) reviewed 97 bioavailability studies of phenolic compounds and reported that the absorption of gallic acid and isoflavones was the highest, whereas proanthocyanidins, epigallocatechin gallate (EGCG), and anthocyanin showed the least absorption. Hollman et al. (1999) reported that the absorption of quercetin glucosides, apart from rutin, was higher than quercetin itself. Moreover, the bioavailability of quercetin differs depending on the type of glycoside from the food source e.g. onions are a better source than apples or tea. The half-lives of quercetin metabolites ranged from 11 to 28 h, which means that continuous intake of quercetin/quercetin glycosides may lead to accumulation in the plasma (Manach et al., 2005). Meanwhile, resveratrol can be absorbed well, however it has low bioavailability. Walle et al. (2004) studied the absorption, bioavailability, and metabolism of resveratrol after an oral dose of 25 mg in six human volunteers. Although 491 ± 90 ng/mL of resveratrol and its metabolites were detected in the plasma after 1 h of 25 mg oral dose, unchanged resveratrol was detected only in trace amounts of < 5 ng/mL; the half-lives ranged from 7 to 14 h.

There are several strategies to improve the bioavailability of nutraceuticals such as encapsulation (microemulsion-based systems) and chemical modification (lipophilisation) (Davidov-Pardo and McClements, 2014; Ting et al., 2014; Torres et al., 2010). Encapsulation is a technique to trap one or more components within some form of a matrix (Davidov-Pardo and McClements, 2014). This technique can protect the ingredients from the environmental conditions which may cause chemical degradation during storage. However, this technique may need a high amount of encapsulant material or surfactant which can restrict its use due to sensory acceptability, economic or legal

issues (Davidov-Pardo and McClements, 2014). Another possible way is a chemical modification. This technique can improve the bioavailability via modifying chemical structures to more favourable forms to be absorbed. Since the nutraceuticals may lose their physiological or therapeutic benefits due to structural changes, the structural change has to be bio-reversible (Ting et al., 2014).

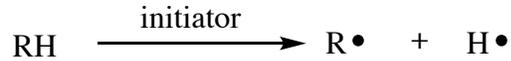
2.2 Lipid oxidation and phenolics as antioxidants

2.2.1 Lipid oxidation

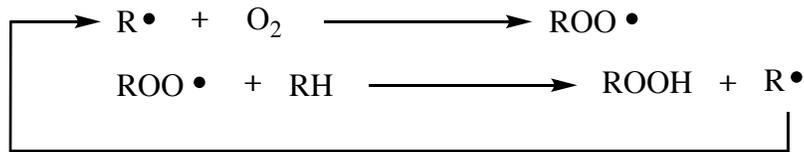
Lipids are one of the major food components, serving as a condensed source of energy, providing both favourable and objectionable flavour notes, and affecting the texture of products. They can also serve as structural components of cell membranes in biological systems (Shahidi and Zhong, 2010). However, lipids containing unsaturated fatty acids are prone to oxidation due to several factors such as the presence of heat, light, metal ions, enzymes, and photosensitizers; these can lead to food quality deterioration (Shahidi and Zhong, 2010). Lipid oxidation not only causes generation of off-flavours and decreases the nutritional value of foods, but it can also lead to the formation of toxic compounds (Shahidi and Zhong, 2015). Lipid oxidation in the human body can cause loss of structure and function of healthy cells, which eventually leads to ageing and non-communicable ailments such as cardiovascular disease and cancer (Percival, 1998). There are different types of oxidation mechanisms based on the specific factors that initiate lipid oxidation, namely autoxidation, photooxidation, thermal oxidation, and enzymatic oxidation. Among these, autoxidation is the most common oxidation process that follows a free

radical chain mechanism typically involving three steps of initiation, propagation, and termination (Figure 2.4).

Initiation:



Propagation:



Termination

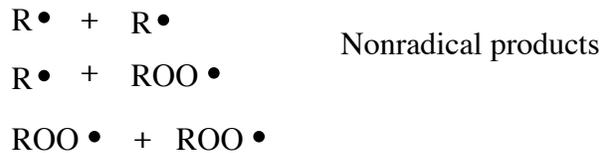


Figure 2.4. Mechanism of lipid oxidation involving initiation, propagation, and termination steps.

In order to initiate autoxidation, the presence of initiators such as heat, light, or metal ions is necessary. Autoxidation is generally initiated by the abstraction of a hydrogen atom attached to an allylic or bis-allylic carbon (carbon next to the double bond) due to their low required dissociation energies. The dissociation energy of bis-allylic, allylic and alkyl

hydrogens are 65, 77, and 100 kcal/mol, respectively (Shahidi and Zhong, 2010). This process is called the initiation step of autoxidation. After losing its hydrogen atom, the alkyl radical formed is stabilised by delocalization (double bond shifting), along with possible formation of *trans* as well as conjugated double bonds of polyunsaturated fatty acids (Shahidi and Zhong, 2010). In the propagation step, the alkyl radical reacts with triplet oxygen ($^3\text{O}_2$) to form a peroxy radical (Frankel, 1984). Subsequently, the lipid peroxy radical abstracts a hydrogen atom from another lipid/fatty acid molecule and forms a hydroperoxide, known as the primary product of oxidation. As shown in Figure 2.5, linoleic acid, for example, can produce a mixture of 9- and 13-hydroperoxides. Hydroperoxides are decomposed to alkoxy radical ($\text{RO}\cdot$), peroxy radical ($\text{ROO}\cdot$), and hydroxyl radical ($\cdot\text{OH}$) and the radicals so produced can further participate in the oxidation process (Shahidi and Zhong, 2010). The O-O bond cleavage energy of hydroperoxides is 44 kcal/mol, which is easily achieved and yields secondary oxidation products such as aldehydes, ketones, alcohols, and hydrocarbons (Choe and Min, 2006). Most of the secondary oxidative products are responsible for off-flavours, and some of them such as alkanals and vinyl ketones have very low threshold values. Possible formation mechanisms of secondary oxidative products are shown in Figure 2.6. Finally, during the termination step the radicals produced are stabilised by binding to one other to form stable non-radical species.

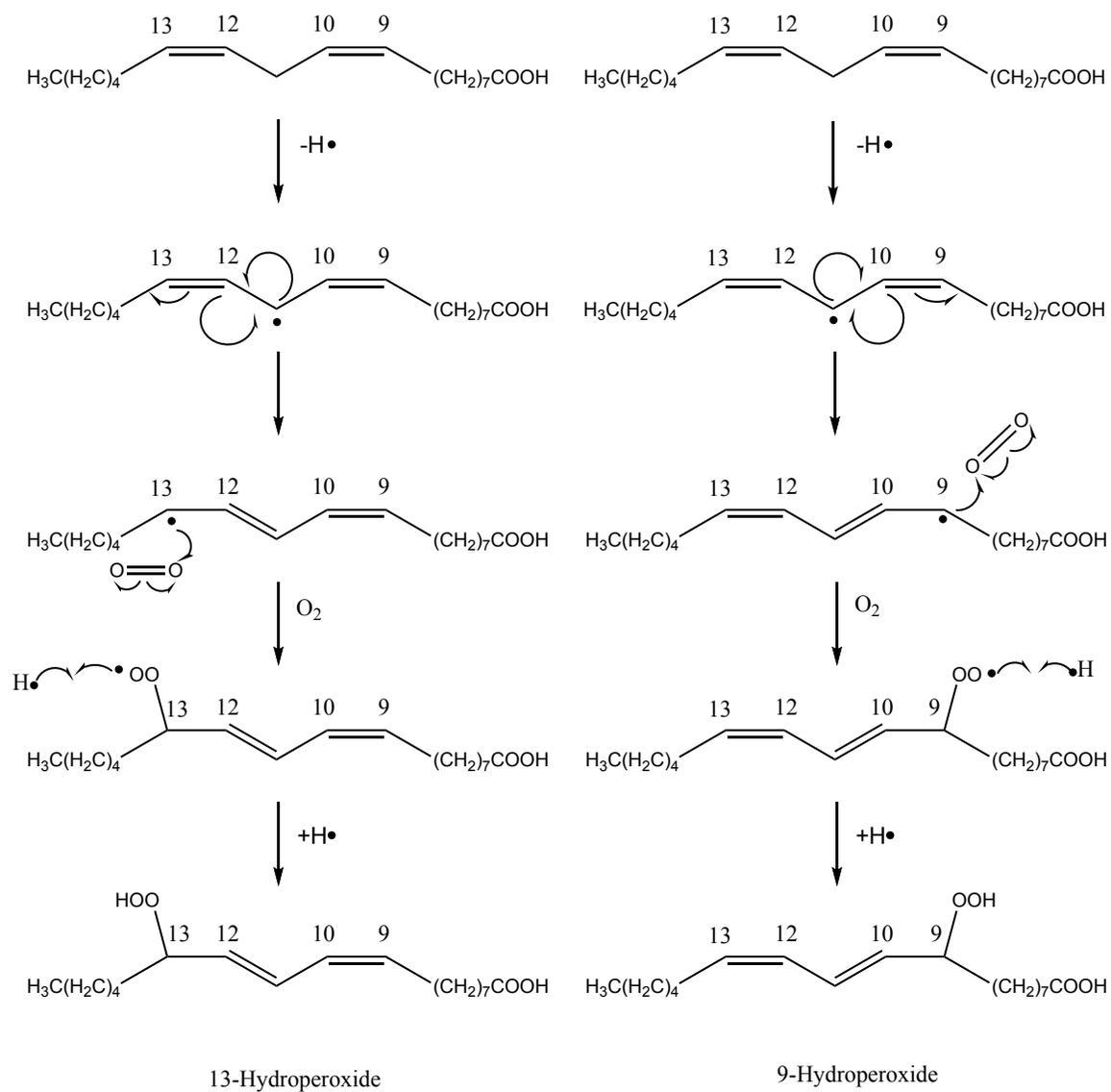


Figure 2.5. Formation of hydroperoxides in the autoxidation of linoleic acid (adopted from Choe and Min, 2006).

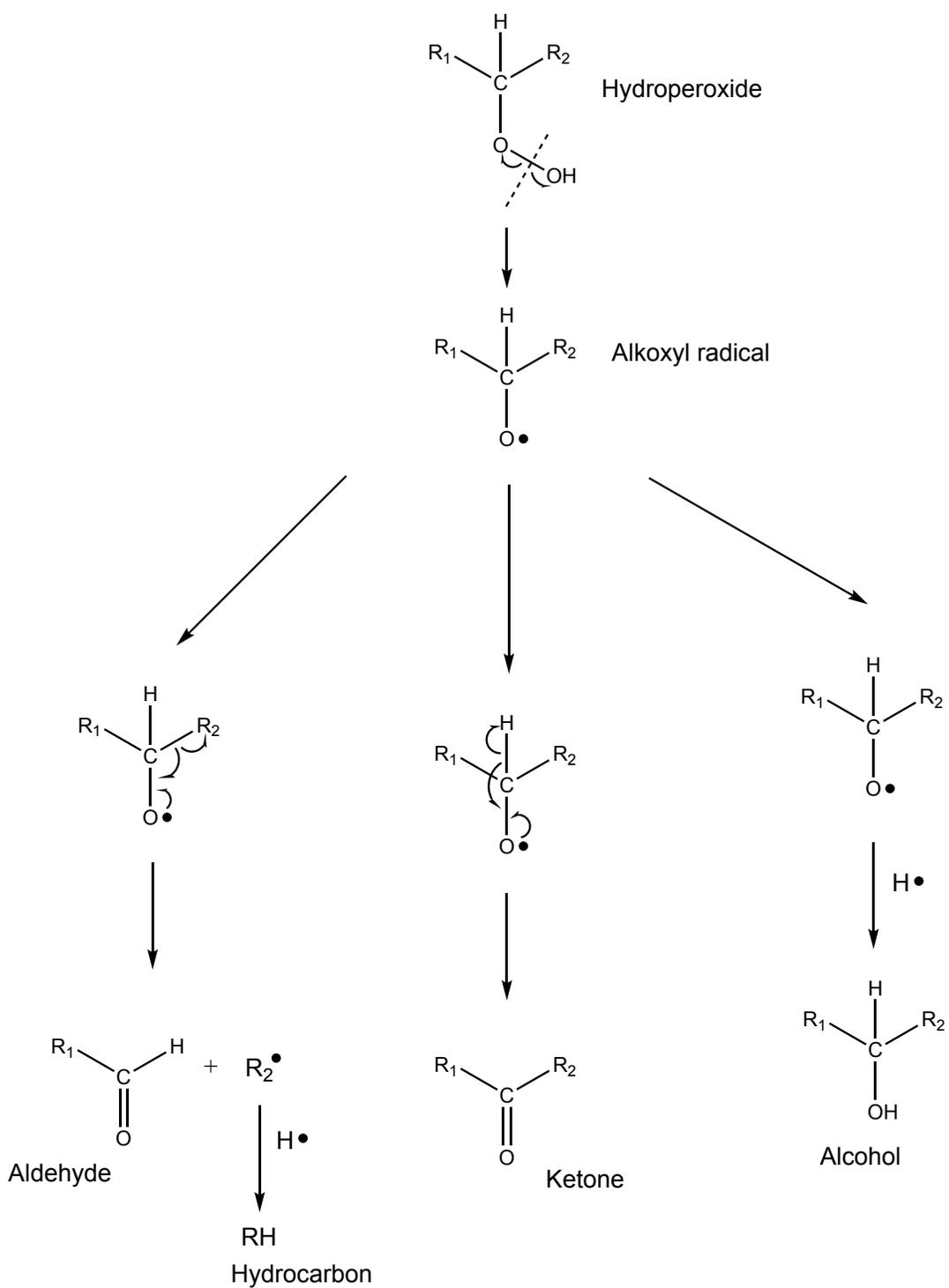


Figure 2.6. Possible mechanisms of formation of secondary oxidation products.

2.2.2 Phenolic compounds as antioxidants

Controlling lipid oxidation is of interest to the food industry for extending the shelf life and maintaining food quality of products. In addition, it is very important because the oxidation can affect biological systems. Free radicals, believed to be a major contributor to cause oxidative damage, can also attack different cells in the body, thus potentially causing a myriad of diseases such as cancer and cardiovascular ailments (Percival, 1998). Antioxidants, known as a most effective means to control oxidation, can delay the onset of oxidation or slow down its rate once it starts (Reische et al., 2002). Antioxidants can be classified into primary antioxidants as free radical chain breakers and secondary antioxidants such as metal ion chelators and singlet oxygen quenchers (Reische et al., 2002).

Phenolic compounds, which are primary antioxidants, can interfere and interrupt the free radical chain reaction by donating a hydrogen atom or transferring an electron to the free radicals (Equations 1,2, and 3) (Frankel, 1980; Shahidi and Ambigaipalan, 2015).



In order to serve as an antioxidant, the resultant antioxidant radical ($A\bullet$) must not be involved in further oxidation (Shahidi and Wanasundara, 1992). The phenoxyl radical produced is not readily available for further oxidation process, because it is stabilised by resonance. The stability of a phenoxyl radical may be enhanced by substitution with a bulky group in the ortho and para positions due to the steric hindrance where the radical is present (Gordon, 1990).

In terms of antioxidant activity, phenol itself is inactive, however substitution can increase its activity. Another hydroxyl group in the ortho or para position of phenol increases its antioxidant potential due to an intramolecular hydrogen bond (ortho position) and possible formation of quinone (para position) (Gordon, 1990). For example, the antioxidant activity of 1,2-dihydroxybenzene is higher than 2-methoxyphenol, because 2-methoxyphenol is unable to form an intramolecular hydrogen bond (Gordon, 1990). Furthermore, substitution in the ortho or/and para position with an alkyl group or other electron donating groups such as amines (-NH₂, -NHR, and -NR₂) and ethers (-OR) improves the antioxidant activity via the inductive effect. Due to the increased electron density, the substituted phenolic antioxidant has a better reactivity toward lipid radicals (Rajalakshmi and Narasimhan, 1996). For example, the strong antioxidant activity of butylated hydroxyanisole (BHA) is closely related to the methoxylation (Gordon, 1990). Substitution in the para position with ethyl, propyl, or butyl groups increases the antioxidant activity compared to a methyl group. However, substitution with longer chain or electron withdrawing groups such as trihalides (-CF₃, -CCl₃) and nitro groups (-NO₂) may decrease the antioxidant activity (Rajalakshmi and Narasimhan, 1996).

Some phenolics may also act as chelators of metal ions such as those of ferrous and copper ions. Metal ions, which have two or more valence states can reduce the induction period and accelerate the rate of oxidation by producing radicals via interaction with intact lipid molecules or interaction with lipid hydroperoxides (Gordon, 1990; Reische et al., 2002). Metals can be involved in the oxidation process even at 10 ppb levels, hence all food should be considered in order to control metal ion-assisted oxidation. The most commonly used metal chelators are citric acid, phosphoric acid, and

ethylenediaminetetraacetic acid (EDTA). EDTA, which is the most powerful metal chelator, forms thermodynamically stable complexes with all metal ions, therefore it can effectively prevent oxidation during storage (Gordon, 1990). Some phenolic compounds can also chelate metal ions (Figure 2.7).

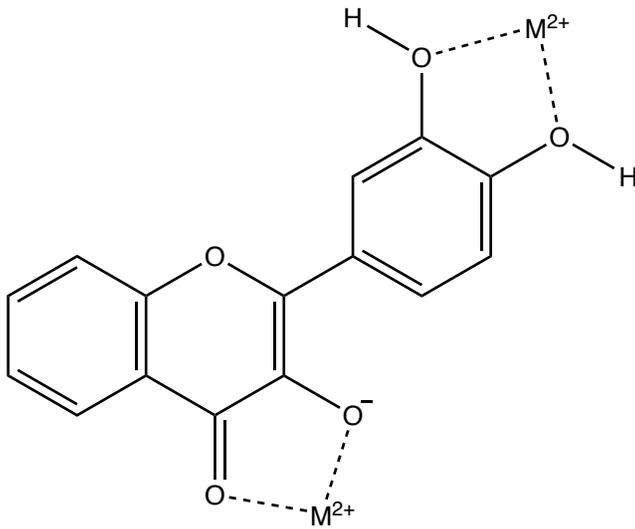


Figure 2.7. A possible mechanism of metal chelation of phenolic compounds (flavonol).

2.3 Resveratrol

2.3.1 Resveratrol in foodstuffs

Resveratrol, a stilbenoid (Figure 2.8), is present in grapes, especially grape skins, and is also expected to be present in grape products and processing by-products (Siemann and Creasy, 1992). According to Siemann and Creasy (1992), resveratrol itself had already

been reported to have lipid-lowering effects and wine also reduced serum lipid levels in humans, thus it was hypothesised that resveratrol in wine would have beneficial effects.

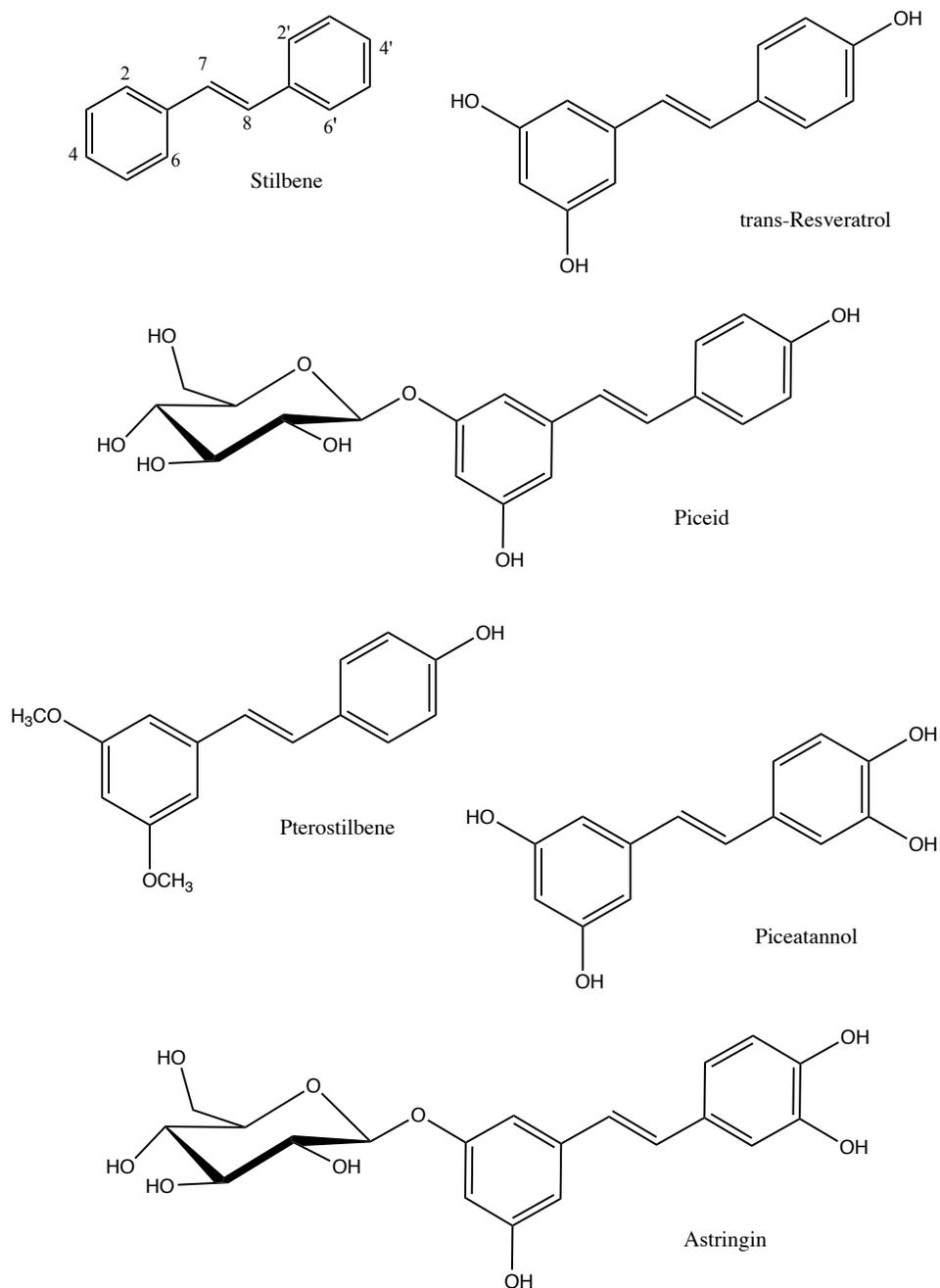


Figure 2.8. Structures of representative stilbenoids.

Resveratrol has been known since 1940, however it was not of interest to scientists until 1992, when it was used to explain the protective effects of red wines on CHD (Baur and Sinclair, 2006).

Resveratrol is produced by the plant as a phytoalexin, which is believed to exert an important disease resistance mechanism in higher plants (Langcake and Pryce, 1977). It is synthesized in response to stress, injury, infection, or UV-irradiation (Soleas et al., 1997). However, resveratrol is not well distributed in the plant kingdom and its presence has been limited to a few edible plants. The primary source of resveratrol from edible plant parts is grape, wine, and to a lesser extent peanuts (Soleas et al., 1997). Siemann and Creasy (1992) studied the concentration of resveratrol in wine and found that resveratrol was present in red wines from less than 0.003 to 2.861 $\mu\text{mol/L}$ and white wine from less than 0.001 to 0.438 $\mu\text{mol/L}$. In addition, they found that resveratrol concentration differed by geographical origins (e.g. New York and California). The concentration of resveratrol in wine may vary depending on the type of grape, fungal infection, growing methods, climatic factors, and wine processing. The level of resveratrol in wine is also affected by species. For example, Pinot Noir grapes have greater ability to synthesize resveratrol regardless of other factors such as soil and climate (Soleas et al., 1997). This might be due to its thin skin compared to other types of grapes. The thin skin makes it susceptible to damage from infection and UV-irradiation. Resveratrol plays an important role against fungal infection, therefore its concentration is higher in colder and moister growing climates (Siemann and Creasy, 1992; Kopp, 1998). As already mentioned, resveratrol is present in higher concentrations in red wine than white wine; a possible reason is the

different processing between red and white wines. After pressing of grapes for wine making, the skins remain in the red wine processing, whereas the skins are removed in the white wine processing (Siemann and Creasy, 1992).

Resveratrol is also found in peanuts, but only in infected tissues. The level of resveratrol can be varied by stimuli, which can be a fungal infection or mechanical damage (Sanders et al., 2000). In addition, immature peanut kernels contain a high concentration of stilbenes, the capability of producing stilbene could be reduced with maturity (Sobolev and Cole, 1999). Sobolev and Cole (1999) reported that resveratrol concentrations in commercially available peanut products such as roasted peanuts, peanut butter, boiled peanuts were 0.055 ± 0.023 , 0.324 ± 0.129 , and 5.138 ± 2.849 $\mu\text{g/g}$, respectively. Sanders et al. (2000) studied the concentration of resveratrol in five different peanut cultivars and peanuts were stored for up to 3 years in cold temperatures. They found that the concentrations of resveratrol were 0.02-1.79 $\mu\text{g/g}$ of peanuts. They suggested that the discrepancy in the concentration of resveratrol with other studies might be due to the different levels of fungal invasion.

Medicinal herbs serve as another source of resveratrol. Burns et al. (2002) compared the concentration of resveratrol in wine, peanut products, and Itadori plants (*Polygonum cuspidatum*), which has been used as a traditional herbal remedy for heart disease and strokes. They reported that grapes and peanuts had a low concentration of stilbenes, however Itadori tea and red wine had a high concentration of resveratrol, suggesting that Itadori tea could serve as a good source of resveratrol for non-alcohol drinkers.

2.3.2 Resveratrol as an antioxidant

As a phenolic compound, resveratrol has been of interest to many researchers to explore its antioxidant capacity. It has three hydroxyl groups, which makes it a good free radical scavenger. Gülçin (2010) reported that resveratrol exhibited the highest inhibition of lipid peroxidation, followed by BHA, BHT, trolox, and α -tocopherol (in decreasing order) at 30 $\mu\text{g/mL}$. In reducing power assay, BHA showed the highest activity using ferric ion (Fe^{3+}) and cupric ion (Cu^{2+}). Resveratrol exhibited the second highest Fe^{3+} reducing power followed by trolox, BHT and α -tocopherol; BHT had the second highest Cu^{2+} reducing ability followed by resveratrol, α -tocopherol, and trolox. However, according to Pulido et al. (2000), resveratrol showed the lowest reducing power compared to tannic acid, selected flavonoids (quercetin, rutin, and catechin), selected phenolic acids (gallic acid, caffeic acid, and ferulic acid), and reference compounds (ascorbic acid, trolox, and BHA). They suggested that the lowest reducing power of resveratrol might be due to the lack of *o*-dihydroxy phenolic structures compared to flavonoids. Gülçin (2010) reported that resveratrol showed the highest superoxide anion radical scavenging activity, followed by BHT, BHA, trolox, and α -tocopherol. On the other hand, resveratrol had the lowest hydrogen peroxide scavenging ability (in the order of BHT > BHA > α -tocopherol > trolox > resveratrol). Gülçin (2010) also compared the scavenging activities of DPPH radical and ABTS radical cation. In the DPPH radical scavenging activity, BHA showed the highest radical scavenging activity, followed by BHT, resveratrol, trolox, and α -tocopherol. However, resveratrol showed a slightly higher ABTS radical cation scavenging activity than BHA and BHT and this decreased in the order of trolox > α -tocopherol. Gülçin (2010) explained that resveratrol had monophenol and diphenol

moieties which were linked by an ethylene bridge and donating hydrogen from the monophenol moiety could easily occur. Caruso et al. (2004) also reported that the para position was more stable after than the meta position upon donating a hydrogen atom, therefore donating hydrogen from 4'-OH was preferred. Hussein (2011) studied the hydroxyl radical scavenging activity of resveratrol and reference compounds such as trolox, ascorbic acid, BHT, and BHA; resveratrol showed the highest radical scavenging activity (resveratrol > trolox > ascorbic acid > BHT > BHA) which was concentration-dependent in the range of 20-80 µg/mL. Hussein (2011) discussed that the antioxidant activity of phenolic compounds was related to their structural features such as substitutions, numbers of hydroxyl groups and their positions. Soares et al. (2003) studied the antioxidant activity of BHT, propyl gallate (PG), resveratrol, α-tocopherol, and ascorbic acid against ABTS radical cation, DPPH radical, and hydroxyl radical. BHT and resveratrol showed a higher ABTS radical cation scavenging activity than PG and ascorbic acid. However, resveratrol showed lower DPPH radical scavenging activity than BHT, PG, and ascorbic acid. In both ABTS radical cation and DPPH radical scavenging activity, α-tocopherol did not show any measurable radical scavenging activity, possibly due to the solvent (Tris-HCl buffer) used for dissolving the samples. BHT and resveratrol exhibited the highest inhibition of β-carotene oxidation against hydroxyl radical (H₂O₂ and Fe²⁺). They also studied the survival of *Saccharomyces cerevisiae* against hydrogen peroxide, in which resveratrol was able to 100% protect yeast cells even at the highest concentration of hydrogen peroxide (33mM), whereas PG did not show any protective effect. Resveratrol also inhibited copper-catalyzed LDL oxidation. Frankel et al. (1993) reported that resveratrol inhibited hexanal formation of copper-catalyzed LDL oxidation

by 70-81%. They also reported that resveratrol exhibited a much higher inhibition of hexanal formation than α -tocopherol, whereas epicatechin and quercetin showed better efficacy than resveratrol. However, others have reported a higher inhibition of copper-induced LDL oxidation for resveratrol compared to flavonoids due to a better ability to bind with copper (Frémont et al., 1999; Caruso et al., 2004). Belguendouz et al. (1998) studied the interaction of resveratrol with plasma lipoproteins and found that the concentration of resveratrol increased as lipid content of lipoprotein increased (high-density lipoprotein < LDL < very low-density lipoprotein); they suggested that resveratrol had a lipophilic character. In addition, they studied the inhibition of formation of thiobarbituric acid reactive substances (TBARS) in unilamellar liposomes containing phospholipids and found that resveratrol was more effective than trolox but was as effective as α -tocopherol.

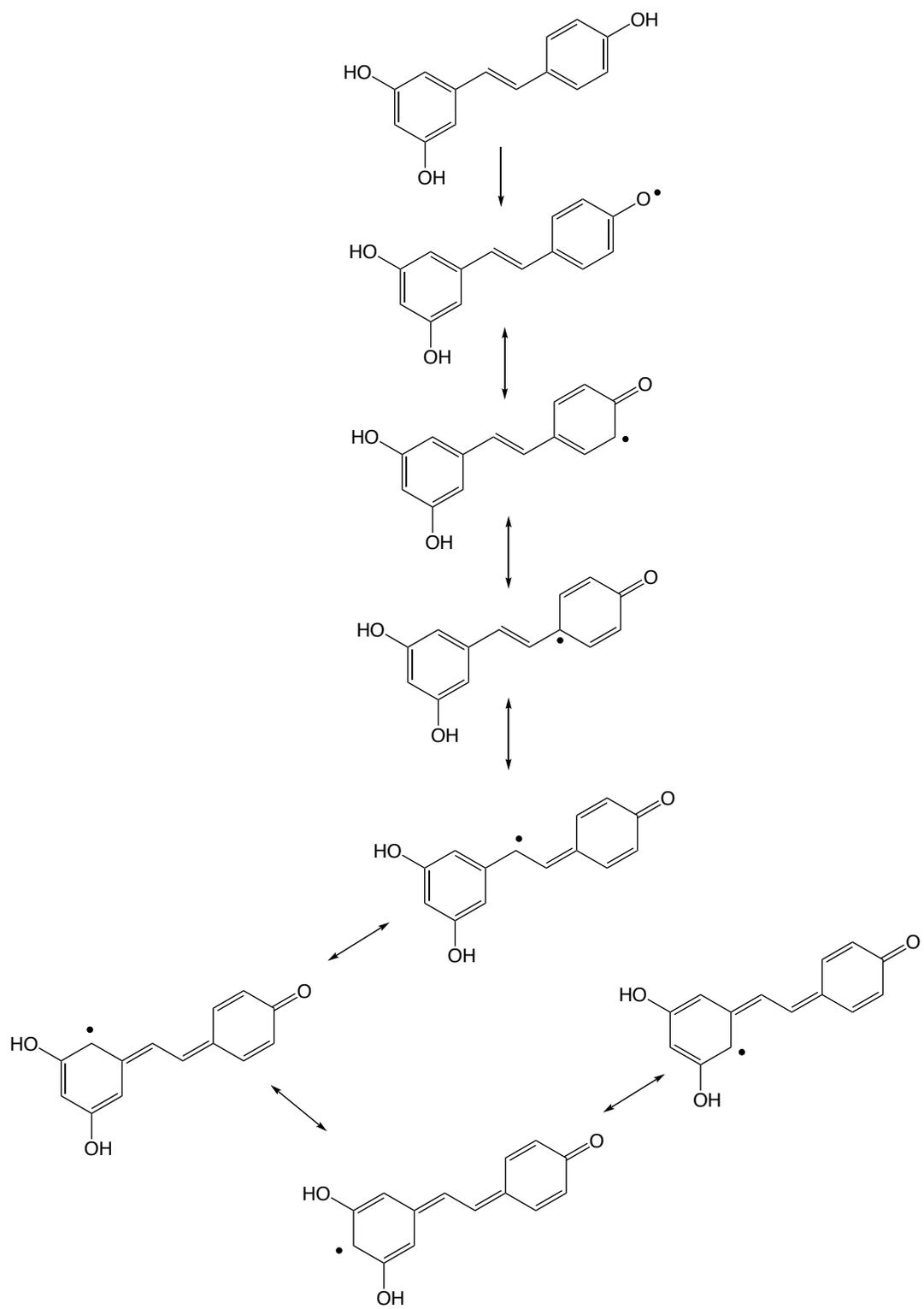


Figure 2.9. Resonance structures of resveratrol radical.

2.3.3 Other properties of resveratrol

Resveratrol is speculated to play a role against atherosclerosis and CHD. One possible way to prevent heart disease is the inhibition of LDL cholesterol oxidation which may be achieved by resveratrol (Holvoet, 2004, Frankel et al., 1993). Inappropriate platelet aggregation can also cause heart disease due to thrombus formation and consequently blood vessel blockage (Baur and Sinclair, 2006). Pace-Asciak et al. (1995) reported that resveratrol and quercetin might protect the heart from diseases via blocking platelet aggregation and synthesis of eicosanoids such as thromboxane B₂ (TxB₂), hydroxyheptadecatrienoate (HHT), and 12-hydroxyeicosatetraenoate (12-HETE). They found that while resveratrol and quercetin inhibited platelet aggregation induced by thrombin and adenosine diphosphate (ADP) in a dose-dependent manner, ethanol was able to inhibit only thrombin-induced platelet aggregation. In addition, resveratrol was able to inhibit the synthesis of the three aforementioned eicosanoids, but the synthesis of 12-HETE was less affected compared to other eicosanoids. Wang et al. (2002) also reported that resveratrol inhibited platelet aggregation induced by collagen as well as thrombin and ADP in a concentration-dependent manner *in vitro*. In addition, they found that resveratrol (4 mg/kg/day) was able to decrease platelet aggregation rate of high-cholesterol fed rabbits from 61.0 ± 7.0 to $35.7 \pm 6.3\%$ (platelet aggregation rate of control rabbit, $39.5 \pm 5.9\%$) without having any effect on serum lipid levels *in vivo*. Another way to decrease the risk of atherosclerosis related to blood pressure, serum total cholesterol and triacylglycerol levels is the caloric restriction (Fontana et al., 2004). The caloric restriction is consuming 60-70% calorie of normal diet without affecting any of the nutrients (Guarente and Picard, 2005). Resveratrol has been shown to activate Sir2, which

is a key to regulate the beneficial effects of caloric restriction to extend the lifespan in yeast (Baur et al., 2006; Guarente and Picard, 2005) and SIRT1, which is the closest homologue to Sir2 based on amino acid sequence, among sirtuins in mammals (Baur and Sinclair, 2006; Howitz et al., 2003). Baur et al. (2006) studied the effects of resveratrol in mice fed a high-calorie diet. They categorized mice into three groups of standard diet, high-calorie diet, and high-calorie diet with 0.04% of resveratrol. Changes related to longer lifespan such as insulin sensitivity, motor function, and mitochondrial number were then monitored. They found that resveratrol could change the physiology of high-calorie diet in mice towards standard diet mice and increase survival of mice on the high-calorie diet.

Anti-inflammatory effect of resveratrol has also been reported. Although inflammation is a normal reaction to the injury or infection, chronic inflammation can lead to various diseases such as cardiovascular ailment and cancer (Zhong et al., 2012; Steinberg, 2002). Immune cells such as neutrophils, macrophages, and monocytes can initiate inflammation in the damaged tissue via the release of various pro-inflammatory cytokines such as interleukin (IL)-1 and tumour necrosis factor (TNF)- α , and reactive oxygen/nitrogen species (ROS/RNS) (Zhong et al., 2012; Wiseman and Halliwell, 1996). In addition, cyclooxygenase (COX) may also play a crucial role in inflammation via producing pro-inflammatory molecules (Simmons et al., 2004; Baur and Sinclair, 2006). According to Jang et al. (1997) resveratrol inhibited COX-1 such as cyclooxygenase activity and hydroperoxidase activity. However, resveratrol showed an inhibition only for hydroperoxidase activity of COX-2. They also reported that resveratrol was able to

reduce both acute and chronic phases of pedal edema. This effect was greater than phenylbutazone, an anti-inflammatory drug. Khanduja et al. (2004) studied the hepatic and pulmonary COX activity induced by N-nitrosodiethylamine (NDEA) via measuring the level of prostaglandins (PGE₂, PGD₂, and PGF_{2α}) and reported that resveratrol significantly reduced the prostaglandin levels via inhibiting pulmonary COX activity. Although NDEA did not affect the hepatic COX activity to produce prostaglandins, resveratrol reduced its synthesis. Bi et al. (2005) reported that resveratrol was able to inhibit the generation of nitric oxide (NO) and TNF-α induced by LPS-stimulated microglia and suggested that it had a potent anti-inflammatory activity on microglia, brain macrophage. Furthermore, Tsai et al. (1999) reported that resveratrol inhibited the production of NO and reduced the amount of inducible NO synthase (iNOS) in the murine macrophage via suppression of iNOS mRNA.

Resveratrol is known to have anticancer activity. Jang et al. (1997) stated that anticancer activity of resveratrol was related to several stages of carcinogenesis such as initiation, promotion, and progression. Resveratrol showed antioxidant and anti-mutagenic activity, which is related to tumour initiation. In addition, resveratrol was able to reduce inflammation (anti-promotion) and induce differentiation of human promyelocytic leukemia cell (anti-progression). According to Sgambato et al. (2001), resveratrol showed anti-proliferative activity on different types of cell lines such as rat fibroblast and mouse mammary epithelial cells as well as human breast, colon, prostate, and cervix cancer cells. Moreover, they reported that resveratrol was able to reduce nuclear DNA fragmentation. Patel et al. (2010) studied the level and anticancer activity of resveratrol and its

metabolites in the colorectal tissue of cancer patients. They gave 0.5 or 1.0 g of resveratrol for eight days to the twenty patients and took colorectal tissues and reported that the highest resveratrol concentration was 18.6 and 674 nmol/g for the 0.5 and 1.0 g dose, respectively. In addition, tumour cell proliferation was reduced by 5%. Aires et al. (2013) examined the anticancer activity of resveratrol metabolites (resveratrol-3-*O*-sulphate, resveratrol-3-*O*-glucuronide, and resveratrol-4'-*O*-glucuronide) on human metastatic colon cancer cells. Among metabolites, only resveratrol-3-*O*-sulphate inhibited cell proliferation and accumulation of cells in the S phase. Surprisingly, the mixture of these three metabolites exerted a synergistic effect on cell proliferation, which was better than resveratrol itself.

2.3.4 Antioxidant and biological activity of modified resveratrol

It is a well-known fact that utilizing resveratrol for health benefits has limitations due to its fast metabolism (Walle et al., 2004; Yu et al., 2002). Many attempts have been made to increase the bioavailability of resveratrol, and this may be achieved by structure modification which may disturb glucuronidation and/or sulphation (Torres et al., 2010). Moreover, increased lipophilicity may enhance affinity with lipophilic cell membranes so that cellular uptake may be increased (Cardile et al., 2005). Torres et al. (2010) studied the antioxidant activity of resveratrol derivatives using trolox equivalent antioxidant capability (TEAC) assay and compared the results with trolox and α -tocopherol. They reported that resveratrol showed better antioxidant activity than trolox and α -tocopherol. They observed that the antioxidant activity of resveratrol derivatives decreased as the

acylation increased (resveratrol > monoesters > diester > triester). In addition, resveratrol with vinyl acetate showed better antioxidant activity than resveratrol with vinyl stearate (C2 > C18:0).

Several biological activities of resveratrol derivatives have been reported. Fragopoulou et al. (2007) studied the effects of resveratrol and its acetylated derivatives (mono-, di-, and triester) on platelet aggregation. They reported that the diesters showed the highest inhibition of platelet aggregation, whereas resveratrol as well as its mono-, and triester exhibited similar inhibitions. Cardile et al. (2005) prepared resveratrol derivatives using a chemo-enzymatic synthesis and studied the cell growth inhibition activity on DU-145 human prostate cancer cells as well as toxicity on non-tumourigenic human fibroblast cells. Resveratrol derivatives showed similar or better cell growth inhibition than the parent molecule. Moreover, resveratrol derivatives exhibited very low toxicity in non-tumourigenic cells. Mazué et al. (2010) modified *trans*- and *cis*-resveratrol via hydroxylation and methylation and then examined their inhibitory activity on the proliferation of human colorectal tumour cells (SW 480). They reported that *cis*-3,5,4'-trimethoxystilbene showed the highest inhibition of tumour cell growth after 48 h of treatment and this was much higher than both *trans*- and *cis*-resveratrol. Cichocki et al. (2008) studied the effect of pterostilbene on nuclear factor (NF)- κ B, activator protein (AP)-1, COX-2, and iNOS induced by 12-O-tetradecanoylphorbol-13-acetate. They reported that pterostilbene showed equal or better inhibition than resveratrol in most of the assays and suggested that this might be due to the increased lipophilicity, which might elicit better bioavailability. Pan et al. (2007) studied the cytotoxic effect of pterostilbene

on COLO205, AGS, HL-60, and HepG2 cell lines and reported that pterostilbene (100 μ M) had the highest cytotoxic effect on AGS cells, among others. Moreover, they confirmed that the cytotoxic effect was due to the apoptotic cell death.

2.4 Quercetin

2.4.1 Quercetin in foodstuffs

Quercetin is a flavonoid belonging to the flavonol subgroup. Flavonoids are widespread in the plant kingdom and are generally present in daily diet (Formica and Regelson, 1995). In addition, quercetin can be found in high concentrations in certain plant-based foodstuffs. Hertog et al. (1992) reported that quercetin in the edible portion of vegetables was usually present in less than 10 mg/kg, however onions, kale, broccoli, French beans, and slicing beans were found to contain high amounts of quercetin, at 284-486, 110, 30, 32-45, and 28-30 mg/kg, respectively. In addition, they also studied the concentration of quercetin in fruit and reported that the average amount of quercetin was 15 mg/kg, but apple contained 21-72 mg/kg of quercetin depending on the variety. Hertog et al. (1993) expanded their study from vegetables and fruits to tea infusion, wine, and fruit juice. They reported that quercetin in white wine, beer, coffee, and chocolate milk was less than 0.5 to 1.3 mg/L. Quercetin in fruit juices such as apple juice, grape juice, and grapefruit juice was less than 5 mg/L, whereas quercetin in fresh lemon juice and tomato juice was 7.4 and 13 mg/L, respectively. Red wine contained quercetin at 4-16 mg/L and black tea infusion had 10-25 mg/L.

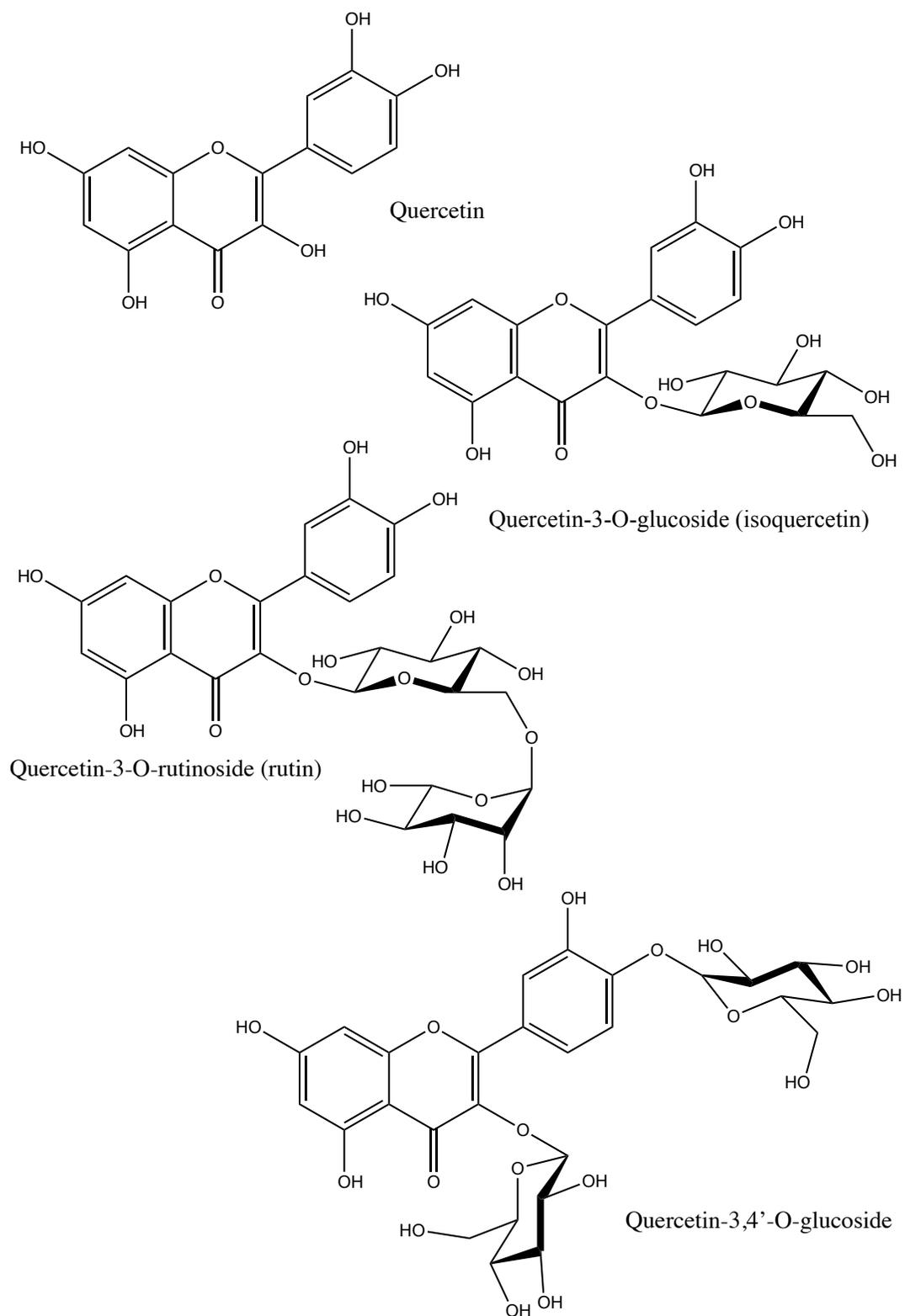


Figure 2.10. Quercetin and its glycosides found in plant-based foods.

Quercetin can also exist as a glucoside. According to Zielinska et al. (2008), the dominant flavonoids in the flesh of two onion varieties, Sochaczewska and Szalotka, were quercetin-4'-O- β -glucoside (61 and 54%), quercetin-3,4'-di-O- β -glucoside (37 and 44%), quercetin-3-O- β -glucoside (1.4 and 1.1%), and quercetin (1.1 and 0.7%). Hempel and Böhm (1996) reported that quercetin-3-O-glucuronides, quercetin-3-O-rutinosides, kaempferol-3-O-glucuronides, and kaempferol-3-O-rutinosides were the main flavonoid glycosides present in six varieties of yellow and green French beans. The total contents of quercetin glucosides and kaempferol glucosides were 19.1-183.5 and 5.6-14.8 $\mu\text{g/g}$, respectively. Stewart et al. (2000) studied the flavonol contents of 20 varieties of tomato and 10 commonly consumed tomato products. They reported that most flavonols were found in the tomato skin (98%) and the main quercetin conjugate was rutin. Among tomato products, tomato purée and tomato juice contained the highest level of quercetin and rutin.

2.4.2 Quercetin as an antioxidant

As shown in Figure 2.11, the antioxidant activity of flavonoids, as reflected in their radical scavenging activity, may increase if there is a 3',4'-dihydroxyl group in the B ring, and a 2,3-double bond and a 4-oxo moiety in the heterocycle (C ring); these allow for conjugation between A and B rings, and 3- and 5-hydroxyl groups in the A and C rings, respectively (Bors et al., 1990; Rice-Evans et al., 1996; Pulido et al., 2000). Quercetin, taxifolin, and catechin have a 5-hydroxyl group, however, taxifolin has no 2,3-double bond and catechin lacks both the 2,3-double bond and the 4-oxo moiety in the C ring.

Quercetin (4.7 ± 0.1 mM) showed a roughly 2-fold higher radical scavenging activity compared to taxifolin (1.9 ± 0.0 mM) and catechin (2.4 ± 0.1 mM) in the TEAC assay. This might be due to the structural effect, which may allow the phenoxyl radical to be more stabilised via electron delocalization across the molecule (Rice-Evans et al., 1996). In addition, a 3-hydroxyl group in the C ring is important in terms of antioxidant activity. Compared to rutin (3-O-glycoside) or luteolin (lack of 3-OH), quercetin showed approximately 2 times more antioxidant activity than the other compounds (Rice-Evans et al., 1996). The influence of a 2,3-double bond on antioxidant activity can be limited when there is no ortho-diphenolic structure in the B ring. Kaempferol (lack of 3'-OH) exhibited similar antioxidant activity in the TEAC assay compared to aromadendrin (lack of both 3'-OH and 2,3-double bond). The antioxidant activity of apigenin (lack of 3'-OH) and chrysin (lack of 3'-OH and 4'-OH), both with similar antioxidant activity to kaempferol, also supported the effect of ortho-diphenolic structural effect in antioxidant activity (Rice-Evans et al., 1996). The effect on the antioxidant activity of an ortho-diphenolic structure might be due to the possible formation of either semiquinone or quinone, which

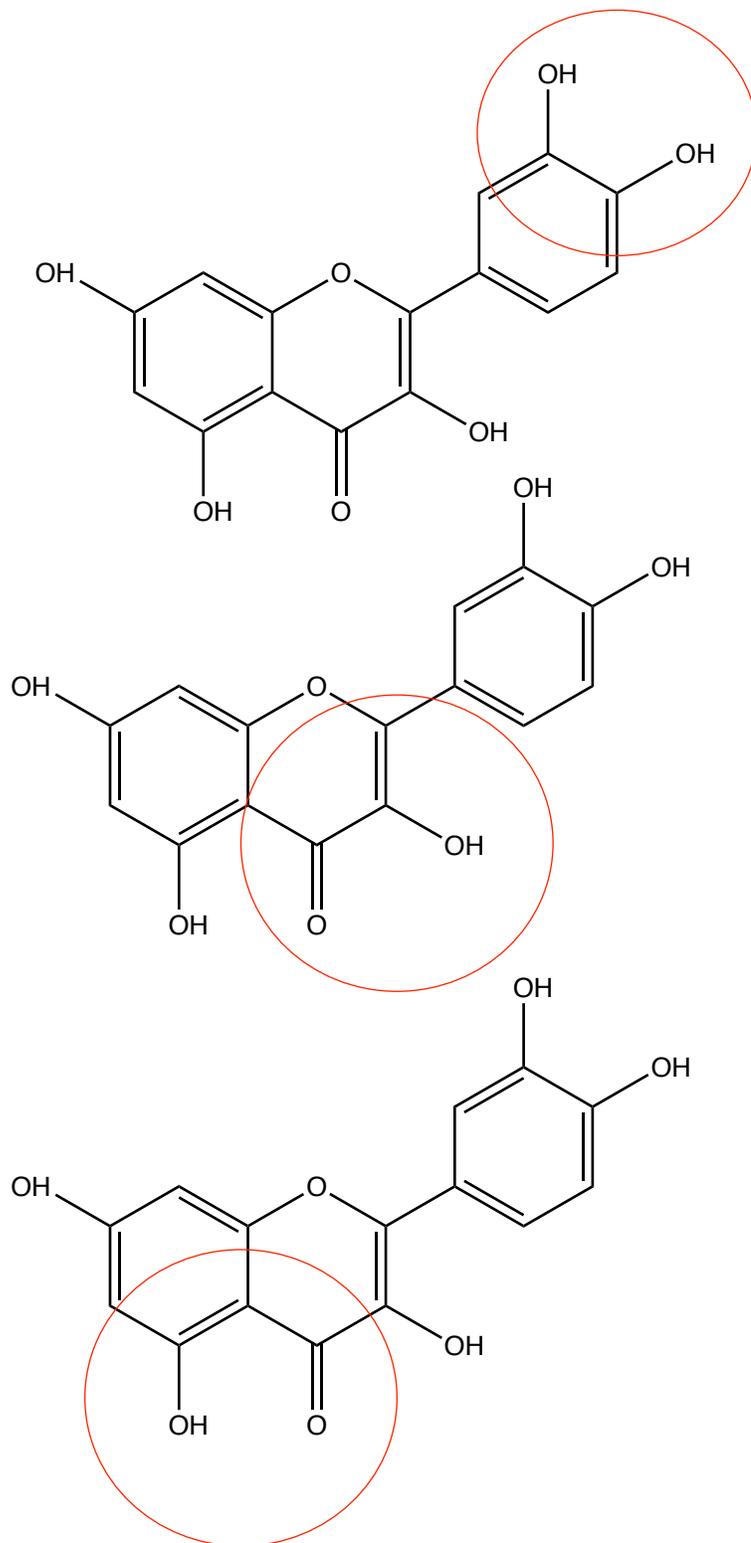


Figure 2.11. Important parts of quercetin for radical scavenging activity.

could stabilise the electron (Bors et al., 1990). Pulido et al. (2000) examined the solvent effect (methanol vs. distilled water) toward ferric reducing/antioxidant power (FRAP) assay using tannic acid, flavonoids (quercetin and catechin), phenolic acids (gallic acid and caffeic acid), and reference compounds (ascorbic acid and trolox). Test compound with ferrous ion in methanol showed higher FRAP values than test compound with ferrous ion in distilled water, however the order of FRAP values was the same. Quercetin exhibited the highest FRAP value among all tested samples. Quercetin also showed inhibition against LDL cholesterol oxidation and it was 2-fold more effective than resveratrol (Frankel et al., 1993). Frémont et al. (1999) compared to inhibition against LDL cholesterol oxidation induced by copper and AAPH (radical initiator) using quercetin, resveratrol, catechin, epicatechin, and trolox. While quercetin showed the highest inhibition in the presence of AAPH, resveratrol exhibited the best protection in the presence of copper. Meyer et al. (1998) compared inhibition activity of catechin, cyanidin, caffeic acid, quercetin, and ellagic acid against LDL oxidation and reported that the inhibition was in the order of catechin > cyanidin \approx caffeic acid > quercetin > ellagic acid. Huber et al. (2009) studied the antioxidant activity of quercetin and its glycosides (quercetin-3-O-glucoside, quercetin-3-O-galactoside, quercetin-3-O-rhamnoside, and quercetin-3-O-rutinoside) in omega-3 polyunsaturated fatty acids and bulk fish oil compared with that of BHT and α -tocopherol. They reported that quercetin showed similar or better antioxidant activity than quercetin glycosides in the oil-in-water emulsion system and also displayed better antioxidant activity compared to α -tocopherol. On the other hand, quercetin and quercetin-3-O-glucoside showed better antioxidant activity than BHT in bulk oil. Zielinska et al. (2008) reported that the antioxidant activity

of quercetin and its glycosides evaluated by TEAC assay was in the order of quercetin > quercetin-3-O- β -glucoside > quercetin-4'-O- β -glucoside > quercetin-3,4'-di-O- β -glucoside. Zhang et al. (2011) observed that quercetin exhibited better reduction potential than curcumin at pH 3, 7, and 9.5. In examining the total antioxidant capacity using phosphomolybdenum, quercetin showed 3.5 times stronger effect compared to curcumin. In addition, they reported that quercetin was able to reduce NO and ROS production induced by LPS in the human acute monocytic leukemia cell. Gordon and Roedig-Penman (1998) studied the antioxidant activity of quercetin and myricetin in a small unilamellar liposome and α -tocopherol was employed as a reference. Myricetin showed the highest antioxidant activity, followed by quercetin > α -tocopherol at pH 5.4 and a phospholipid concentration of 0.01 mol/mol. They also compared the effect of cupric chloride and ferric chloride on oxidation and found that cupric chloride could cause more oxidation than ferric chloride. While quercetin showed better antioxidant activity than myricetin in the presence of cupric chloride, myricetin exhibited a greater antioxidant activity than quercetin in the presence of ferric chloride. Mira et al. (2002) studied the metal chelation of the following flavonoids: flavonols (quercetin, kaempferol, myricetin, and rutin); flavones (apigenin and luteolin); a flavanol (catechin); flavanones (taxifolin, naringenin, and naringin); and isoflavones (daidzein and genistein). The result showed that all flavonoids had a better reducing activity for Cu^{2+} than Fe^{3+} . They reported that only myricetin and quercetin were able to reduce Fe^{3+} , concluding that this might be due to the presence of ortho-diphenolic structure in the B ring and 3-OH, 2,3-double bond, and 4-oxo group in C ring. In Cu^{2+} reducing study, myricetin showed the highest reducing activity followed by taxifolin, catechin, quercetin > rutin > naringin

> luteolin, kaempferol. They suggested the reducing activity of Cu^{2+} might depend on the number of hydroxyl groups of flavonoids. According to Mira et al. (2002), a 2,3-double bond in the flavonoids renders the molecule more planar and more rigidity so that the A and C rings can be in a more coplanar position. Hence the 3-OH, 4-oxo, and 5-OH group of flavonoids can be closer to each other. Mira et al. (2002) studied the metal chelation via a spectrophotometric method and observed the occurrence of bathochromic shifts in the interaction between flavonoids and metal ions. The peaks of Cu^{2+} with kaempferol, luteolin, and apigenin from spectra were around 408 nm at both pH 5.5 and 7.4. This implied that the metal chelation might occur at the same site, possibly between the 5-OH and 4-oxo group. On the other hand, the peaks of Cu^{2+} with myricetin or quercetin at pH 7.4 from the spectra were around 436 nm. This might be due to the 3 possible metal chelating sites of these two flavonoids, thus myricetin and quercetin had the peaks at the longer wavelength. However, the spectral peaks of Cu^{2+} with myricetin or quercetin at pH 5.5 were around 412 nm, which is similar to kaempferol, luteolin, and apigenin. This result implies that myricetin and quercetin might have more metal chelating sites at pH 7.4 than at pH 5.5. In fact, metal chelation ability of catechol (orthodiphenolic structure) can be enhanced by the increase of pH. Leopoldini et al. (2006) reported that metal chelation between 4-oxo and 5-OH showed minimum energy (0.0 kcal/mol), whereas metal chelation between 3'-OH and 4'-OH showed the highest energy (12.3 kcal/mol).

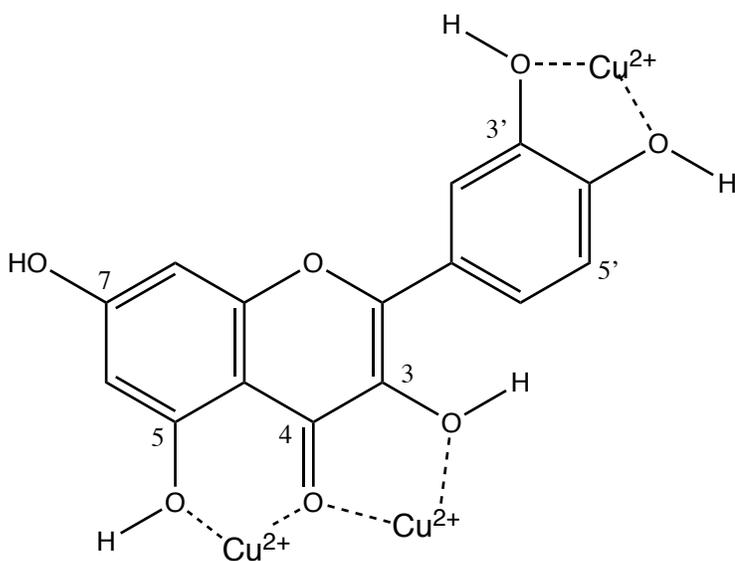


Figure 2.12. Possible sites of quercetin for cupric ion chelation.

2.4.3 Other properties of quercetin

Quercetin has been known to have anti-inflammatory activity. Kaidama and Gacche (2015) studied the anti-inflammatory activity of quercetin in guinea pigs and reported its significant inhibition ($P < 0.05$) in both acute and chronic inflammation in carrageenan-induced paw edema. Bureau et al. (2008) studied the effect of resveratrol and quercetin on neuroinflammation. They reported that both compounds were able to decrease the level of interleukin 1- α and tumour necrosis factor- α induced by lipopolysaccharide (LPS). Kim et al. (1998) reported that quercetin showed inhibition of cyclooxygenase and lipoxygenase, whereas apigenin was unable to do so.

Quercetin has been reported to have protective effects on cardiovascular disease. Pace-Asciak et al. (1995) reported that resveratrol and quercetin might have a protective effect

on CHD via blocking platelet aggregation induced by thrombin and ADP as well as eicosanoid synthesis (12-hydroxyeicosatetraenoate). Egert et al. (2009) studied the effect of quercetin on blood pressure, lipid metabolism, oxidative stress, inflammation, and body composition in 93 overweight or obese subjects. They found that the quercetin treatment group decreased both systolic blood pressure by 2.6 mmHg ($P < 0.01$) and plasma concentration of atherogenic oxidized LDL, compared to the placebo group. However, quercetin did not alter total cholesterol, triacylglycerol (TAG), LDL: HDL ratio, and TAG: HDL ratio.

Quercetin has also been shown to have anticancer activity. Lu et al. (2006) studied the anticancer activity of quercetin and myricetin by inhibiting mammalian thioredoxin reductase and found that both quercetin and myricetin showed powerful inhibitory activity on mammalian thioredoxin reductase. They suggested that one of the anticancer mechanisms of quercetin might be due to enzyme inhibition. Agullo et al. (1994) studied the cytotoxic effect of quercetin on colon carcinoma (HT-29, Caco-2 cells) and reported its cytotoxicity on active proliferating cells in a dose-dependent manner.

According to Vessal et al. (2003), quercetin showed antidiabetic effects in diabetic rats induced by streptozocin. While quercetin was able to reduce plasma glucose level of diabetic rats, it was unable to reduce plasma glucose level in normal rats. Plasma cholesterol and triacylglycerol levels in diabetic rats decreased significantly. Furthermore, they reported that quercetin was able to increase hepatic glucokinase activity. Coskun et al. (2005) studied the protective effect of quercetin on oxidative stress and β -cell damage in diabetic rat pancreas induced by streptozotocin. Quercetin was able to reduce the level

of malondialdehyde and NO production and increase antioxidant enzyme activity. Furthermore, quercetin increased insulin staining and preserved islet cells in diabetic rats.

2.4.4 Antioxidant and biological activity of modified quercetin

Low bioavailability of quercetin has been an issue of concern to many scientists. Many attempts have been made to enhance its bioavailability while maintaining its beneficial effects; a possible way is by structure modification. Mainini et al. (2013) prepared quercetin triesters, tetraesters, and pentaesters using stearic, oleic, linoleic, and linolenic acids and evaluated antioxidant activity of quercetin esters by TEAC and DPPH radical scavenging assays. They reported that quercetin showed the highest antioxidant activity in both assays. Among esters, triesters exhibited a stronger antioxidant activity than pentaesters. Guardia et al. (2001) studied the effect of rutin (quercetin-3-O-glycoside), quercetin, and hesperidin on acute and chronic inflammation in the rat. They induced arthritis in the rat using carrageenan; phenylbutazone was a positive control. They reported that rutin and quercetin were able to decrease inflammation in the acute phase and rutin showed the most potential in the chronic phase. Montenegro et al. (2007) prepared quercetin-3-O-acyl esters and evaluated their water stability, solubility, susceptibility to enzymatic hydrolysis, and permeability. They observed quercetin with acetic acid and caproic acid not stable in water. Some esters (quercetin with propionic acid, butyric acid, and lauric acid) showed more susceptibility to hydrolysis by human plasma. Furthermore, quercetin with propionic acid and butyric acid not only had a better solubility in water than quercetin itself but also exhibited greater permeability than the

parent molecule. Hence, they suggested that quercetin with propionic acid and butyric acid could serve as quercetin prodrugs. Biasutto et al. (2007) studied the effect of esterification on transepithelial transport by measuring permeation of three different epithelial cell monolayers, namely MDCK-1, MDCK-2, and Caco-2 cells. They reported that all types of cells extensively metabolized the parent molecule. Some esters (pentaacetylquercetin, 3'-*t*-butyl-oxycarbonyl-D-alanine tetraacetylquercetin ester, 3'-[4-(*t*-butyl-oxycarbonyl-amino)butanoic acid] tetraacetylquercetin ester, and 3'-[6-(*t*-butyl-oxycarbonyl-amino)hexanoic acid] tetraacetylquercetin ester) passed through monolayer and underwent partial deacylation. They suggested that ester derivatives might provide a useful means to increase the level of systemic aglycone.

CHAPTER 3

Materials and methods

Part of this chapter has been published in *J. Agric. Food Chem.* (2017), 65, 8617-8625 and *Food Chem.* (2018), 261, 267-273.

3.1 Materials

The materials reported in this chapter are for all studies performed in this thesis.

Resveratrol and DHA single cell oil (DHASCO) were procured from DSM (Columbia, MD, USA). EPA ethyl ester was a product of Mochida Pharmaceutical CO., LTD. (Tokyo, Japan) and was kindly provided by Professor Kazuo Miyashita. Potassium hydroxide was purchased from BDH Inc. (Toronto, ON, Canada). Silica gel and flexible thin layer chromatography (TLC) plates with silica gel 60A (2.5 × 7.5 cm) were bought from Selecto Scientific (Suwanee, GA, USA). Deuterated dimethyl sulphoxide (DMSO-d₆) containing tetramethylsilane (TMS) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Acyl chlorides (propionyl chloride, butyryl chloride, caproyl chloride, capryloyl chloride, capryl chloride, lauroyl chloride, myristoyl chloride, palmitoyl chloride, stearoyl chloride, and oleoyl chloride), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), trolox, and 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), were obtained from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada). Hydrogen chloride, phosphate buffers (sodium phosphate monobasic and sodium phosphate dibasic), sodium chloride, and other solvents were procured from Fisher Scientific Ltd (Ottawa, ON, Canada).

SensoLyte 520 HCV fluorimetric Protease Assay Kit (lot #1028) and HCV NS3/4A protease (lot# 103-075), bought from Anaspec. Com. (San Jose, CA, USA).

The RAW 264.7 cells, derived from murine macrophages, were procured from the American Type Culture Collection (Rockville, MD, USA). Heat-inactivated fetal calf serum was purchased from GIBCO (Grand Island, NY, USA). LPS (*Escherichia coli* O127:E8, molecular weight, 60 kDa) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Both the human AGS and MCF7 cell lines were isolated from gastric adenocarcinoma (ATCC CRL-1739) and breast adenocarcinoma (ATCC HTB-22). Fetal bovine serum (FBS) was bought from Biological Industries (Cromwell, CT, USA). Alpha-glucosidase (from *Bacillus stearothermophilus*, Lot# SLBP7209V), *tert*-butylhydroperoxide (t-BOOH) and dichlorofluorescein-2',7'-diacetate (DCFH-DA) and all other chemicals, not mentioned which were used, were purchased from Sigma-Aldrich (Oakville, ON, Canada).

3.2 Methods

3.2.1 Lipophilisation of resveratrol and effects on antioxidant activities

3.2.1.1 Preparation of EPA and DHA

EPA was produced from its ethyl ester via saponification. Preparation of DHA from DHASCO was carried out as described by Wanasundara and Shahidi (1999). DHASCO (30 g) was saponified using potassium hydroxide (6.9 g), water (13.2 mL), and 95% aqueous ethanol (79.2 mL) under reflux conditions and under a nitrogen atmosphere at 62

± 2 °C. Water (60 mL) and hexane (100 mL x2) were subsequently added, mixed, and separated; the hexane layer was discarded to remove unsaponifiable matter. The pH of the aqueous layer was adjusted to 1 with 3.0 M HCl. Hexane (50 mL x4) was subsequently added to extract the liberated free fatty acids which were then passed through a layer of anhydrous sodium sulphate. The hexane was subsequently removed by using a rotary evaporator. The free fatty acids obtained were stirred with 20% urea in 95% aqueous ethanol (150 mL, w/v) at 60 °C. When a clear solution resulted, the mixture was kept for 24 h at 4 °C to allow the formation of urea-complexed crystals. The crystals were removed by suction filtration followed by the addition of an equal volume of the water to non-urea complexed fraction (NUCF) and the pH was adjusted to 4-5 with 6.0 M HCl. An equal volume of hexane was added and stirred for 1 hour. The hexane layer was passed through anhydrous sodium sulphate and the hexane subsequently removed by rotary evaporation. The identity and relative purity of the DHA so obtained were confirmed by gas chromatography-mass spectrometry (GC-MS) of its fatty acid methyl ester (FAME).

3.2.1.2 Preparation of resveratrol derivatives

Preparations of resveratrol derivatives were carried out according to Zhong and Shahidi (2011). EPA and DHA were converted to eicosapentaenoyl chloride and docosahexaenoyl chloride with thionyl chloride. The mole ratio between PUFA and thionyl chloride was 1:2. Resveratrol was dissolved in ethyl acetate and then pyridine was added at a mole ratio of 1:1. Acyl chlorides (propionyl chloride, butyryl chloride, caproyl chloride, capryloyl chloride, capryl chloride, lauroyl chloride, myristoyl chloride, palmitoyl chloride, stearoyl chloride, oleoyl chloride, eicosapentaenoyl chloride, and

docosahexaenoyl chloride) were added dropwise to a solution of resveratrol in ethyl acetate. The reaction proceeded under reflux heating and a blanket of nitrogen at 50 °C. Upon completion of the reaction, the mixture was allowed to stand until it reached room temperature and was then washed 3 times with distilled water (60 °C). The ethyl acetate layer was then passed through a layer of anhydrous sodium sulphate followed by the removal of the solvent.

3.2.1.3 Purification of resveratrol derivatives

The resveratrol derivatives were purified using column chromatography on silica gel. The solvent ratios used were gradients of hexane / ethyl acetate / formic acid (90/ 10/ 2; 80/ 20/ 2; 70/ 30/ 2; 60/ 40/ 2: v/v/v). Each fraction was collected and monitored by TLC (hexane / ethyl acetate / formic acid: 3/ 3/ 0.12: v/v/v). Each purified compound was procured following solvent removal.

3.2.1.4 Identification of resveratrol derivatives

The chemical structures of resveratrol derivatives were determined by high-performance liquid chromatography-diode array detector-mass spectrometry (HPLC-DAD-MS) using an Agilent 1100 HPLC unit (Agilent Technologies, Palo Alto, CA, USA). A C18 column (4.6 mm × 250 mm × 5 µm with guard column; Sigma-Aldrich, Oakville, ON, Canada) was used for separation. The mobile phase was methanol/ 5% acetonitrile in water at different ratios (60:40-95:5, v/v; see results and discussion) varying from 0 to 70 min at 0.8 mL/min and the compounds were detected at 306 nm.

Proton nuclear magnetic resonance ($^1\text{H-NMR}$) spectroscopy, carbon-13 nuclear magnetic resonance ($^{13}\text{C-NMR}$) spectroscopy, correlation spectroscopy (COSY), single-quantum correlation (HSQC) spectroscopy, nuclear Overhauser effect spectroscopy (NOESY), and heteronuclear multiple-bond correlation (HMBC) spectroscopy were performed to identify their molecular structures. All NMR data were collected on a Bruker Avance 300 MHz (Bruker Biospin Co., Billerica, MA, USA) and data interpretation was performed with Topspin 3.0 with ICON. The compounds were dissolved in perdeuterated dimethyl sulphoxide (DMSO-d_6) containing tetramethylsilane (TMS). The results were confirmed by comparing the chemical shifts of resveratrol and its derivatives.

3.2.1.5 Determination of lipophilicity

The lipophilicity of resveratrol derivatives was computationally obtained by using ALOGPS 2.1. established by Tetko et al. (2005). The structures of resveratrol derivatives in simplified molecular input line entry (SMILE) format were drawn using ChemDraw Std 14.0.

3.2.1.6 DPPH radical scavenging assay

The antioxidant activities of the parent resveratrol molecule and its derivatives were determined according to their DPPH scavenging capacity using electron paramagnetic resonance (EPR) according to Madhujith and Shahidi (2006) with slight modification. Trolox in ethanol was used as a standard (0-750 μM) and resveratrol derivatives (500 μM) were also dissolved in ethanol. Test compounds (0.10 mL) were added to 0.90 mL of DPPH (0.20 mM) in ethanol. The mixtures were mixed well and placed in the dark for 30

min. The DPPH radical scavenging capacities were expressed as micromoles of trolox equivalents (TE) per micromole of test compound.

3.2.1.7 ABTS radical cation scavenging assay

The ABTS radical cation scavenging assay of resveratrol and its derivatives was conducted according to the method described by Miller et al. (1993) and as reported by Ambigaipalan et al. (2015) with slight modifications.

A mixture of 1.25 mM ABTS and 1.25 mM AAPH was prepared in a 0.1 M phosphate buffer containing 0.15 M sodium chloride (pH 7.4) and subsequently heated for 20 min at 60 °C to prepare the ABTS radical cation solution. The container was covered with aluminum foil and stored at room temperature. The samples to be tested (500 µM in ethanol, 40 µL) and blank (ethanol, 40 µL) were mixed with the ABTS radical cation solution (1.96 mL). The absorbance of the mixture was read after 6 min at 734 nm. ABTS radical cation scavenging ability was expressed as micromoles of trolox equivalents per micromole of test compound.

3.2.2 Antioxidant activity of resveratrol ester derivatives in food and biological model systems

3.2.2.1 Preparation of resveratrol derivatives

Resveratrol derivatives were prepared as described in section 3.2.1.1 and 3.2.1.2 (Oh and Shahidi, 2017). Briefly, resveratrol was esterified with acyl chlorides of propionic acid

(C3:0), butyric acid (C4:0), caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), eicosapentaenoic acid (C20:5, EPA), and docosahexaenoic acid (C22:6, DHA). The chemical structures of resveratrol derivatives, after HPLC separation, were determined by MS and different types of NMR. The mono- and diesters of crude products were used to assess their antioxidant potential in selected food and biological model systems.

3.2.2.2 Antioxidant activity of resveratrol derivatives in oil-in-water emulsion (β -carotene bleaching assay)

The β -carotene bleaching assay was used to determine antioxidant activity of resveratrol and its derivatives in the oil-in-water emulsion according to Zhong and Shahidi (2012). An aliquot (1.2 mL) of β -carotene (10 mg) dissolved in 10 mL of chloroform was transferred into a flask containing linoleic acid (40 mg) and Tween 40 (400 mg). A blank was also prepared (40 mg of linoleic acid + 400 mg of Tween 40 without β -carotene). Chloroform was removed under a stream of nitrogen and then 100 mL of oxygenated distilled water were added and stirred vigorously. One millimole of resveratrol and its derivatives in 100% ethanol (0.5 mL) as well as the control (0.5 mL, 100% ethanol) were mixed with aliquots (4.5 mL) of the above emulsion. Each sample blank (40 mg of linoleic acid + 400 mg of Tween 40 + 0.5 mg of samples without β -carotene) was also prepared. The absorbance of the contents of each tube was read immediately at 470 nm and then incubated in a shaking water bath at 50 °C. The UV-vis absorbance was read over a 105 min period at 15 min intervals. The antioxidant activity of resveratrol and its

derivatives in the oil-in-water emulsion was subsequently calculated using the following equation.

$$\text{Antioxidant activity (\%)} = [1 - (S_0 - S_t) / (C_0 - C_t)] * 100$$

where S_0 and S_t are corrected absorbance values for test compounds measured at zero time and after incubation, respectively; while C_0 and C_t are corrected absorbance values for the control at zero time and after incubation, respectively.

3.2.2.3 Antioxidant activity of resveratrol derivatives in bulk oil

Stripped corn oil, which is free of minor compound, was prepared according to a simplified stripping method. The stripped oil (1 g) was weighed into 10 mL clear glass vials and loosely capped in order to induce oil oxidation. Resveratrol and its derivatives (75 μM , 100 μL) in ethanol were added, and the solvent was then removed under a stream of nitrogen. The vials were wrapped in aluminum foil to protect samples from light and stored in a forced air oven (Precision Scientific Co., Chicago, IL, USA) at 60 ± 0.5 °C. Samples were withdrawn on day 0, 1, 3, and 6 for analyses. Conjugated dienes and *p*-anisidine values were used for monitoring oxidative products according to the AOAC (Conjugated dienes, 1980; *p*-anisidine values, 1990) methods.

3.2.2.4 Antioxidant activity in a meat model system (thiobarbituric acid reactive substances, TBARS)

A ground meat model system was used to determine the antioxidant activity of resveratrol and its derivatives (Wettasinghe and Shahidi, 1999). Forty grams of ground pork were

mixed with 10 mL of deionized water in Mason jars. One millilitre of test compounds (1 mM) or a positive control (butylated hydroxytoluene, BHT, 1 mM) was added directly to the meat. A blank with no antioxidant was also prepared. The meat samples were mixed and cooked for 40 min in a water bath at 80 °C with intermittent stirring. The contents were transferred into plastic bags and stored for 7 days at 4 °C. Meat samples were analyzed on day 0, 3, 5, and 7 for their oxidative state using the TBARS test as described by Shahidi and Hong (1991) with slight modification. Trichloroacetic acid (10 %, w/v, 2.5 mL) was added to meat samples (1 g) in centrifuge tubes which were then vortexed for 2 min. Subsequently, TBA reagent (0.02 M, 2.5 mL) was added and vortexed again for 30 sec. After centrifugation at 3000g for 10 min, the supernatants were filtered through a Whatman No.3 filter paper. The samples were kept in a water bath at 95 °C for 45 min, then cooled to room temperature. A precursor of malondialdehyde (MDA), 1,1,3,3-tetramethoxypropane, was used for constructing a standard curve. The absorbance of pink TBA-MDA adducts was read at 532 nm. TBARS values were calculated using the standard curve and expressed as milligrams of MDA equivalents per kilogram of sample.

3.2.2.5 Hydrogen peroxide (H₂O₂) scavenging activity

The H₂O₂ scavenging activity of resveratrol and its derivatives was determined according to the method explained by Chandrasekara and Shahidi (2011) with slight modification. The standard (ascorbic acid) and test compounds were dissolved in 100% ethanol (1 mM, 0.4 mL) were added to 0.6 mL of 40 mM H₂O₂ (in 45 mM sodium phosphate buffer solution, pH 7.4) and 1 mL of buffer solution. The absorbance was read at 230 nm after 40 min of incubation at 30 °C. Blanks were run for each sample; buffer solution was

added instead of H₂O₂. A standard curve was prepared with ascorbic acid (AA). The results were expressed as micromoles of AA equivalents per micromoles of each compound.

3.2.2.6 Inhibition of copper-induced low-density lipoprotein (LDL) oxidation

The inhibition of LDL oxidation induced by copper was determined according to Chandrasekara and Shahidi (2012). Human LDL was dialyzed in 10 mM phosphate buffer (pH 7.4, 0.15 M NaCl) using a dialysis tube (molecular weight cutoff of 12–14 kDa; Fisher Scientific, Nepean, ON, Canada) at 4 °C under a nitrogen blanket in the dark for 12 h. Diluted LDL (0.04 mg LDL/ mL, 0.8 mL) was mixed with resveratrol and its derivatives (5 µM, 100 µL). The samples and cupric sulphate (CuSO₄) were pre-incubated at 37 °C for 15 min. The reaction was initiated by adding CuSO₄ (50 µM, 100 µL) and measured immediately at 234 nm. The samples were then incubated at 37 °C for 8 h. The formation of conjugated dienes (CD) was monitored at 234 nm using a diode array spectrophotometer (Agilent, Palo Alto, CA, USA). The blanks were run for each sample by replacing LDL and CuSO₄ with phosphate buffer for background correction.

3.2.2.7 Inhibition of hydroxyl radical-induced DNA scission

Inhibitory activity of resveratrol and its derivatives against DNA scission induced by hydroxyl radical was determined according to the method described by Ambigaipalan and Shahidi (2015) with slight modification. Supercoiled pBR 322 DNA (50 µg/mL) was dissolved in 10 mM phosphate buffer (pH 7.4), and resveratrol and its derivatives dissolved in ethanol were diluted with phosphate buffer. In an eppendorf tube, test compound (12.5 µM, 2 µL), phosphate buffer (2 µL), DNA (50 µg/mL, 2 µL), FeSO₄ (0.5

mM, 2 μ L), and H₂O₂ (1 mM, 2 μ L) were added. A control (DNA with radicals) and a blank (DNA only) were also prepared. After incubation at 37 °C for 1 h, 1 μ L of the loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, and 50% glycerol) was added. The mixture was loaded onto 0.7% agarose gel (50 mL). The gel was prepared in Tris–acetic acid–EDTA (ethylenediaminetetraacetic acid) buffer (TAE, 40 mM, pH 8.5) with SYBR safe gel stain (5 μ L). Electrophoresis was performed at 80 V for 90 min in TAE buffer using a horizontal mini gel electrophoresis system (Owl Separation Systems Inc., Portsmouth, NH, USA) with a 300 V power supply (VWR International Inc., West Chester, PA, USA). The DNA bands were visualized under UV light using the Alpha-Imager gel documentation system (Cell Biosciences, Santa Clara, CA, USA) and the data processing was performed with the Chemi-Imager 4400 software (Cell Biosciences). The retention of supercoiled DNA strand (%) was calculated according to the following equation:

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

3.2.3 Effect of lipophilisation of resveratrol on reactive nitrogen/oxygen species generation in murine macrophages and human cancer cell lines

3.2.3.1 Preparation of resveratrol esters

Resveratrol derivatives were prepared as described in sections 3.2.1.1 and 3.2.1.2 (Oh and Shahidi, 2017). Resveratrol was lipophilised with propionyl chloride and docosahexaenoyl chloride. The monoesters were identified by HPLC-MS and several

types of NMR (^1H NMR, ^{13}C NMR, COSY, HSQC, NOESY, and HMBC). Resveratrol and its esters were subjected to NO production, cell viability, and ROS generation.

3.2.3.2 Cell culture

The RAW 264.7 cells, derived from murine macrophages, were cultured in Dulbecco's minimal essential medium (DMEM) containing 10% endotoxin-free, heat-inactivated fetal calf serum, 100 units/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin. Human hepatoma carcinoma cells (HepG2) in DMEM containing 10% FBS and 100 units/mL penicillin and streptomycin were maintained in humidified atmosphere of 5% CO_2 at 37 °C. The AGS cell lines in DMEM-F12 were grown at 37°C in 5% CO_2 atmosphere. A431 cell lines and MCF7 cell lines were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum, 100 units/mL of penicillin, and 100 $\mu\text{g}/\text{mL}$ of streptomycin. Those cell lines were kept at 37°C in a humidified atmosphere of 5% CO_2 .

3.2.3.3 Nitrite assay

The cells were seeded into 24 well plates, after reaching a density of 1×10^6 cells/mL and were activated by changing their medium to serum-free DMEM without phenol red containing lipopolysaccharide (LPS; Zhong et al., 2012). The RAW 264.7 cells were treated with different concentrations of compounds and LPS or LPS only for 24 h. The supernatants (100 μL) were mixed with the Griess reagent (100 μL , 1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) in duplicate on 96-well plates. The absorbance was then read at 570 nm with an ELISA reader. Sodium nitrite (NaNO_2) was employed as a standard.

3.2.3.4 Determination of cytotoxicity and ROS generation on human hepatoma carcinoma (HepG2) cells

The cell viabilities and cytotoxic properties of resveratrol and its esters were evaluated by using MTT assay (Mosmann, 1983). The 5000 cells were seeded each well in 96-well plate and incubated for 16 hours. After that, the samples (10 µg/mL) were added to each well and incubated for 24 or 48 hours and 20 µL MTT (5 mg/mL) were then added and continued incubating for 3 hours. The medium was then removed and DMSO (100 µL) was added to dissolve the crystals. The absorbance was measured at 570 nm.

HepG2 cells were seeded in 96-well black plate (at a rate of 30000 cells per well) and incubated for 14 h (Wang et al., 2016). After that, the samples at various concentrations were added and incubated for 4 h, and then the medium was removed. The cells were washed with PBS, added 10 µM DCFH-DA, and continued incubating for 30 min. After removing the medium, the cells were washed with PBS again and 400 µM *tert*-butyl hydroperoxide (t-BOOH) was added with subsequent incubation for 90 min. For control, FBS-free medium was added instead of t-BOOH. The cells were washed with PBS and the fluorescence (excitation 485 nm and emission 535 nm) was then measured.

3.2.3.5 Cell viability of A431, MCF7, HT-29, and AGS

Resveratrol and its esters were dissolved in dimethyl sulphoxide (DMSO). Cell viability of various cancer cell lines was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay (Mosmann, 1983). The cells were plated at a density of 2×10^5 cells/mL into 96 well plates and incubated for 24 h. The cells were then

pretreated with test compounds at different concentration for 24 h. The final concentration of DMSO in the culture medium was <0.05% (v/v). MTT (100 μ L) was added, and the cells were incubated for a further 1 h. Then the medium was aspirated and 100 μ L of DMSO was added to dissolve the MTT-formazan crystals formed by metabolically viable cells. Cell viability was scanned with an enzyme-linked immunosorbent assay reader with a 570 nm filter.

3.2.4 Preparation esterified quercetin derivatives and effects on antioxidant activity

3.2.4.1 Preparation of EPA and DHA

Saponification of EPA ethyl ester was carried out to prepare EPA. DHA was prepared from DHASCO via urea complexation as described by Wanasundara and Shahidi (1999). For saponification, DHASCO was stirred with potassium hydroxide, water, and 95% aqueous ethanol at 62 ± 2 °C under reflux condition and a nitrogen blanket. In order to remove unsaponified materials, water and hexane were mixed then the hexane was discarded. Hydrochloric acid (HCl, 3 M) was added to the aqueous layer to adjust to pH 1 and then hexane was added to extract the liberated free fatty acids. The solvent was subsequently passed through a layer of anhydrous sodium sulphate then removed by a rotary evaporator.

The free fatty acids were mixed with 20% urea in 95% aqueous ethanol (w/v) and this was heated at 60 °C until a clear solution resulted. The clear solution was then in the cold room (4 °C) for 24 h to form urea complexed crystals, subsequently suction filter was

carried out to remove the crystals. The resultant solution was added an equal volume of water and the pH was adjusted to 4-5 with 6 M HCl. An equal volume of hexane was added and mixed. The solvent was then passed through anhydrous sodium sulphate and subsequently removed by rotary evaporation.

3.2.4.2 Preparation of quercetin derivatives

Preparation of quercetin derivatives was conducted according to Zhong and Shahidi (2011). Eicosapentaenoyl chloride and docosahexaenoyl chloride was prepared with thionyl chloride as well as EPA and DHA. Quercetin (in ethyl acetate) was stirred with pyridine at a molar ratio of 1:1. Then acyl chlorides (propionyl chloride, butyryl chloride, caproyl chloride, capryloyl chloride, capryl chloride, lauroyl chloride, myristoyl chloride, palmitoyl chloride, stearoyl chloride, oleoyl chloride, eicosapentaenoyl chloride, and docosahexaenoyl chloride) were added dropwise under a blanket of nitrogen at 50 °C while stirring constantly. After the reaction was completed, the mixture was cooled down to room temperature and washed 3 times with distilled water (60 °C). The ethyl acetate layer was subsequently passed through a layer of anhydrous sodium sulphate. The product mixture was obtained by the removal of the solvent.

3.2.4.3 Identification of quercetin derivatives

The chemical structures of quercetin derivatives were determined by high-performance liquid chromatography-electrospray ionization-time of flight-mass spectrometry (HPLC-ESI-TOF-MS) using an Agilent 1260 HPLC unit (Agilent Technologies, Palo Alto, CA, USA). A C18 column (4.6 mm × 250 mm × 5 µm; Sigma-Aldrich, Oakville, ON, Canada)

was used. The mobile phase was methanol/ 5% acetonitrile in water at 80:20-90:10 (v/v) and the runtime varied from 0 to 100 min at 1 mL/min. The compounds were detected at 370 nm. The compounds were then dissolved in perdeuterated dimethyl sulphoxide (DMSO-d₆) containing tetramethylsilane (TMS) in order to record their proton nuclear magnetic resonance (¹H NMR, Bruker Avance 300 MHz; Bruker Biospin Co., Billerica, MA, USA) for structure identification. The results were compared with the chemical shifts of quercetin and literature values.

3.2.4.4 Separation of quercetin and its derivatives

Intact quercetin was removed from different mixtures of quercetin and derivatives using column chromatography on silica gel. The gradients of hexane / ethyl acetate / formic acid (90: 10: 2, 80: 20: 2, 70: 30: 2, 60: 40: 2, v/v/v) were employed and each collected fraction was monitored by TLC (hexane / ethyl acetate / formic acid, 3: 3: 0.12, v/v/v).

3.2.4.5 Determination of lipophilicity

The lipophilicity of quercetin derivatives was theoretically calculated by using ALOGPS 2.1. established by Tetko et al. (2005). The simplified molecular input line entry (SMILE) system of quercetin derivatives was obtained from ChemDraw Standard 16.0.

3.2.4.6 ABTS radical cation scavenging assay

The procedure of ABTS radical cation scavenging assay followed was described by Miller et al. (1993) as described by Ambigaipalan et al. (2015) with slight modifications. Solutions of ABTS (2.5 mM) and AAPH (2.5 mM) in a 0.1 M phosphate buffer containing 0.15 M sodium chloride (pH 7.4) were mixed at a ratio of 1:1 (v/v). The

mixture (ABTS radical cation) was then heated for 20 min at 60 °C and subsequently stored in the dark at room temperature. Quercetin and its derivatives (0.5 mg/mL) were dissolved in ethanol. The compounds and a blank (40 µL) were vortexed with the ABTS radical cation solution (1.96 mL). Six minutes later, the samples were measured at 734 nm. The ABTS radical cation scavenging ability of quercetin and its derivatives was expressed as mmoles of trolox equivalents per mg of sample.

3.2.5 Antioxidant activity of quercetin derivatives in food and biological model systems

3.2.5.1 Preparation of quercetin derivatives

Quercetin derivatives were prepared as described in 3.2.4.1 and 3.2.4.2. Briefly, quercetin was esterified with 12 different fatty acids, propionic acid (C3:0), butyric acid (C4:0), caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), eicosapentaenoic acid (C20:5, EPA), and docosahexaenoic acid (C22:6, DHA), after the fatty acids were converted to their corresponding acyl chlorides. The chemical structures of quercetin derivatives were determined by HPLC-MS and ¹HNMR. Intact quercetin from the crude product mixture was removed and used to evaluate their antioxidant potential in selected food and biological model systems.

3.2.5.2 DPPH radical scavenging assay

The DPPH scavenging capacity of quercetin and its derivatives was determined according to Madhujith and Shahidi (2006) with slight modifications. The samples (40 μ L, 0.5 mg/mL, in ethanol) were mixed with 60 μ L of ethanol and 0.9 mL of DPPH (0.2 mM, in ethanol), kept in the dark for 30 min and monitored using electron paramagnetic resonance (EPR). The DPPH scavenging capacities of quercetin and its derivatives were expressed as mmoles of trolox equivalents (TE) per mg of sample.

3.2.5.3 Hydroxyl radical scavenging assay

The hydroxyl radical scavenging capacity of quercetin and its derivatives was evaluated according to Chandrasekara and Shahidi (2011) with modifications. The samples in ethanol were diluted with 75 mM phosphate buffer (pH 7.2) and added with H₂O₂ (200 μ L, 10 mM), DMPO (400 μ L, 17.6 mM), and FeSO₄ (200 μ L, 10 mM). After 3 min, the sample was recorded using EPR spectrometer. The hydroxyl radical scavenging activity of quercetin and its derivatives was expressed as micromoles of catechin equivalents per milligram of sample.

3.2.5.4 Reducing power

The antioxidant activities of quercetin and its derivatives were determined according to their reducing power according to Zhong et al. (2012) with slight modification. The sample (1 mL, 0.5 mg/mL in 100% ethanol) was added 0.2 M phosphate buffer solution (2.5 mL, pH 6.6) and 1% potassium ferricyanide solution (2.5 mL), then incubated at 50 °C for 20 min. Subsequently, 10% trichloroacetic acid (2.5 mL) was added. An aliquot

of the mixture (2.5 mL) was transferred to a tube and mixed with distilled water (2.5 mL) and 0.1% ferric chloride solution (0.5 mL). The absorbance of the mixture was then read at 700 nm. Reducing power was expressed as μ moles of ascorbic acid equivalents per mg of quercetin and its derivatives.

3.2.5.5 Antioxidant activity of quercetin derivatives in oil-in-water emulsion (β -carotene bleaching assay)

An oil-in-water emulsion system was used to determine antioxidant activity of quercetin and its derivatives according to Zhong and Shahidi (2012). Linoleic acid (40 mg), Tween 40 (400 mg), and an aliquot (1.2 mL) of β -carotene in chloroform (10 mg/10 mL) were transferred to a flask. A blank without β -carotene was also prepared. The flask was under a stream of nitrogen in order to remove chloroform then oxygenated distilled water (100 mL) was added and stirred vigorously. Aliquots (4.5 mL) were added to the samples in ethanol (0.5 mL, 0.25 mg/mL) and the control. Samples without β -carotene were also prepared. The reading at 470 nm was monitored immediately and subsequently the tubes were placed in a shaking water bath at 50 °C. The measurements were conducted every 15 min over 105 min period. The antioxidant activity of quercetin and its derivatives was calculated as follows:

$$\text{Antioxidant activity (\%)} = [1 - (S_0 - S_t) / (C_0 - C_t)] \times 100$$

where S_0 and S_t are corrected values for the sample measured at zero time and after incubation, respectively, while C_0 and C_t are corrected values of the control at zero time and after incubation, respectively.

3.2.5.6 Antioxidant activity of quercetin derivatives in bulk oil

Stripped corn oil was prepared according to a simplified stripping method. The stripped oil (1 g) was weighed into test tube. Quercetin and its derivatives (12.5 µg/mL, 100 µL) in ethanol were added and stored in a forced air oven (Precision Scientific Co., Chicago, IL, USA) at 60 ± 0.5 °C. The samples were taken on day 0, 2, 4, 6, and 8 for analyses. Conjugated dienes and *p*-anisidine values were monitored according to the AOAC (Conjugated dienes, 1980; *p*-anisidine values, 1990) methods.

3.2.5.7 Inhibition of copper-induced low-density lipoprotein (LDL) oxidation

The inhibition of LDL oxidation induced by copper was determined following the method described by Chandrasekara and Shahidi (2012). Human LDL obtained from Sigma was dialyzed in 10 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl using a dialysis tube (molecular weight cutoff of 12–14 kDa; Fisher Scientific, Nepean, ON, Canada) at 4 °C under a nitrogen atmosphere in the dark for 12 h. The dialyzed LDL was then diluted to 0.04 mg LDL/ mL. An aliquot of diluted LDL (0.8 mL) was added with the sample (5 µg/mL, 100 µL). The blanks (without LDL and CuSO₄) were run for each sample for background correction. The mixture and cupric sulphate (CuSO₄) were then pre-incubated at 37 °C for 15 min. The oxidation was initiated by the addition of 100 µL of CuSO₄ (50 µM, 100 µL) and formation of conjugated dienes (CD) was measured immediately at 234 nm for 0 h. The samples were then kept at 37 °C for 12 h and the absorbance read again at 234 nm.

3.2.5.8 Inhibition of hydroxyl radical-induced DNA scission

Inhibitory activity of quercetin and its derivatives against DNA scission induced by

hydroxyl radical was determined according to Ambigaipalan and Shahidi (2015) with modification. Supercoiled pBR 322 DNA was diluted to 50 µg/mL using 10 mM phosphate buffer (pH 7.4). The sample (0.5 mg/mL) dissolved in ethanol was also diluted 200 times with phosphate buffer. Sample (2 µL), phosphate buffer (2 µL), DNA (2 µL), 0.5 mM FeSO₄ (2 µL), and 1 mM H₂O₂ (2 µL) were mixed and incubated at 37 °C for 1 h. DNA with radical (control) and DNA only (blank) were also prepared. During incubation, 50 mL of 0.7 % agarose gel in Tris–acetic acid– ethylenediaminetetraacetic acid (TAE) buffer (40 mM, pH 8.5) with SYBR safe gel stain (5 µL) was prepared and placed in a horizontal mini gel electrophoresis system (Owl Separation Systems Inc., Portsmouth, NH, USA) filled with TAE buffer. After incubation, loading dye (1 µL, mixture of 0.25% bromophenol blue, 0.25% xylene cyanol, and 50% glycerol) was added. The sample prepared was loaded onto the agarose gel, which was then subjected to electrophoresis at 80 V for 2 h using with a 300 V power supply (VWR International Inc., West Chester, PA, USA). The visualization of DNA bands was achieved by Alpha-Imager gel documentation system (Cell Biosciences, Santa Clara, CA, USA) and the data processing was conducted by the Chemi-Imager 4400 software (Cell Biosciences). The inhibition of supercoiled DNA strand oxidation (%) was calculated according to the following equation:

$$\text{Inhibition (\%)} = (\text{DNA}_{\text{sample}} / \text{DNA}_{\text{blank}}) * 100$$

3.3 Statistical analysis

Tukey's honest significant difference (HSD) test was performed with one way analysis of variance (ANOVA) to calculate the significance of differences using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA) for Chapters 4, 5, 7, 8 ($P < 0.05$). For Chapter 6, t-test was employed ($P < 0.05$). All determinations were repeated three times using the same combined starting materials and mean values and standard deviations were reported. Values with different letters were significantly different at $P < 0.05$ as performed by ANOVA or t-test.

RESULTS AND DISCUSSIONS

CHAPTER 4

Lipophilisation of resveratrol and effects on antioxidant activity

This chapter has mainly been published in *J. Agric. Food Chem.* (2017), 65, 8617-8625.

4.1 Introduction

Resveratrol, as a stilbenoid, has a C₆-C₂-C₆ structure with three hydroxyl groups. It serves as a UV-protectant and acts as a phytoalexin in plants (Vermerris and Nicholson, 2008). Resveratrol is speculated to play a role in human health due to its biomedical aspects due to its potential ability to prevent oxidation, inflammation, cardiovascular disease, and cancer, which were observed *in vitro* (Jang et al., 1997; Wang et al., 2002; Donnelly et al., 2004). However, its application to biological systems is a real challenge because of its fast metabolism in the body (Walle et al., 2004). Moreover, the hydrophilicity of phenolic compounds restricts their application in lipophilic systems, therefore resveratrol may exhibit reduced antioxidant activity in oily foods, drugs, and cosmetics (Zhong and Shahidi, 2011; Laguerre et al., 2013). Thus, structure modification was carried out to increase lipophilicity of resveratrol using different fatty acids with varying chain lengths and degrees of unsaturation (Baur and Sinclair, 2006; Torres et al., 2010; Zhong and Shahidi, 2011).

Polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA, C₂₀:5n-3) and docosahexaenoic acid (DHA, C₂₂:6n-3), have their own reported health benefits, such as anticancer and cardioprotective effects (Tapiero et al., 2002). Therefore, it is of interest to examine structurally-modified resveratrol with PUFA, since they may display an additive,

synergistic or antagonistic effect when present in the same molecule (Zhong and Shahidi, 2011; Zhong et al., 2012; Zhong and Shahidi, 2012). Moreover, short-chain fatty acids, such as propionic acid (C3:0) and butyric acid (C4:0), may inhibit cholesterol synthesis and prevent colon cancer (Wong et al., 2006). Medium-chain fatty acids such as caprylic acid (C8:0), capric acid (C10:0) and lauric acid (C12:0), are also of interest because they can directly serve as an energy source (Bach and Babayan, 1982). Saturated fatty acids, such as myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0), are most stable in terms of oxidative processes. Thus, esterification of resveratrol with such fatty acids may increase both the lipophilicity and stability of the final products. By using different fatty acids, one can explore which degree of lipophilicity provides the optimum condition for best effects in test systems, both *in vitro* and *in vivo*.

In this work, resveratrol was structurally modified via esterification in order to improve its application in food and biological systems. Lipophilised resveratrols were obtained by acylation of resveratrol with C3:0-C22:6 fatty acyl chlorides, namely propionyl chloride (C3:0) to docosahexaenoyl chloride (DHA, C22:6n-3).

4.2 Preparation of resveratrol derivatives

There have been several attempts to improve the biological effects and performance of resveratrol by modifying its structure. Chao et al. (2010) methylated resveratrol to examine possible enhancement of its neuroprotective effects. Biasutto et al. (2008) used membrane-permeable lipophilic triphenylphosphonium cation to improve pathophysiological relevance of resveratrol. In this study, fatty acyl chlorides with varying chain length and degree of unsaturation (C3:0 to C22:6), were employed to

expand the application of resveratrol in food and non-food commodities. To the best of our knowledge, this is the first work incorporating short-, medium-, and long-chain fatty acids, especially the omega-3 PUFA into the resveratrol molecule.

To prepare resveratryl docosahexaenoate (RDHA), as an example, DHA was esterified with resveratrol, and the esterification was monitored using TLC. Following esterification, two monoesters, two diesters, and one triester were produced. The crude products of RDHA were identified using HPLC-MS and the esterification yield was 37.7%. On the other hand, the yield of RC3:0 was 74%. The structural characteristic of DHA (long carbon chain and nonlinear) may be responsible for its low incorporation. Zhong and Shahidi (2011) reported that the incorporation yields of epigallocatechin gallate (EGCG) with stearic acid, EPA and DHA was 56.9, 42.7 and 30.7%, respectively.

4.3 Identification of resveratrol derivatives

For the HPLC-MS ion source, atmospheric-pressure chemical ionization (APCI) was employed in this study, since APCI can be applied to small and low polarity molecules (Herderich et al., 1997). Wang et al. (2002) reported that resveratrol might be ionized in both positive and negative modes. In this study, only the positive mode was employed. The crude product of RC3:0 ester was subjected to HPLC-MS with methanol/ 5% acetonitrile in water (90:10, v/v) as the mobile phase. Intact resveratrol, monoester, diester, and triester were detected at m/z 229.1, 285.1, 341.1, and 397.3, respectively (spectra not shown). However, using TLC plates, the esters appeared as two monoesters, one diester, and one triester components. Although the HPLC chromatogram showed only a single peak for RC3:0 monoesters at 4.064 min, two monoesters were expected in

HPLC-MS (Figure 4.1). This might be due to the high proportion of methanol in the mobile phase, which made it difficult to stay in the column. Thus, the 5% acetonitrile in the water portion of the mobile phase of the HPLC-MS solvent was increased to 60:40 (methanol/ 5% acetonitrile in water) to obtain better separation. Although RC3:0 monoesters were not fully separated, two different retention times were noted at 8.958 and 9.544 min with the same m/z at 285.1, which implies the presence of two monoesters of RC3 (Figure 4.1 and Figure 4.2). Interestingly, the diester appeared as only a single spot on the TLC; however, it was resolved into two spots with retention times at 26.640 and 31.374 min, respectively (Figure 4.1 and Figure 4.2) by the HPLC-MS. RC3:0 triester was not detected by HPLC-MS (mobile phase 60:40, 70 min runtime), although it was detected with the mobile phase of 90:10 (v/v) and was visualized on the TLC plate under UV light (254 nm). This might be related to its increased lipophilicity of the molecule.

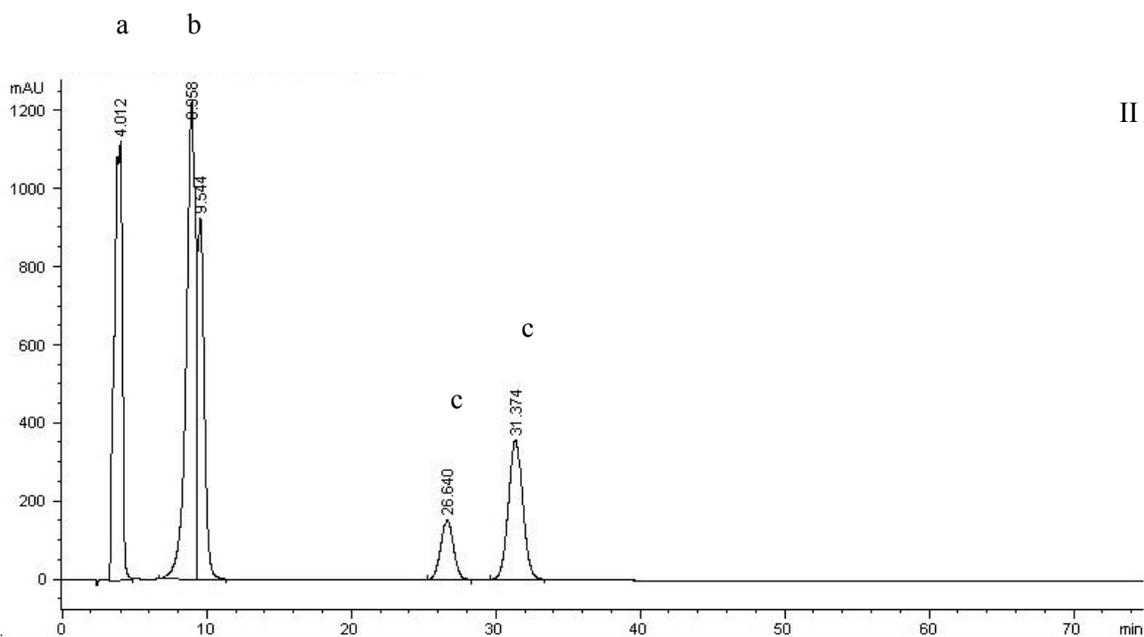
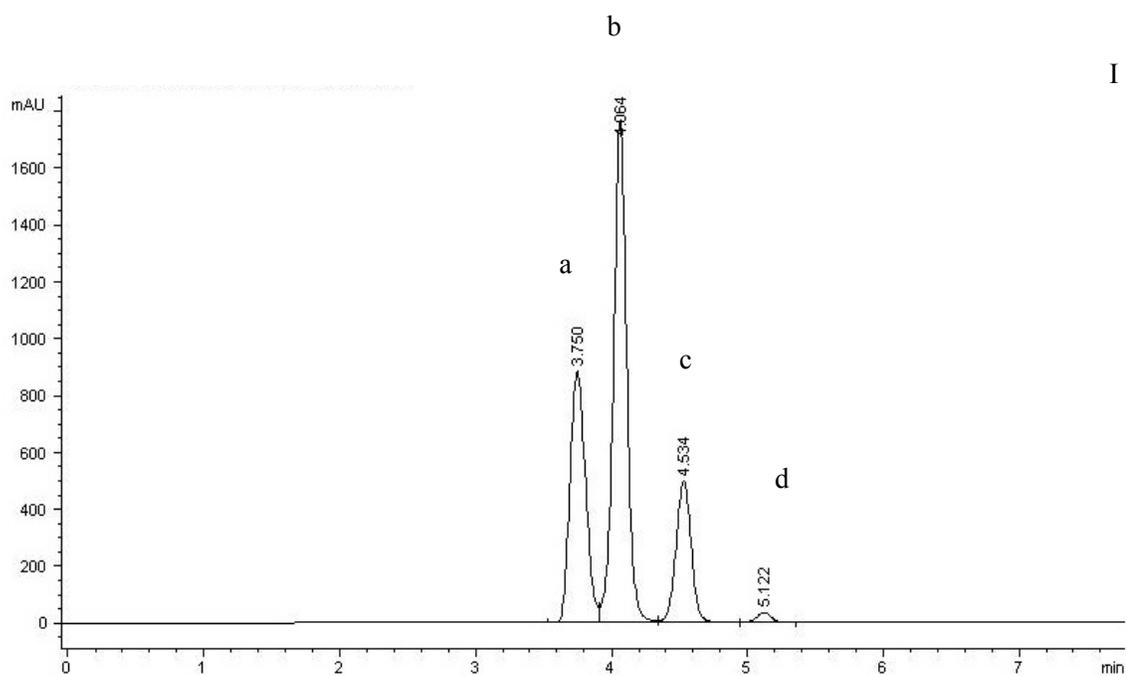


Figure 4.1. HPLC chromatogram of RC3:0 esters with mobile phase methanol/ 5% acetonitrile in water 90:10 (I) and 60:40 (II), respectively (a, resveratrol; b, RC3:0 monoesters; c, RC3:0 diesters; and d, RC3:0 triester).

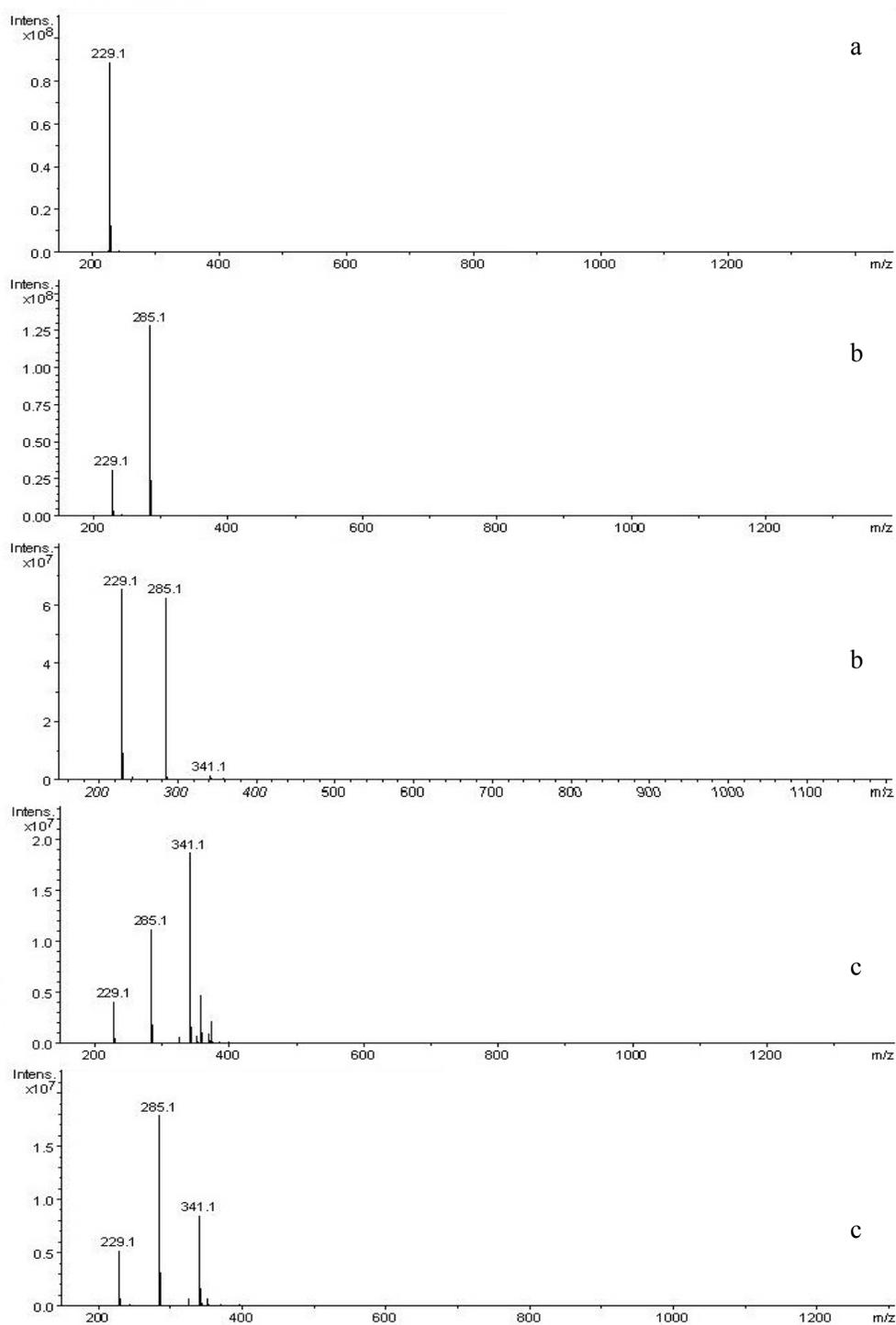


Figure 4.2. Mass spectra of RC3:0 esters (a, resveratrol; b, RC3:0 monoesters; and c, RC3:0 diesters).

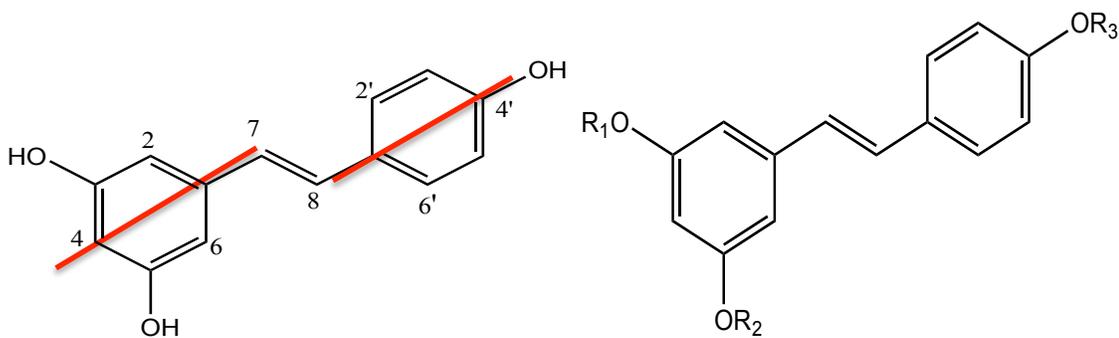
In addition to the HPLC-MS analysis, it was necessary to investigate the location of esterification due to the presence of three hydroxyl groups in the resveratrol molecule. Meanwhile, the esterification positions of the phenolic compounds with different fatty acyl chlorides remained the same as it was also observed for EGCG (Zhong and Shahidi, 2011). Therefore, RDHA, with the most complicated structure among the synthesised fatty acid esters, was selected for further structure elucidation. The crude RDHA product was separated by flash column chromatography using silica gel as the stationary phase and gradient elution with hexane, ethyl acetate, and formic acid as the mobile phase; each fraction was monitored using TLC (hexane/ ethyl acetate/formic acid at 3:3:0.12, v/v/v). The formulas of the esterified resveratrol were confirmed by different types of NMR (¹H-NMR, ¹³C-NMR, COSY, HSQC, NOESY, and HMBC) spectroscopy after being separated by flash column chromatography. The position of esterification was determined by comparison with the literature data and its parent resveratrol (Jayatilake et al., 1993; Mannila et al., 1993; Lancake and Pryce, 1977; Mattivi and Korhammer, 1995). The H-2 and H-6, H-2' and H-6', and H-3' and H-5' of the parent resveratrol had the same chemical shifts at δ 6.38, δ 7.39, and δ 6.75 ppm, respectively (Table 4.1). This means they are chemically equivalent as resveratrol is a symmetric molecule (Torres et al., 2010; Figure 4.3).

R-4'-O-DHA was easily identified as the position of monoesterification (Figure 4.3 and Table 4.1). First of all, H-2 and H-6 still had the same chemical shift at δ 6.43 ppm, which means it remained a symmetric molecule. Moreover, large downfield shifts were observed at H-3' and H-5' ($\Delta\delta= 0.32$ ppm) and H-2', H-6' ($\Delta\delta= 0.22$ ppm). Because of the

distance from the acylation site, downfield shifts of H-2' and H-6' ($\Delta\delta= 0.22$ ppm) were lower than those for H-3' and H-5' ($\Delta\delta= 0.32$ ppm). In addition, the 4'-OH peak of resveratrol (δ 9.54 ppm) disappeared in the spectrum.

Table 4.1. ^1H chemical shifts (δ in ppm) of resveratrol (R), R-3 and R-4'-O-DHA (DHA signals omitted for clarity)

^1H position	resveratrol	R-4'-O-DHA	R-3-O-DHA
2	6.38	6.43	6.79
4	6.11	6.16	6.36
6	6.38	6.43	6.72
7	6.81	overlapping	6.90
8	6.93	overlapping	7.05
2'	7.39	7.61	7.40
3'	6.75	7.07	6.76
5'	6.75	7.07	6.76
6'	7.39	7.61	7.40
3-OH	9.19	9.25	
5-OH	9.19	9.25	9.60
4'-OH	9.54		9.7



Resveratrol: $R_1=R_2=R_3=H$

Monoesters: $R_1=X, R_2=R_3=H$ and $R_1=R_2=H, R_3=X$

Diesters: $R_1=R_2=X, R_3=H$ and $R_1=R_3=X, R_2=H$

Triester: $R_1=R_2=R_3=X$

X: C_3H_6O (C3:0)

C_4H_8O (C4:0)

$C_6H_{12}O$ (C6:0)

$C_8H_{16}O$ (C8:0)

$C_{10}H_{20}O$ (C10:0)

$C_{12}H_{24}O$ (C12:0)

$C_{14}H_{28}O$ (C14:0)

$C_{16}H_{32}O$ (C16:0)

$C_{18}H_{36}O$ (C18:0)

$C_{18}H_{34}O$ (C18:1)

$C_{20}H_{30}O$ (C20:5)

$C_{22}H_{32}O$ (C22:6)

Figure 4.3. Structures of resveratrol and its esters. Red lines represent the symmetry in resveratrol itself.

For the R-3 or 5-*O*-DHA, there were several evidences indicating that either the 3-OH or 5-OH group was esterified with the DHA (Figure 4.3 and Table 4.1). First, resveratrol originally showed one chemical shift for H-2 and H-6 because of the symmetry, however after esterification, the peak of H-2 and H-6 was split into two chemical shifts (δ 6.79 and δ 6.72 ppm). This means the compound was no longer symmetric due to mono-esterification. In addition, large downfield shifts were observed at H-2 and H-6 ($\Delta\delta=0.34-0.41$ ppm). In addition, there were two hydroxyl group peaks at δ 9.60 and 9.70 ppm in the spectrum. Integration of the peak at δ 9.60 was reduced to one, which means either 3-OH or 5-OH was esterified. The 3-OH and 5-OH of resveratrol are chemically equivalent, hence both resveratrol-3-DHA and resveratrol-5-DHA are possibly the same. According to Lee et al. (2003), the specific substituted position can be determined by HMBC. In this study, ^{13}C -NMR, COSY, HSQC, NOESY and HMBC were employed but none of them gave any information about the substitution at either H-3 or H-5. This could be caused by the difference between resveratrol-3-*O*- β -D-glucopyranoside and R-3-*O*-DHA. It could be determined by showing correlations between H-1" of glucopyranose and C-3 of R-3-*O*- β -D-glucopyranoside, however no hydrogen is attached at C-1 of DHA in R-3-*O*-DHA. Theoretically, HMBC detects correlations between protons and carbons within 2~3 bonds (Richards and Hollerton, 2011).

The diesters of resveratrol could be confirmed as R-3,4'-*O*-DHA and R-3,5-*O*-DHA without NMR assignment due to their symmetric nature. The monoesters and the diesters were subsequently used in antioxidant activity studies.

4.4 Lipophilicity of resveratrol derivatives

There are many relevant reported examples that increased lipophilicity of antioxidants leads not only to their better bioactivity but also improved performance in food systems. For example, ascorbic acid is one of the most powerful antioxidants, although it can hardly penetrate the phospholipid bilayers of membranes. However, ascorbyl palmitate, a lipophilic form of vitamin C, can be considered as a carrier of ascorbate into the tissues (Pokorski et al., 2003). Ascorbyl palmitate and ascorbyl stearate have already been used as antioxidant food additives. Furthermore, Zhong and Shahidi (2012) demonstrated that structurally modified EGCG showed not only excellent antioxidant activity but better performance in biological model systems. Thus, we also expected additional potential for use as nutraceuticals or even as pharmaceuticals for resveratrol esters.

In this study, fatty acyl chlorides (C3:0-C22:6) were used to prepare esters to enhance the lipophilicity of resveratrol. This investigation may provide an ideal degree of lipophilicity for the best outcome of the test system. According to Medina et al. (2010), the lipophilicity of resveratrol was 1.85 ± 0.46 . In addition, our preliminary data showed that lipophilicity of resveratrol was 1.84 ± 0.12 , as determined by octanol-water partition coefficient measurement using a shake flask method. The shake flask method is the most classical method to measure partition coefficient (P). However, the lipophilicity of resveratrol derivatives was calculated by using a computer program in this work due to the difficulty of measuring the large value of partition coefficients by shake flask method (Ayouni et al., 2008; Giaginis and Tsantili-Kakoulidou, 2008; Finizio et al., 1997). Therefore, the lipophilicity calculator (ALOGPS 2.1.) was employed for reporting the lipophilicity of resveratrol derivatives. As expected, resveratrol derivatives showed higher

lipophilicity than resveratrol (Table 4.2) and their corresponding values increased according to their chain length extension. Tan et al. (2012) reported that the retention time of phytosteryl docosahexaneates was increased compared to the phytosterol itself. However, lipophilicity decreased as the degree of unsaturation of the fatty acids increased. A similar tendency was observed on TLC. This might be due to the property of the double bond with pi bond. Alkanes are made up of single-bonded carbon and hydrogen atoms and are nonpolar because carbon and hydrogen atoms have similar electronegativities. Alkenes also consist of carbon and hydrogen atoms, but they are slightly more polar than alkanes due to the high density of electrons, with a partial negative charge (Bruice, 2011).

Table 4.2. Lipophilicity of resveratrol and its monoesters^a.

Compounds	Lipophilicity (Log P)
Resveratrol(R)	2.57
RC3:0	4.28
RC4:0	4.55
RC6:0	5.31
RC8:0	5.85
RC10:0	6.45
RC12:0	7.28
RC14:0	8.06
RC16:0	8.72
RC18:0	9.30
RC18:1	9.07
REPA	8.47
RDHA	8.59
DPPH radical	4.35
ABTS radical cation	0.43

^aThe lipophilicity of resveratrol derivatives was calculated using ALOGPS 2.1. The structures of resveratrol derivatives in simplified molecular input line entry system (SMILE) were drawn by ChemDraw Std 14.0.

4.5 DPPH radical scavenging assay

The DPPH radical is a stable organic radical and is commercially available so that there are no preparation steps involved for its generation. The DPPH radical scavenging assay is frequently used as a first approach for evaluating antioxidant activity (Shahidi and Zhong, 2015). Antioxidants can neutralize the DPPH radical via electron and/or hydrogen atom donation (Prior et al., 2005). The purple colour of DPPH gets lighter (from purple to yellow) upon radical neutralization. The efficacy of antioxidant capacity can be measured by using UV-Vis spectrometry at 517 nm or electron paramagnetic resonance (EPR) spectroscopy.

In this study, the resveratrol derivatives showed decreased DPPH scavenging capacity compared to resveratrol (Figure 4.4). It is possible that losing one or more hydroxyl group plays a major role in decreasing the antioxidant activity of resveratrol (Medina et al., 2010; Torres et al., 2010). Chao et al. (2010) reported that antioxidant activity of methylated derivatives of resveratrol was in the order of R > R-3-C1 (monoester) > R-4'-C1 (monoester) > R-3,4'-C1 (diester); the triester (R-3,5,4'-C1) lacked any antioxidant activity because it does not carry any free hydroxyl groups. In addition, RC10:0 showed the lowest antioxidant activity, whereas RC18:1 showed the highest antioxidant activity among all resveratrol derivatives tested. The short-chain and medium-chain fatty acid substituents (RC3:0 to RC12:0) exhibited scavenging of around 55~61% compared to the antioxidant activity of the parent resveratrol molecule, but this was increased up to 95% for RC12:0 to RC18:1. DPPH is a lipophilic compound which may have better affinity to the lipophilic compounds tested. Therefore, resveratrol with long-chain fatty acids, which

have more lipophilicity than resveratrol with short-chain fatty acids, might have better accessibility to DPPH and this could lead to a better antioxidant activity. Zhong and Shahidi (2011) reported that EGCG esters showed better DPPH radical scavenging activity than EGCG itself and suggested that DPPH radical is lipophilic so that the substituted compounds have more accessibility/affinity to it than the parent compound. Moreover, the antioxidant activities of resveratrol substituted with C18:0, EPA and DHA showed a similar pattern (RC18:0 > RDHA > REPA) with the results for EGCG esters (EGCG-C18:0 > EGCG-DHA > EGCG-EPA). However, some studies have reported that the antioxidant activity increases with the increase of alkyl chain length to a maximum point and then begin to reverse with further chain extension. The maximum point is generally reached for medium chain fatty acids. This phenomenon is known as a nonlinear theory, or cutoff effect. Laguerre et al. (2009) observed collapse in antioxidant activity for longer chain esters than dodecyl ester of chlorogenic acid. In addition, Panya et al. (2012) observed a drastic decrease after dodecyl chain in DPPH radical scavenging activity using rosmarinic acid and its alkyl esters.

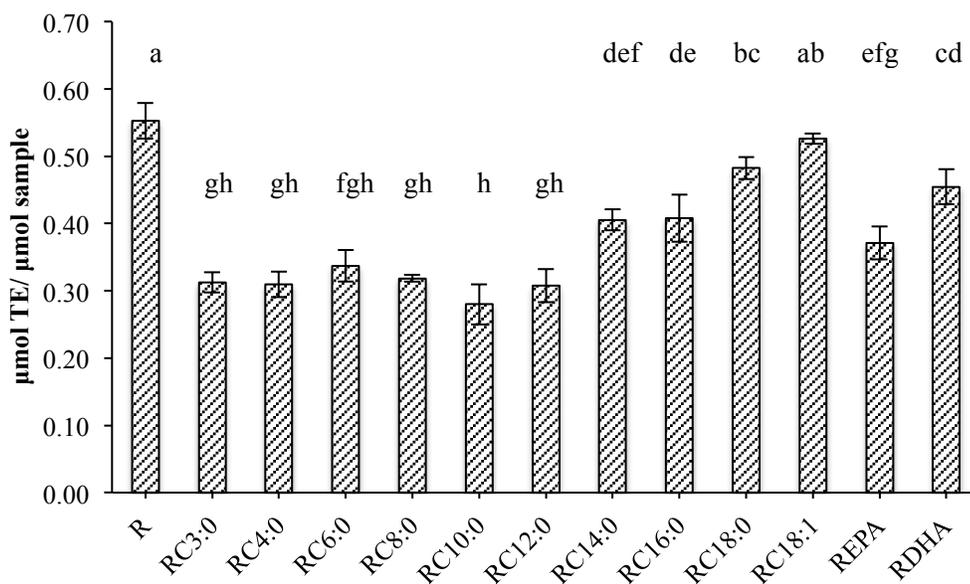


Figure 4.4. DPPH radical scavenging capacity in micromoles of trolox equivalents (TE) per micromole of resveratrol (R) or its derivatives. All determinations were repeated three times using the same combined starting materials and mean values and standard deviations reported. Bars with different letters are significantly different at $P < 0.05$ performed by Tukey's HSD test. Bars with same letters such as "a" and "ab" are not significantly different.

4.6 ABTS radical cation scavenging assay

The ABTS radical cation scavenging assay was developed by Miller et al. (1993) and then improved by Re et al. (1999) and van den Berg et al. (1999). The ABTS radical cation was prepared by heating with the azo compound AAPH and measuring the

absorbance of the blue-green colour can be read at 734 nm. The ABTS radical cation scavenging assay is based on single electron transfer (SET) as well as hydrogen atom transfer (HAT) mechanisms. The extent of decreasing absorbance can be monitored either every few minutes or at an endpoint (Chandrasekara and Shahidi, 2010; Ambigaipalan et al., 2015; Shahidi and Zhong, 2015).

In this study, the ABTS radical cation scavenging effect of resveratrol derivatives was lower than that of resveratrol, similar to that observed for DPPH radical scavenging activity (Figure 4.5). As mentioned earlier, substitution of hydroxyl group(s) appears to negatively affect the antioxidant activity of resveratrol derivatives. The compound RC4:0 showed the highest ABTS radical cation scavenging effect among all resveratrol derivatives tested by exhibiting 83.8% scavenging compared to that of resveratrol. Meanwhile, RC8:0 and RC16:0 showed the lowest ABTS radical cation scavenging effect among all resveratrol derivatives tested and exhibited scavenging of 34.8 and 33.6%, respectively, compared to the scavenging activity of resveratrol. In the DPPH radical scavenging assay, resveratrol with long-chain fatty acids showed better radical scavenging activity than with short-chain fatty acids. On the other hand, resveratrol with short-chain fatty acids showed better antioxidant capacity than other derivatives in the ABTS radical cation scavenging assay. The result of the ABTS radical cation scavenging assay was similar to that reported by Torres et al. (2010). These author reported that the ABTS radical cation scavenging effect of resveratrol (R), its acetate ester (RC2) and resveratrol esterified with stearic acid (RC18:0) was in the order of $R > RC2 > RC18:0$. This finding might also be due to the increased lipophilicity of the resveratrol derivatives.

According to lipophilicity calculations conducted in this work, the lipophilicity of DPPH radical was 4.35, whereas the lipophilicity of ABTS radical cation was 0.43 (Table 4.2). Therefore, resveratrol with short-chain fatty acids might have a better affinity towards the ABTS radical cation than the other ester derivatives with long-chain fatty acids. The ABTS radical cation scavenging effects of resveratrol derivatives with short- (C3:0 to C6:0), medium- (C8:0 to C12:0), and long- (C14:0 to C18:0) chain fatty acids decreased as their lipophilicity increased. The average ABTS radical cation scavenging effects of resveratrol with short-, medium-, long-, and unsaturated fatty acids (C18:1 to DHA) were 78.4, 45.5, 41.2, and 46.4%, respectively. Reyes-Duarte et al. (2011) studied the ABTS radical cation scavenging effects of ascorbyl esters and reported that ascorbic acid showed a better ABTS radical cation scavenging effect than ascorbyl esters (in the order of ascorbic acid > ascorbyl palmitate > ascorbyl oleate). However, some studies showed an opposite trend (enhanced ABTS radical cation scavenging effects as lipophilicity increased). Wang et al. (2016) studied the ABTS radical cation scavenging effects of EGCG derivatives and reported that EGCG derivatives showed increased ABTS radical cation scavenging effects as alkyl chain length increased (EGCG-C18 > EGCG-C8 > EGCG-C6 > EGCG-C12, EGCG-C2 > EGCG-C3, EGCG). Saito et al. (2004) studied the ABTS radical cation scavenging effects of protocatechuic acid and its alkyl esters. They reported that the esterification reaction increased their ABTS radical cation scavenging effects, whereas ABTS radical cation scavenging effects of protocatechuic alkyl esters decreased as alkyl chain length increased (protocatechuyl methyl ester > protocatechuyl ethyl ester > protocatechuyl buthyl ester > protocatechuyl isopropyl ester > protocatechuyl *tert*-butyl ester > protocatechuic acid). In addition, some studies have

demonstrated that the 4'-OH group of resveratrol is necessary for its antioxidant activity (Caruso et al., 2004; Stivala et al., 2001). Stivala et al. (2001) reported that 4'-OH is required more than 3-OH and 5-OH for antioxidant activity. They suggested that the electronic structure and the formation enthalpy caused by three different radicals losing hydrogen atoms at the 3, 5, and 4'-OH groups in resveratrol might account for this behaviour.

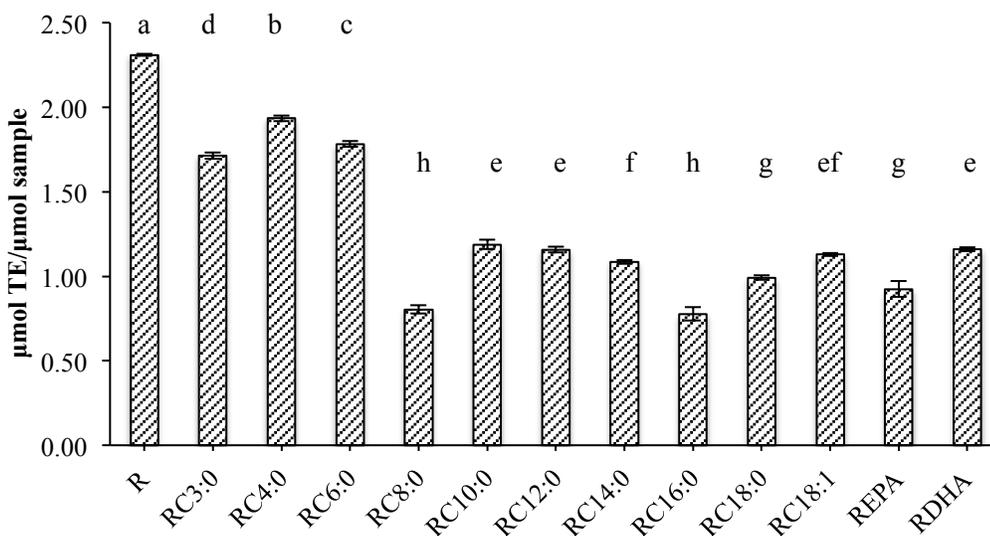


Figure 4.5. ABTS radical cation scavenging assay in micromoles of trolox equivalents (TE) per micromole of resveratrol (R) or its derivatives. All determinations were repeated three times using the same combined starting materials and mean values and standard deviations reported. Bars with different letters are significantly different at $P < 0.05$ performed by Tukey's HSD test. Bars with same letters such as "a" and "ab" are not significantly different.

4.7 Summary

Esterification of resveratrol with 12 different fatty acyl chlorides afforded monoesters as the main products. The monoesters, determined by NMR, were both resveratrol-3-monoester and resveratrol-4'-monoester. Resveratrol was successfully lipophilised via esterification as calculated using ALOGPS 2.1. In the DPPH radical and ABTS radical cation scavenging assays, resveratrol showed the highest antioxidant activity. Among resveratrol derivatives, RC18:0 and RC18:1 showed a higher DPPH radical scavenging activity than the other derivatives tested at 87 and 95%, respectively. On the other hand, RC3:0, RC4:0, and RC6:0 showed higher ABTS radical cation scavenging activity than other derivatives at 74, 84, and 77%, respectively. In this context, it may be of interest to carry out more research using RC3:0, RC4:0, RC18:0, and RC18:1. Moreover, it is hypothesised that resveratrol with PUFA may have synergistic effect in biological systems. Therefore, these molecules may also deserve to be studied further. This suggests that some resveratrol derivatives may have the potential of serving as antioxidants in lipophilic systems.

CHAPTER 5

Antioxidant activity of resveratrol ester derivatives in food and biological model systems

This chapter has been published in *Food Chem.* (2018), 261, 267-273.

5.1 Introduction

Lipid oxidation is a major deteriorative process in food. It is responsible for off-flavour formation, a decrease of nutritional value, and generation of potentially toxic compounds (Frankel, 1980). The most common lipid oxidation process encountered in food is autoxidation in which the free radicals formed lead to further adverse effects once consumed (Choe and Min, 2006). Concerns about lipid oxidation are not limited to food, because the free radicals formed can also attack different cells in the body, thus causing ageing and a myriad of diseases such as cancer, cardiovascular ailments, and immune system deficiencies (Percival, 1998). Therefore, many attempts have been made to improve the oxidative stability of foods by using antioxidants as a most effective means (Shahidi and Ambigaipalan, 2015). Antioxidants are generally phenolic compounds that can interfere with free radical chain reactions by donating a hydrogen atom or an electron to the free radicals (Frankel, 1980; Shahidi and Ambigaipalan, 2015). By retarding oxidation, they can also prevent certain diseases related to oxidative stress such as skin lesions, atherosclerosis, pulmonary dysfunction, cancer, and inflammatory disorders, among others (Percival, 1998).

Resveratrol is a phenolic stilbenoid compound with powerful antioxidant activity. It also has preventive effects on inflammation, cardiovascular disease, and cancer (Jang et al., 1997; Wang et al., 2002; Donnelly et al., 2004). However, some studies have failed to reflect these beneficial effects, possibly due to its high absorption but low bioavailability (Baur and Sinclair, 2006; Bove et al., 2002; Walle et al., 2004). In addition, like some other phenolic compounds, application of resveratrol could be compromised due to its hydrophilicity when used in lipophilic systems (Zhong and Shahidi, 2011). However, this problem could be overcome by structural modification. In fact, there are several studies reporting maximizing antioxidant activities via structure modification, such as those for chlorogenic acid (Laguerre et al., 2011), epigallocatechin gallate (EGCG) (Zhong and Shahidi, 2011), caffeic acid (Alemán, et al., 2015), and rosmarinic acid (Laguerre et al., 2010), among others. Moreover, EGCG esterified with polyunsaturated fatty acids (PUFA), especially docosahexaenoic acid (DHA) provided a novel bioactive product that was a composite of the two bioactive compounds since DHA itself possesses health benefits (Zhong et al., 2012; Zhong and Shahidi, 2012). For this reason, resveratrol was esterified with 12 different acyl chlorides (propionyl chloride, butyryl chloride, caproyl chloride, capryloyl chloride, capryl chloride, lauroyl chloride, myristoyl chloride, palmitoyl chloride, stearoyl chloride, oleoyl chloride, eicosapentaenoyl chloride, and docosahexaenoyl chloride) as described in Chapter 3 (see sections 3.2.1.1 and 3.2.1.2; Oh and Shahidi, 2017).

Vlachogianni et al. (2015) demonstrated that 4'-acetylated resveratrol (R) had a better DPPH radical scavenging activity than its 3-acetylated isomer (R > 4'-acetylated R > 3-

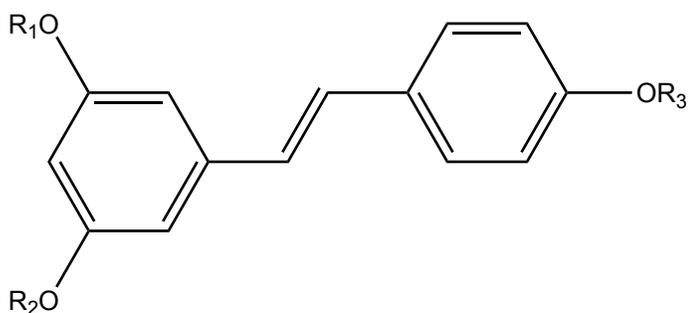
acetylated R > 3,5-diacetylated R). However, in the non-enzymatic linoleic acid peroxidation assay, they reported a better antioxidant activity for 3-acetylated R than that for 4'-acetylated R (3,5-diacetylated R > 3-acetylated R > R > 4'-acetylated R > 3,4'-diacetylated R) isomer. Moreover, they reported that 3,5-diacetylated R exhibited the most powerful effect in the non-enzymatic linoleic acid peroxidation assay even though it had lost two of its hydroxyl groups. The effect of esterification position and degree of esterification is still unknown. Since the resultant esters still have antioxidant activity, it is of interest to see whether the mixture of mono- and diesters show an additive, synergistic, or antagonistic effect. In the case of oral administration of ester compounds, the ester bond might undergo hydrolysis. Biasutto et al. (2007) studied the bioavailability of ester-based quercetin using Madin-Darby Canine Kidney (MDCK)-1, MDCK-2, and human colon adenocarcinoma (Caco-2) cells. They reported that some quercetin esters underwent partial hydrolysis when they passed through a monolayer of MDCK-1, MDCK-2, and Caco-2 cells. Hydrolysis of the ester was dependent on the particular ester examined. Pokorski et al. (2003) compared the amount of ascorbate in the brain of cats when they were untreated (control), treated with ascorbyl acid, or treated ascorbyl palmitate. They could not recover ascorbyl palmitate as is; they found accumulation of ascorbate in the brain of the ascorbyl palmitate-treated cat. According to Pokorski et al. (2003), ascorbyl palmitate-treated cats showed more recovery of ascorbate in their brain tissues compared to ascorbic acid-treated cats. Therefore, it might be hypothesised that resveratrol esters would show a beneficial effect even if they underwent hydrolysis.

In this work, selected food and biological model systems were used to fill the existing knowledge gap in the literature on antioxidant potential of resveratrol derivatives in food

and for potential health promotion and disease risk reduction. Potential synergistic effects of resveratrol with PUFA were also examined in this contribution.

5.2 Antioxidant activity of resveratrol derivatives in oil-in-water emulsion (β -carotene bleaching assay)

A β -carotene bleaching assay was used to evaluate the antioxidant activity of resveratrol and its derivatives (Figure 5.1) in an oil-in-water emulsion. When two immiscible liquids exist together, one liquid can form a dispersion of small droplets in the other liquid, known as emulsion (McClements, 2007). Tween 40 and linoleic acid, used in this assay, are amphiphilic with a hydrophilic head and a lipophilic tail, thus can form small spherical micelles in the water (McClements, 2002). Meanwhile, β -carotene, a lipophilic compound, stays inside the micelle. Heat induces oxidation, and linoleic acid forms hydroperoxides and free radicals as a result of losing a hydrogen atom (Chandrasekara and Shahidi, 2010). Beta-carotene is attacked by those oxidative products, therefore it loses its yellow-orange colour (Ambigaipalan and Shahidi, 2015). The change of colour could be monitored spectrometrically at 470 nm.



Resveratrol: $R_1=R_2=R_3=H$

Monoesters: $R_1=X, R_2=R_3=H$ and $R_1=R_2=H, R_3=X$

Diesters: $R_1=R_2=X, R_3=H$ and $R_1=R_3=X, R_2=H$

X : C_3H_5O (C3:0)

C_4H_7O (C4:0)

$C_6H_{11}O$ (C6:0)

$C_8H_{15}O$ (C8:0)

$C_{10}H_{19}O$ (C10:0)

$C_{12}H_{23}O$ (C12:0)

$C_{14}H_{27}O$ (C14:0)

$C_{16}H_{31}O$ (C16:0)

$C_{18}H_{35}O$ (C18:0)

$C_{18}H_{33}O$ (C18:1)

$C_{20}H_{29}O$ (C20:5, EPA)

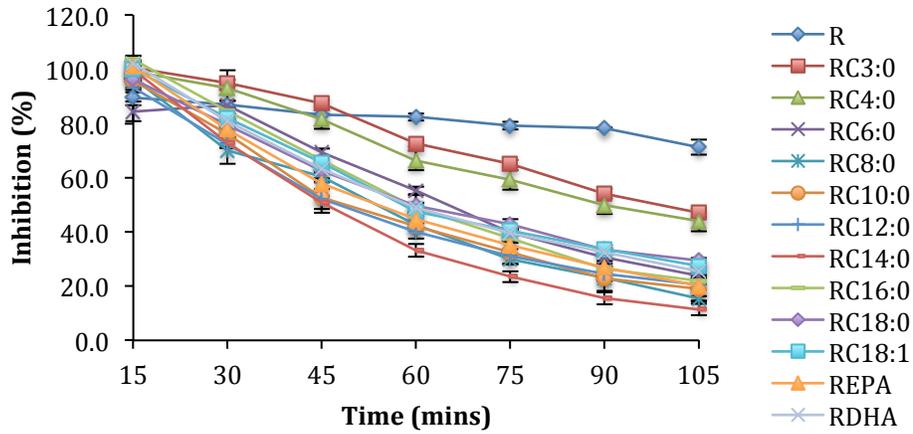
$C_{22}H_{31}O$ (C22:6, DHA)

Figure 5.1. Structures of resveratrol derivatives.

In this study, all test compounds prevented decolouration of β -carotene compared to the control (Figure 5.2). RC3:0 and RC4:0 exhibited a better inhibition of oxidation than resveratrol after 30 min of incubation, but this started to decrease and showed a lower inhibition than resveratrol from 45 to 105 min. While resveratrol had the highest antioxidant activity among all tested compounds from 60 to 105 min, RC14:0 showed the

lowest effect over the same period (Figure 5.2A). Figure 5.2B indicates that resveratrol and its derivatives inhibited the decolouration of β -carotene by 11.4-71.2 % over the 105 min incubation period. Resveratrol had the highest inhibition among all resveratrol-based compounds. Among derivatives, resveratrol with short-chain fatty acids (RC3:0 and RC4:0) showed better antioxidant activities than other derivatives which were tested. The antioxidant activity of resveratrol derivatives in the oil-in-water emulsion was well correlated with ABTS radical cation scavenging activity with a correlation coefficient of 0.84 (Oh and Shahidi, 2017). The antioxidant activity of resveratrol derivatives in the oil-in-water system did not correspond with the polar paradox. According to the polar paradox, nonpolar antioxidants tend to be more effective than polar ones in the oil-in-water emulsion systems. This is because nonpolar antioxidants may stay around the lipid droplet where oxidation occurs (Shahidi and Zhong, 2011). However, several studies have demonstrated that not all antioxidants follow the trend that can be explained by the polar paradox (Torres de Pinedo et al., 2007; Stöckmann et al., 2000). Torres de Pinedo et al. (2007) studied antioxidant activity of phenolic compounds containing alcohols with different alkyl chain lengths such as protocatechuic alcohol (C1), hydroxytyrosol (C2), and dihydrocaffeoyl alcohol (C3). They found an increase in antioxidant activity in bulk oil ($C3 > C2 > C1$), which contradicts the polar paradox theory. Stöckmann et al. (2000) observed different trends of antioxidant activity using various emulsifiers in the oil-in-water system and suggested that the application of the polar paradox hypothesis might need to be narrowed down to the emulsions containing phospholipids as emulsifiers.

(a)



(b)

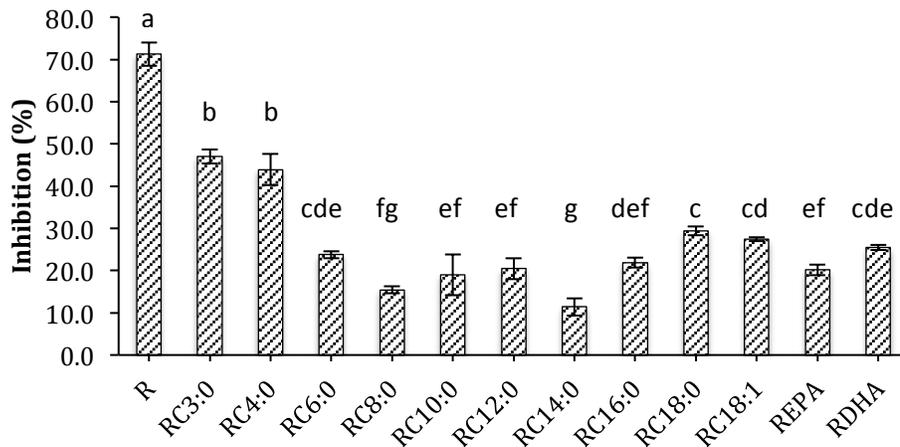


Figure 5.2. Antioxidant activities of resveratrol and its derivatives against β -carotene bleaching over a 105 min period (a) and inhibition against β -carotene bleaching after 105 min incubation (b) by resveratrol and its derivatives. All determinations were repeated three times using same combined starting materials and the mean values and standard deviation values were reported. Bars with different letters mean significantly different at

$P < 0.05$ performed by Tukey's HSD test. Bars with same letters such as "a" and "ab" mean insignificantly different.

5.3 Antioxidant activity in bulk oil

In the food industry, many different techniques such as degumming, refining, bleaching, and deodorization have been used to refine oils by removing a variety of minor compounds that influenced the oxidative stability of oils (McClements and Decker, 2007). For example, the refining process effectively removes FFAs accelerate lipid oxidation of oils, leading to an enhancement of oxidative stability (Choe and Min, 2006). However, purified oils are highly susceptible to oxidation. Thus, one may recover the tocopherols and put them back into oils, since tocopherols can improve the oxidative stability of oils by acting as electron or hydrogen atom donors, therefore retarding lipid oxidation (Choe and Min, 2006). Thus, commercial oils generally contain some tocopherols. In order to use the oils (either bought from the market or extracted in the laboratory) for experimental work, further processing to remove their minor components is needed, and this is known as stripping.

Corn oil used in this study was stripped of its endogenous antioxidants in order to eliminate their influence on oxidative stability of the oil containing the test compounds. Major fatty acids in corn oil are oleic acid (C18:1, 34.1 %) and linoleic acid (C18:2, 47.9 %) (Ferrari et al., 1996). Generally, oxidation is initiated by the abstraction of a hydrogen atom attached to an allylic or a bis-allylic carbon atom (carbon next to the double bond) due to their low dissociation energy (Zhong and Shahidi, 2009). After losing its hydrogen

atom, the alkyl radical formed is stabilised by delocalisation, along with possible formation of trans isomers as well as conjugated dienes (polyunsaturated fatty acids) (Choe and Min, 2006; McClements and Decker, 2007). The conjugated diene content was measured at 233 nm. However, conjugated dienes could dissociate to secondary oxidation products, such as aldehydes, ketones, alcohols and hydrocarbons (Choe and Min, 2006). For secondary oxidation products, the *p*-anisidine assay was employed. The *p*-anisidine can react with aldehydes (2-alkenals and 2,4-alkadienals) and form a yellowish chromophore, which can be measured at 350 nm (Shahidi and Wanasundara, 2002).

Conjugated diene formation of corn oil in this study is shown in Table 5.1. The oils with test compounds exhibited a similar lag phase of 3 days. On day 6, most of the test compounds were able to retard the formation of conjugated dienes significantly except for RC3:0 and RDHA ($p < 0.05$). Although resveratrol derivatives lost their hydroxyl groups due to esterification, some derivatives (RC6:0, RC8:0, RC10:0, RC12:0, RC16:0) showed significantly better antioxidant activity than resveratrol itself ($p < 0.05$). This result also contradicts polar paradox, as polar antioxidants generally are expected to be more efficacious than nonpolar ones in bulk oil systems. In addition, we hypothesised that resveratrol with polyunsaturated fatty acids (PUFA), namely EPA and DHA might produce the same or even a higher level of conjugated dienes than the control because PUFA connected to resveratrol could also be oxidized and form conjugated dienes. However, REPA inhibited the oxidation to nearly the same extent as resveratrol itself (no significant difference). RC3:0 had very little effect on antioxidant activity but the antioxidant activity of other resveratrol derivatives from RC4:0 to RC8:0 increased as the lipophilicity increased, but after reaching a maximum point (RC8:0), a decrease was

noted. This has been observed in several studies; this phenomenon was coined as the cutoff effect, or nonlinear theory (Laguerre et al., 2009; Panya et al., 2011; Laguerre et al., 2015). Laguerre et al. (2009) studied the antioxidant activities of chlorogenic acid and its alkyl esters in emulsion systems in order to examine the effect of chain length on antioxidant activity. They observed a drastic decrease in antioxidant activity after the dodecyl chain (C12:0). Furthermore, Panya et al. (2011) observed a nonlinear phenomenon in DPPH radical scavenging assay using rosmarinic acid and its alkyl esters.

Table 5.1. Conjugated dienes formation of stripped corn oil over a 6-day period in the presence of resveratrol and its derivatives.

Sample	Storage period			
	Day 0	Day 1	Day 3	Day 6
Control	0.21 ± 0.02 e	0.50 ± 0.01 bcd	0.62 ± 0.01 ab	5.46 ± 0.03 a
R	0.22 ± 0.01 e	0.48 ± 0.02 de	0.57 ± 0.00 d	4.42 ± 0.08 ef
RC3:0	0.24 ± 0.00 e	0.48 ± 0.02 cde	0.63 ± 0.00 a	5.44 ± 0.02 a
RC4:0	0.22 ± 0.01 e	0.49 ± 0.01 bcde	0.61 ± 0.00 bc	4.95 ± 0.02 b
RC6:0	0.23 ± 0.00 e	0.49 ± 0.02 bcde	0.55 ± 0.00 de	4.04 ± 0.01 g
RC8:0	0.25 ± 0.03 de	0.48 ± 0.00 de	0.57 ± 0.01 d	3.78 ± 0.01 h
RC10:0	0.29 ± 0.02 cd	0.48 ± 0.01 bcde	0.56 ± 0.01 de	3.99 ± 0.03 g
RC12:0	0.31 ± 0.01 bc	0.46 ± 0.01 e	0.54 ± 0.00 e	4.02 ± 0.08 g
RC14:0	0.35 ± 0.03 ab	0.49 ± 0.02 bcde	0.57 ± 0.01 d	4.57 ± 0.01 cd
RC16:0	0.37 ± 0.01 a	0.50 ± 0.01 abcd	0.56 ± 0.01 de	4.08 ± 0.01 g
RC18:0	0.36 ± 0.01 a	0.51 ± 0.01 abcd	0.54 ± 0.01 e	4.34 ± 0.03 f
RC18:1	0.35 ± 0.02 ab	0.52 ± 0.00 abc	0.56 ± 0.01 de	4.64 ± 0.01 c
REPA	0.37 ± 0.00 a	0.52 ± 0.01 ab	0.55 ± 0.01 de	4.53 ± 0.01 de
RDHA	0.36 ± 0.00 a	0.54 ± 0.01 a	0.59 ± 0.01 c	5.53 ± 0.04 a

All determinations were repeated three times using the same combined starting materials and mean values and standard deviations reported. Values in the same column with

different letters are significantly different at $P < 0.05$ performed by Tukey's HSD test. Columns with same letters such as "a" and "ab" are not significantly different.

The *p*-anisidine value of the oils with test compounds (RC6:0, RC8:0, RC10:0, RC12:0, RC16:0, RC18:0) on day 6 showed a significantly better antioxidant activity than the parent molecule ($P < 0.05$, Table 5.2). RC8:0 showed the highest antioxidant activity among all compounds tested while RC3:0 and RDHA had no effect compared to the control. This result correlated well with conjugated dienes and their correlation coefficient was 0.96.

Table 5.2. *p*-Anisidine values of stripped corn oil over a 6-day period in the presence of resveratrol and its derivatives.

Sample	Storage period			
	Day 0	Day 1	Day 3	Day 6
Control	22.4 ± 9.1 a	11.8 ± 0.2 cde	17.7 ± 0.4 bcd	165 ± 0.8 a
R	16.9 ± 6.5 ab	12.0 ± 0.3 cd	16.5 ± 0.3 d	84.8 ± 0.3 e
RC3:0	10.0 ± 1.5 bc	12.6 ± 1.1 bc	17.0 ± 0.3 cd	163.0 ± 1.2 a
RC4:0	10.9 ± 1.5 b	11.1 ± 0.2 cde	17.0 ± 0.3 cd	112.9 ± 0.7 b
RC6:0	9.5 ± 0.4 bcd	10.6 ± 0.4 de	16.8 ± 0.3 d	70.2 ± 0.4 g
RC8:0	14.6 ± 1.1 ab	10.4 ± 0.3 e	17.5 ± 0.1 bcd	65.0 ± 0.6 h
RC10:0	10.3 ± 0.2 bc	10.7 ± 0.2 de	17.0 ± 0.7 cd	71.6 ± 0.7 g
RC12:0	9.1 ± 0.4 bcd	10.4 ± 0.2 e	18.6 ± 0.3 ab	66.7 ± 1.5 h
RC14:0	8.7 ± 0.8 bcd	10.9 ± 0.2 de	19.6 ± 0.3 a	87.5 ± 0.6 d
RC16:0	8.9 ± 0.7 bcd	13.7 ± 1.0 ab	18.9 ± 0.2 ab	70.5 ± 0.8 g
RC18:0	1.5 ± 0.8 cd	14.2 ± 0.6 a	17.8 ± 0.7 bcd	78.0 ± 0.5 f
RC18:1	0.8 ± 0.2 d	13.8 ± 0.4 ab	18.4 ± 0.9 abc	91.4 ± 0.5 c
REPA	1.4 ± 0.1 cd	14.5 ± 0.5 a	18.3 ± 0.4 abc	85.0 ± 0.3 e
RDHA	1.6 ± 0.4 cd	15.0 ± 0.3 a	18.3 ± 0.9 abc	163.8 ± 1.1 a

All determinations were repeated three times means and standard deviations were reported. Values in the same column with different letters are significantly different at $P < 0.05$ performed by Tukey's HSD test. Columns with same letters such as "a" and "ab" are not significantly different.

5.4 Antioxidant activity in a meat model system (thiobarbituric acid reactive substances, TBARS)

The thiobarbituric acid (TBA) method was employed to assess the effect of resveratrol and its derivatives in the control of oxidation in a meat model system. The secondary oxidative products, mainly aldehydes and ketones, are responsible for off-flavour development and loss of nutrients in muscle foods (Cesa, 2004). MDA, one of degradation products from polyunsaturated fatty acids, reacts with TBA. The MDA-TBA so produced has a pink colour, which can be measured at 530-535 nm, therefore TBARS are usually reported as MDA equivalents (Shahidi and Zhong, 2015). In this study, BHT was employed as a positive control. As shown in Table 5.3, all samples showed an increasing trend of TBARS during cold storage (4 °C). The ground pork system containing resveratrol with unsaturated fatty acids (C18:1, EPA, DHA) had the lowest TBARS values at the beginning of the storage after cooking (day 0). From day 3 to day 7, most of the test compounds except RC14:0 displayed lower TBARS values compared to the control. RC3:0 and RC6:0 had the lowest TBARS value among all tested compounds after 3 days of storage, significantly lower than resveratrol itself ($p < 0.05$). Meanwhile RC3:0, RC6:0, REPA, and RDHA showed some, but insignificant differences with resveratrol after 5 days of storage (in the following order $RC3:0 > R > RDHA > RC6:0 > REPA$, $p > 0.05$). However, REPA and RDHA were able to inhibit the production of TBARS significantly compared to resveratrol after 7 days of storage ($p < 0.05$). According to Zhong and Shahidi (2012), inhibition of β -carotene bleaching by EGCG and its derivatives was correlated with the inhibition of TBARS production and their lipophilicity (EGCG-C18:0 > EGCG-DHA > EGCG-EPA > EGCG). However, there was

no correlation between TBARS values and lipophilicity in this study. There was a possibility that the ester bonds could be hydrolysed during the cooking (80 °C), which might lead to the insignificant differences with resveratrol itself. Moreover, REPA and RDHA carrying PUFA, are able to produce aldehydes that could increase TBARS. However, REPA and RDHA showed the lowest TBARS values among all tested compounds after 7 days of storage ($p < 0.05$). Moreover, most of the test compounds, except resveratrol with medium chain fatty acids, exhibited a better inhibition of the formation of TBARS when compared with BHT during 7 days of storage. The amount of BHT used was low (equivalent to 5 ppm) compared to other similar studies (Ambigaipalan and Shahidi, 2015), therefore the positive control might show lower antioxidant activity than other studies. However, some of the resveratrol esters were able to inhibit TBARS formation and this was stronger statistically than the positive control at the same concentration. Therefore, the results so obtained suggest that resveratrol derivatives might be used as potential antioxidants in cooked muscle foods in order to provide better oxidative stability to the products.

Table 5.3. TBARS values of pork model system over a 7-day period in the presence of resveratrol and its derivatives (MDA eq. /kg).

Sample	Storage period			
	Day 0	Day 3	Day 5	Day 7
Control	1.03 ± 0.00 ^b	2.25 ± 0.05 ^{ab}	2.84 ± 0.09 ^{ab}	2.94 ± 0.09 ^a
BHT	1.19 ± 0.06 ^a	2.10 ± 0.04 ^{bc}	2.74 ± 0.04 ^b	2.74 ± 0.04 ^b
R	0.86 ± 0.01 ^c	1.56 ± 0.02 ^h	1.86 ± 0.07 ^{fg}	2.35 ± 0.05 ^{cde}
RC3:0	0.46 ± 0.01 ^{fg}	1.25 ± 0.06 ^j	1.89 ± 0.05 ^{fg}	2.19 ± 0.03 ^{ef}
RC4:0	0.62 ± 0.01 ^{de}	1.60 ± 0.04 ^h	2.12 ± 0.03 ^e	2.27 ± 0.05 ^{cde}
RC6:0	0.68 ± 0.01 ^d	1.35 ± 0.03 ^{ij}	1.78 ± 0.03 ^g	2.24 ± 0.06 ^{def}
RC8:0	0.89 ± 0.03 ^c	1.76 ± 0.02 ^{fg}	2.34 ± 0.03 ^d	2.74 ± 0.09 ^b
RC10:0	0.83 ± 0.05 ^c	1.62 ± 0.04 ^{gh}	2.10 ± 0.05 ^e	2.81 ± 0.04 ^{ab}
RC12:0	1.04 ± 0.01 ^b	1.95 ± 0.03 ^{cde}	2.41 ± 0.10 ^{cd}	2.80 ± 0.03 ^{ab}
RC14:0	0.86 ± 0.02 ^c	2.26 ± 0.05 ^a	2.99 ± 0.7 ^a	2.70 ± 0.06 ^b
RC16:0	1.01 ± 0.04 ^b	2.26 ± 0.03 ^{cd}	2.99 ± 0.08 ^c	2.70 ± 0.02 ^b
RC18:0	0.53 ± 0.03 ^{ef}	1.94 ± 0.06 ^{de}	2.47 ± 0.07 ^{cd}	2.39 ± 0.08 ^{cd}
RC18:1	0.36 ± 0.03 ^h	1.47 ± 0.08 ^{hi}	2.03 ± 0.03 ^{ef}	2.44 ± 0.07 ^c
REPA	0.42 ± 0.00 ^{gh}	1.79 ± 0.02 ^{ef}	1.71 ± 0.03 ^g	2.00 ± 0.06 ^g
RDHA	0.42 ± 0.04 ^{gh}	1.56 ± 0.12 ^h	1.82 ± 0.05 ^g	2.08 ± 0.05 ^{fg}

All determinations were repeated three times using the same combined starting materials and mean values and standard deviations reported. Values in the same column with

different letters are significantly different at $P < 0.05$ performed by Tukey's HSD test. Columns with same letters such as "a" and "ab" are not significantly different.

5.5 Hydrogen peroxide (H_2O_2) scavenging activity

Superoxide dismutase in the human body can generate H_2O_2 in order to remove superoxide radical ($O_2^{\bullet-}$) (Halliwell, 1991). In addition, activated phagocytes can produce H_2O_2 to protect the body from invading microorganisms (Chandrasekara and Shahidi, 2011). Although H_2O_2 is one of the non-radical reactive oxygen species (ROS), it can be very toxic to the cells due to its ability to cause DNA damage and membrane disruption (Halliwell, 1991). It is also known that in the presence of iron or copper ions, H_2O_2 can form hydroxyl radical (HO^{\bullet}) which is a highly reactive radical (Halliwell and Aruoma, 1991). Therefore, it is important to keep the level of H_2O_2 in balance. In this study, the H_2O_2 scavenging activity of resveratrol and its derivatives was determined and the results are shown in Figure 5.3. Some resveratrol derivatives (RC3:0 to RC14:0) showed better activity than the parent resveratrol. RC3:0 and RC4:0 were 3- to 3.2-fold and RC6:0 to RC14:0 were 1.6- to 2.4-fold more effective than resveratrol itself. Konyalioglu et al. (2013) reported that resveratrol might be effective against hydrogen peroxide-induced oxidation stress on embryonic neural stem cells. Jang and Surh (2001) stated that pheochromocytoma (PC12) cells in rats may be protected by resveratrol from hydrogen peroxide-induced apoptosis. This study suggests that resveratrol derivatives could render enhanced effects on H_2O_2 scavenging activity so that they may lead to better activities than resveratrol itself in various diseases caused by H_2O_2 . Meanwhile, except for RC3:0

and RC4:0, the compounds beginning with resveratrol to RC10 exhibited an increasing trend of activity in the order of RC10:0 > RC8:0 > RC6:0 > R. However, RC12:0 to RC18:0 followed an opposite trend. Moreover, unsaturated esters showed no H₂O₂ scavenging activity. This might also be related to the so-called nonlinear or cutoff effect. Several studies have reported a cutoff effect in emulsions as well as with other systems (Devínsky et al., 1990; Locatelli et al., 2008; Sugandhi et al., 2007). Devínsky et al. (1990) found a cutoff effect on antimicrobial activity of the homologous series of *N,N*-dimethylalkylamine oxides for *Staphylococcus aureus* and *Escherichia coli*. Locatelli et al. (2008) observed a cutoff effect on the cytotoxic effect toward L1210 leukemia cells.

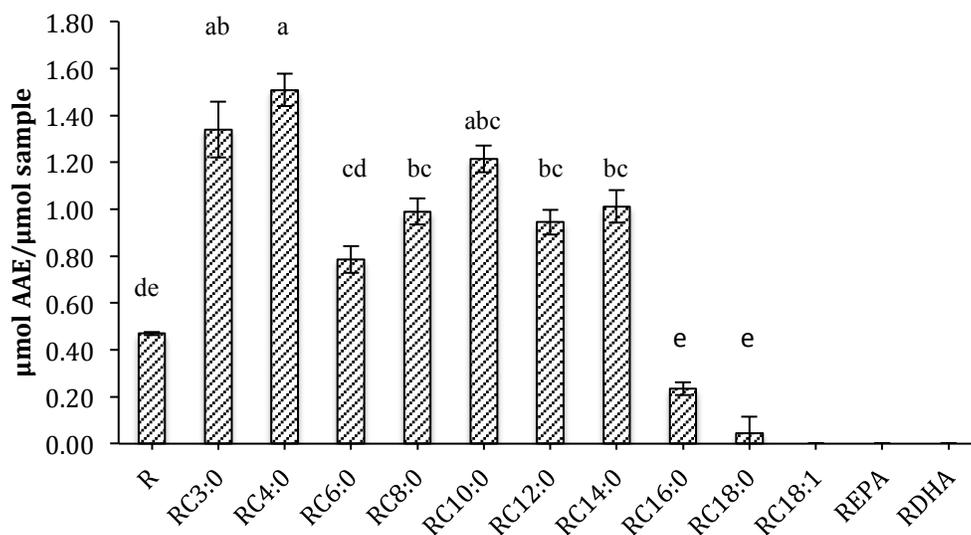


Figure 5.3. Hydrogen peroxide scavenging activities in micromoles of ascorbic acid equivalents (AAE) per micromoles of resveratrol and its derivatives. Resveratrol (R) or its derivatives. All determinations were repeated three times using the same combined starting materials and mean values and standard deviations reported. Bars with different letters mean significantly different at $P < 0.05$ performed by Tukey's HSD test. Bars with same letters such as "a" and "ab" are not significantly different.

5.6 Inhibition of copper-induced low-density lipoprotein oxidation

LDL is an essential particle for transporting cholesterol to cells in the human body. Since LDL is composed of lipids such as phospholipids, cholesterol, and triacylglycerols, among others, it is susceptible to oxidation. The oxidation of LDL might start with phospholipids located on the surface of the LDL and then propagate to the core lipids causing modification of phospholipids, lipids, cholesterol, as well as apolipoprotein B (Witztum, 1994). The oxidized LDL, known as modified LDL, can lead to atherosclerosis.

Therefore, inhibition of LDL oxidation is of interest to many scientists. There are two ways to initiate LDL oxidation outside of the body, one is using transition metal ions such as copper ion (Cu^{2+}) and the other is using AAPH to generate peroxy radical (Magalhães, 2008). In this study, LDL oxidation was induced by CuSO_4 , and the resulting LDL oxidation (formation of conjugated dienes) was monitored spectrophotometrically at 234 nm. Resveratrol is known to be an inhibitor of human LDL oxidation (Frankel et al. 1993). Frankel et al. (1993) measured hexanal formation by copper-catalyzed oxidation, in which resveratrol was able to inhibit LDL oxidation by 70-81%. However, resveratrol and resveratrol with EPA showed a prooxidative activity in this study (Figure 5.4). There are several studies suggesting resveratrol as being responsible for DNA cleaving due to its prooxidant effect (Fukuhara and Miyata, 1998; Fukuhara et al., 2006; Zheng, et al., 2006). Fukuhara and Miyata (1998) reported that resveratrol was able to convert supercoiled DNA to nicked DNA effectively at neutral pH in the presence of O_2 and Cu^{2+} . On the other hand, resveratrol could not damage the DNA in the presence of other metal ions such as Mg^{2+} , Mn^{2+} , Zn^{2+} , Ni^{2+} , Co^{2+} or Fe^{3+} . Although Frankel et al. (1993) did not report the specific conditions for their experiment reporting the inhibitory effect of LDL oxidation, the pro-oxidative activity of resveratrol in this work might originate from different conditions such as pH and presence of O_2 . The behaviour of resveratrol (antioxidant or prooxidant) in LDL oxidation induced by copper needs to be further investigated. Interestingly, most resveratrol derivatives were able to inhibit LDL oxidation. Among resveratrol derivatives, RC16:0 and RC18:0 showed the highest inhibition of LDL oxidation. Structural modification might affect the inhibition ability of test materials. Fukuhara et al. (2006) studied the structural basis of resveratrol and its

analogues for DNA-cleaving activity in the presence of Cu^{2+} . Among hydroxyl groups of resveratrol, the 4'-hydroxyl group was most important for DNA cleavage due to its ability to bind with Cu^{2+} . Therefore, loss of hydroxyl groups via esterification could affect their binding ability with Cu^{2+} , which leads to a better inhibition of LDL oxidation.

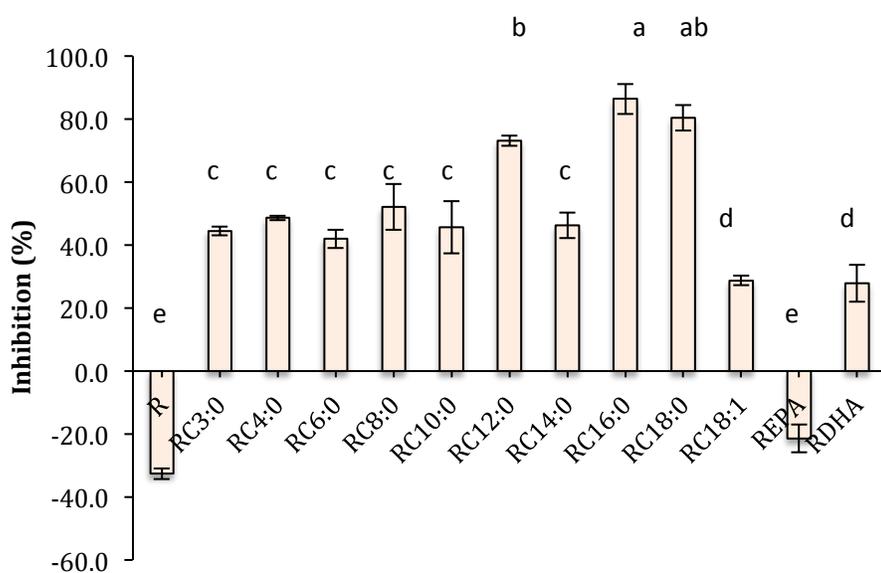


Figure 5.4. Inhibition (%) of resveratrol and its derivatives against LDL oxidation induced by copper. Resveratrol (R) or its derivatives. All determinations were repeated three times using the same combined starting materials and mean values and standard deviations reported. Bars with different letters mean significantly different at $P < 0.05$ performed by Tukey's HSD test. Bars with same letters such as "a" and "ab" are not significantly different.

5.7 Inhibition of hydroxyl radical-induced DNA scission

It is well known that damaged DNA that may occur upon the action of reactive oxygen species (ROS), can lead to mutation and potentially to carcinogenesis. However, ROS is not avoidable to living cells because it is continuously generated via biochemical reactions and external factors (Loft and Poulsen, 1996). For example, hydroxyl radical, one of the ROS, can be generated from degradation of hydrogen peroxide through Fenton reaction and from interaction between superoxide and hydrogen peroxide via a Haber-Weiss reaction (Yu, 1994). Although hydroxyl radical is the most reactive radical of the ROS, it has low diffusion capability due to its short half-life. Antioxidative defense systems to protect from ROS in our body such as superoxide dismutase, GSH peroxidase, and catalase are frequently inadequate to neutralize them (Yu, 1994; Loft and Poulsen, 1996). Therefore, it is of interest to find external sources of antioxidants to serve as critical barriers against free radical and oxidative damage. In this study, hydroxyl radical was employed to determine the inhibition of DNA scission by resveratrol and its derivatives. Hydrogen peroxide and Fe^{2+} were used to generate hydroxyl radical. As shown in Figure 5.5, supercoiled DNA in the control was fully converted into open circular DNA by hydroxyl radicals, however the linear form was not observed. Resveratrol esters were able to inhibit DNA scission, similar to resveratrol itself ($p > 0.05$, Table 5.4). The esterification did not affect the inhibition of DNA scission in this work. However, Zhou et al. (2017) reported that esterification of tyrosol increased the inhibition of DNA scission induced by hydroxyl radical, whereas esterification of hydroxytyrosol decreased the inhibition of DNA scission. According to Zhong and Shahidi (2012), inhibition against DNA scission of EGCG and its derivatives might be due to a

combination of radical scavenging and Fe^{2+} chelation. Some studies have reported that resveratrol also has Fe^{2+} chelating ability (Hussein, 2011; Gülçin, 2010). Hence, inhibition against DNA scission induced by hydroxyl radical might also be due to a cumulative result of radical scavenging, Fe^{2+} chelating ability, and H_2O_2 scavenging ability. Although resveratrol derivatives (RC3:0 to RC14:0) showed a significantly better H_2O_2 scavenging activity than resveratrol, inhibition against DNA scission induced by hydroxyl radical was to various extent, but not significantly different. This might be due to its compromised radical scavenging ability. Resveratrol derivatives showed a lower radical scavenging activity in both DPPH radical and ABTS radical cation scavenging assays (Oh and Shahidi, 2017), suggesting that losing one or more hydroxyl group might compromise their radical scavenging activity. Therefore, decreased radical scavenging activity might counteract the better effect of resveratrol derivatives (RC3:0 to RC14:0) on H_2O_2 scavenging ability. While unsaturated derivatives of resveratrol (RC18:1, REPA, and RDHA) showed no H_2O_2 scavenging activity, their inhibition of DNA scission induced by hydroxyl radical was similar to resveratrol. This might be due to the structural nature (bent structure) of unsaturated fatty acids that might positively influence the metal chelation activity (Zhong and Shahidi, 2012).

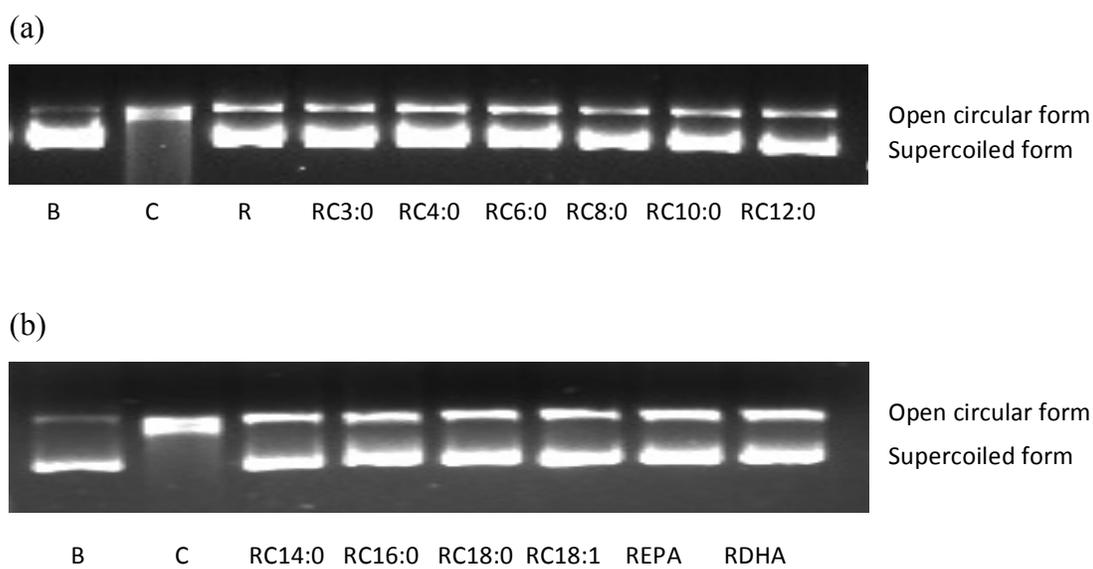


Figure 5.5. Inhibition (%) of hydroxyl radical induced DNA scission by (a) R, RC3:0, RC4:0, RC6:0, RC8:0, RC10:0, and RC12:0 and (b) RC14:0, RC16:0, RC18:0, RC18:1, REPA, and RDHA (B: DNA only, C: DNA + FeSO₄ + H₂O₂). The visualisation of DNA bands was obtained using the Alpha-Imager gel documentation system (Cell Biosciences, Santa Clara, CA, USA) and the data processing was performed with the Chemi-Imager 4400 software (Cell Biosciences).

Table 5.4. Inhibition of hydroxyl radical-induced DNA scission by resveratrol and its derivatives.

	Inhibition (%)
R	79.75 ± 1.86 abc
RC3:0	86.94 ± 1.40 a
RC4:0	79.76 ± 1.49 abc
RC6:0	80.92 ± 1.31 abc
RC8:0	86.15 ± 3.58 ab
RC10:0	84.46 ± 3.63 abc
RC12:0	81.03 ± 1.77 abc
RC14:0	77.94 ± 2.49 abc
RC16:0	75.94 ± 0.70 c
RC18:0	79.07 ± 3.71 abc
RC18:1	77.55 ± 7.67 bc
REPA	77.23 ± 1.18 bc
RDHA	79.38 ± 1.04 abc

All determinations were repeated three times using same combined starting materials and mean values and standard deviations reported. Values with different letters mean significantly different at $P < 0.05$ performed by Tukey's HSD test. Columns with same letters such as "a" and "ab" are not significantly different.

5.8 Summary

This study demonstrated the potential extended use of lipophilised resveratrol derivatives in food and biological systems in order to control oxidative processes. The derivatives showed a range of antioxidant activities depending on the model system employed. We observed disagreement with the polar paradox hypothesis in both oil-in-water emulsion and bulk oil systems. Moreover, a nonlinear phenomenon was observed in the bulk oil system and hydrogen peroxide scavenging activity. The test compounds, except for resveratrol and REPA, were able to inhibit LDL oxidation induced by copper ion effectively. In addition, all test compounds inhibited DNA scission induced by hydroxyl radical. The results clearly demonstrated that resveratrol derivatives might serve as potential antioxidants in food and biological systems.

CHAPTER 6

Effect of lipophilisation of resveratrol on reactive nitrogen/oxygen species generation in murine macrophages and human cancer cell lines

6.1 Introduction

Inflammation, such as redness, warmth, and swelling, is a normal reaction to injuries or infections. However, prolonged inflammation can progress further to various chronic diseases such as cardiovascular disease and cancer (Zhong et al., 2012). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) may explain the link between inflammation and other chronic diseases (Wiseman and Halliwell, 1996). It is well known that ROS and RNS can continuously be generated *in vivo* as a by-product of biological reactions (Halliwell, 1996, Fransen et al., 2012). Superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and nitric oxide (ON^{\bullet}) are the primary ROS/RNS species in a cell and they can readily form other ROS/RNS (Fransen et al., 2012). At a low level, ROS/RNS can serve as mediators for various biological responses such as gene expression and cell proliferation. However, at high levels, they can also cause harmful effects, including inflammation, cardiovascular disease, and cancer (Fransen et al., 2012; Zhong et al., 2012). Antioxidative defense systems are frequently insufficient in our body to keep ROS/RNS level in balance, therefore external sources of antioxidants are needed (Yu, 1994; Loft and Poulsen, 1996).

Resveratrol is a powerful antioxidant and has been found in more than 70 plant species, especially grape skins. Red wine also contains resveratrol that originates from grapes (Dercks and Creasy, 1989; King et al., 2006). Resveratrol had received increasing

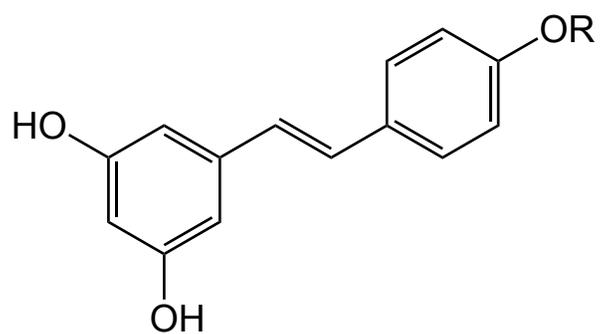
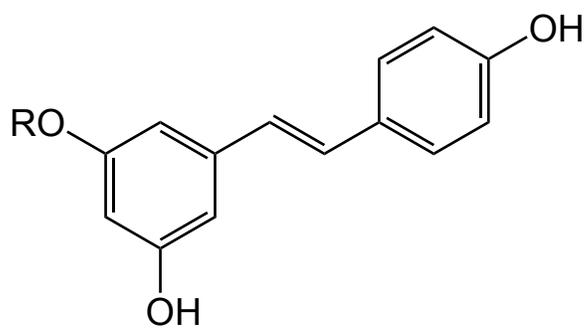
attention since red wine was first shown to display cardioprotection (Baur and Sinclair, 2006). In addition, resveratrol has been known to prevent oxidation, cancer, coronary heart disease and inflammation (Jang et al., 1997; Wang et al., 2002; Donnelly et al., 2004). Although it has beneficial health properties, there are limitations to its bioactivity due to its hydrophilic nature and fast metabolism in the body (Zhong and Shahidi, 2011; Walle et al., 2004). Structural modification may be considered to overcome its limitations in this respect (Torres et al., 2010; Baur and Sinclair, 2006). Several studies on lipophilic derivatives of phenolic compounds, including resveratrol, have demonstrated their better performance in biological model systems. Acetylated resveratrol showed better inhibition of platelet activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine, PAF)-induced washed rabbit platelet aggregation and DU-145 human prostate cancer cell-growth than resveratrol itself (Fragopoulou et al., 2007; Cardile et al., 2005). In addition, Cichocki et al. (2008) reported that pterostilbene, similar to resveratrol, acted as an inhibitor of tumour promotion biomarker. Moreover, esterification of resveratrol with polyunsaturated fatty acids (PUFAs) may provide a synergistic effect due to their own individual bioactivities. In addition, our preliminary data showed that some of the resveratrol esters have a potential effect on ROS/RNS generation in murine macrophages. For this reason, resveratrol was esterified with propionyl chloride (C3:0) and docosahexaenoyl chloride (DHA, C22:6) as described in Chapter 3 (sections 3.2.1.1 and 3.2.1.2; Oh and Shahidi, 2017). In the study reported in this chapter, the monoesters (RC3:0 and RDHA) were evaluated for inhibition against NO production, and RDHA was evaluated further for cell viability and ROS generation in HepG2 cells.

6.2 Nitric oxide (NO) production

Nitric oxide is an important molecule with known beneficial activities such as vascular relaxation and neurotransmission in mammals (Coleman, 2001). The solubility and diffusion properties of NO are similar to those of oxygen and it can freely cross cell membranes and has a long half-life (Coleman, 2001). It is a relatively stable radical, however it can form peroxynitrite anion (ONOO^-) which is a more reactive form of RNS (Kruidenier and Verspaget, 2002). Moreover, an excessive amount of NO in activated immune cells leads to tissue damage during inflammation (Zhong et al., 2012). NO is generally synthesized from L-arginine by three different types of NO synthase (NOS), namely neural NO synthase (nNOS), endothelium NO synthase (eNOS), and inducible NO synthase (iNOS). Unlike nNOS and eNOS, iNOS is continuously active once expressed and produces NO at a sustained high level. Expression of iNOS can be induced by LPS or cytokines such as tumour necrosis factor (TNF)- α and interleukin (IL)-1 (Coleman, 2001).

In this study, inhibition of LPS-induced NO production in murine RAW 264.7 was examined for two different resveratrol esters (Figure 6.1) was evaluated and compared with resveratrol. The nitrite concentration in the culture medium was considered as NO production. As shown in Figure 6.2, the nitrite level of LPS treatment alone (LPS) increased greatly compared to that of the control ($p < 0.005$). All samples were able to attenuate NO production in murine RAW 264.7 cells. Resveratrol and RC3:0 showed a significant decrease of NO production at both 5 and 25 $\mu\text{g/mL}$ ($p < 0.05$). However, RDHA at 5 $\mu\text{g/mL}$ was able to attenuate the NO production slightly but an insignificant

difference was observed as compared to LPS; RDHA at 25 $\mu\text{g/mL}$ was able to inhibit overproduction of NO effectively and it was as effective as resveratrol itself at the same concentration. Tsai et al. (1999) reported that resveratrol inhibited the generation of NO and reduced the level of iNOS protein. They also found almost complete suppression of iNOS mRNA by resveratrol, suggesting that the inhibition of iNOS generation might be due to suppression of iNOS mRNA. Bi et al. (2005) reported that resveratrol inhibited the release of NO as well as TNF- α induced by LPS. Zhong et al. (1999) stated that resveratrol showed inhibition of IL-6 release. TNF- α and IL-6 are involved in pro-inflammatory responses. Resveratrol esters have not been studied on the generation of iNOS protein, iNOS gene expression, the release of IL-6 and TNF- α , however they are expected to exert an effect on pro-inflammatory agents due to possessing inhibitory activity on NO production. This implies that all tested compounds may render an inhibitory effect on NO production and possibly serve as anti-inflammatory agents, but this needs to be further investigated.



R: C₃H₆O (C3:0)

C₂₂H₃₂O (C22:6; DHA)

Figure 6.1. Structures of RC3:0 and RDHA monoesters.

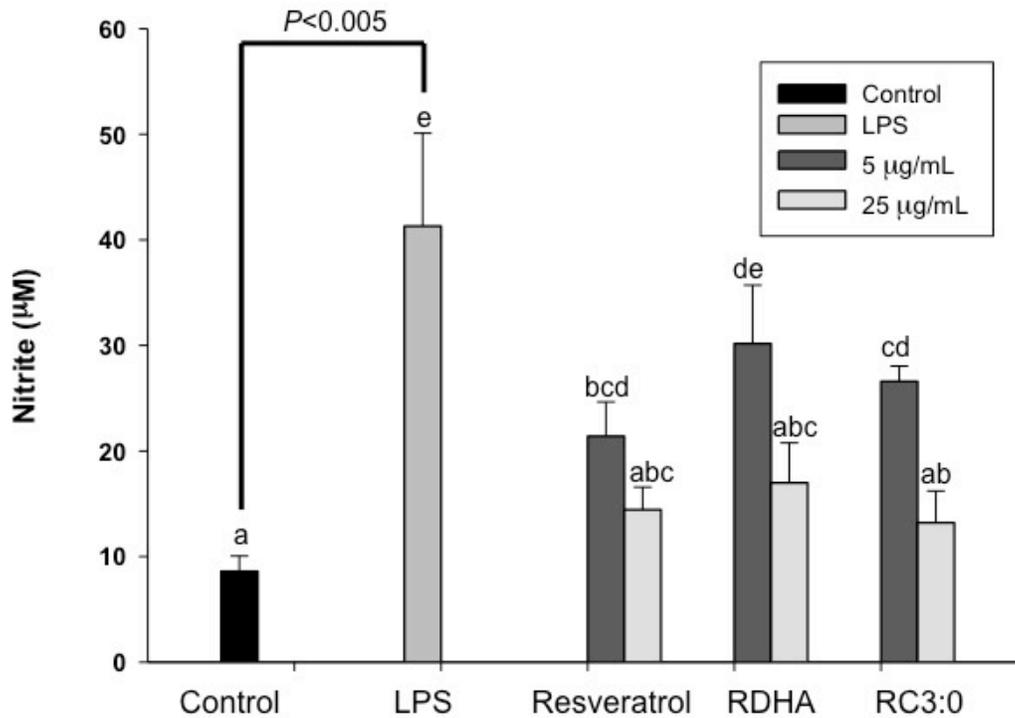


Figure 6.2. Effect of resveratrol and its monoesters on NO production in LPS-induced RAW 264.7 macrophages ($P < 0.001$ compared with the control; $P < 0.05$, compared with the LPS treatment only). All determinations were repeated three times using the same combined starting materials and mean values and standard deviations reported.

6.3 Determination of cytotoxicity and ROS generation on human hepatoma carcinoma (HepG2) cells (cellular antioxidant assay)

The cytotoxicity of resveratrol and its esters (10 µg/mL) on HepG2 cells was determined by using the MTT assay. The MTT assay is a colourimetric assay, which is fast and precise and is based on the reducing ability of mitochondria of living cells. Mitochondria can reduce tetrazolium salt MTT, a pale-yellow compound, to formazan, a dark-blue product. Formazan so obtained can be dissolved in dimethyl sulphoxide (DMSO) and measured at 570 nm and considered as the cell population (Mosmann, 1983). As shown in Table 6.1, the cells treated with resveratrol and its esters were incubated for 24 or 48 h. None of the compounds tested showed any cytotoxicity, thus the ROS generation assay was conducted. A cellular antioxidant assay (CAA), using 2',7'-dichlorofluorescein diacetate (DCFH-DA), was employed to assess ROS generation in cells. DCFH-DA is a stable, non-fluorescent and cell membrane permeable compound due to its hydrophobicity (Le Bras et al., 2005; Pan et al., 2005). When DCFH-DA is taken into the cultured HepG2 cells, cellular esterase forms DCFH by cleaving the acetate groups (Le Bras et al., 2005). DCFH is sensitive to ROS and RNS, thus it can be oxidized to a fluorescent DCF. The measured fluorescent intensity can be considered as an index of the overall oxidative stress in cells (Pan et al., 2005; Shahidi and Zhong, 2015).

Table 6.1. Effect of resveratrol and its DHA ester on HepG2 cell viability

Compound (10 µg/mL)	Cell Viability % (24 h)	Cell Viability % (48 h)
Resveratrol	111.05 ± 9.16 Aa	111.58 ± 10.19 Aa
RDHA	101.08 ± 5.54 Ba	89.47 ± 4.96 Bb

Values in the same column with different uppercase letters were significantly different at $P < 0.05$ performed by a t-test. Values in the same row with different lowercase letters mean significantly different at $P < 0.05$ performed by the t-test. All determinations were repeated three times using the same combined starting materials and mean values and standard deviations reported.

In this study, HepG2 cells were treated with various concentrations of resveratrol and RDHA for 4 h. As shown in Figure 6.3, ROS generation increased in 400 µM t-BOOH compared to the control. All concentrations of resveratrol were able to suppress ROS generation in a concentration-dependent manner. Although 0.5 µg/mL of RDHA showed a slightly higher ROS generation than t-BOOH, ROS generation decreased in the presence of 1-10 µg/mL RDHA in a concentration-dependent manner. Resveratrol showed a higher inhibition of ROS generation than RDHA in this study. However, limitations of using HepG2 should be realized as recently discussed by Kellett et al. (2018). They questioned the use of HepG2 cells because liver cells are not the most suitable for assessing antioxidant activity of dietary phenolics. They reported that catechin at 50 µM showed no cellular antioxidant activity in HepG2 cells, on the other

hand, it reduced fluorescence by 54.1% in Caco-2 cells. Furthermore, the intestinal barrier is the most critical factor because it can affect the function of phenolics *in vivo*. Hence the Caco-2 cell model was suggested as being better for measuring cellular antioxidant activity.

ROS plays a crucial role in cancer cell growth and apoptosis (Juan et al., 2008) and is known to cause cancer by inducing DNA base changes, strand breaks, and rearrangements, among other effects (Wiseman and Halliwell, 1996). On the other hand, an excess amount of ROS in the damaged cell can initiate and execute apoptosis (Juan et al., 2008). Apoptosis of cancer cells can therefore be controlled by the level of ROS by overcoming the antioxidative defense systems (Wenzel et al., 2005). Resveratrol is known as an anticancer agent by inducing apoptosis via ROS generation. Jung and Woo (2016) studied ROS generation in A172 human glioma cells exposed to 100 μM of resveratrol and reported that ROS generation of the cell exposed to resveratrol greatly increased as compared to the control. Juan et al. (2008) studied the production of superoxide radicals in the mitochondria of HT-29 human colorectal carcinoma cells containing 150 μM of resveratrol and reported that superoxide radicals level increased compared to that of the control. Pan et al. (2007) studied ROS generation in human gastric carcinoma AGS cells treated with 80 μM of pterostilbene, and reported an increase in the ROS level. These reports contradict those found in this study, possibly due to the different concentration of resveratrol used. Antioxidant activity may depend on the concentration of antioxidants such as tocopherol (Jung and Min, 1990). According to Ahmad et al. (2003), 50-150 μM hydrogen peroxide-induced apoptosis was blocked by 4 and 8 μM resveratrol. They suggested that resveratrol could trigger or inhibit apoptosis in cancer cells, depending on

the concentration used.

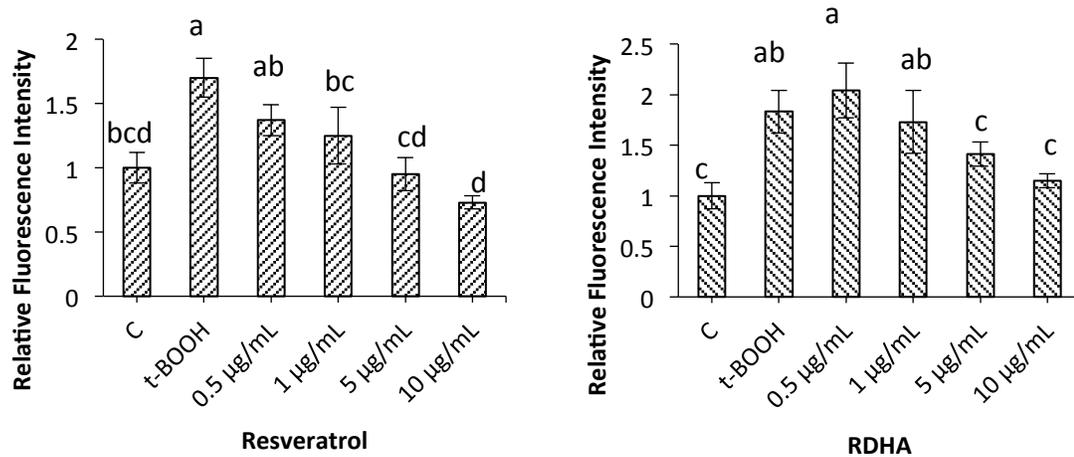
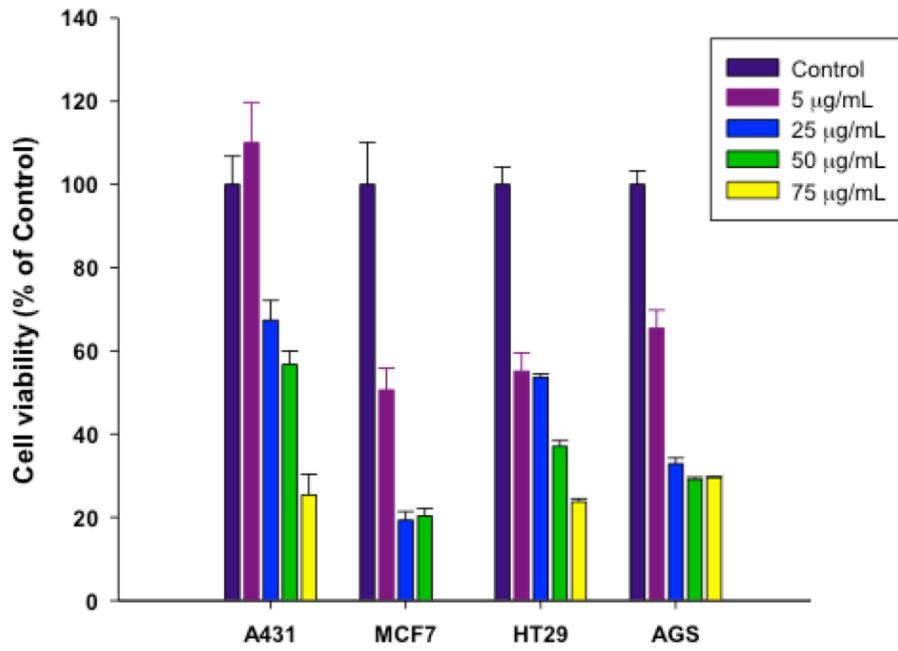


Figure 6.3. Effect of resveratrol and RDHA on intracellular reactive oxygen species (ROS) generation. The different letters mean significantly different ($P < 0.05$) performed by Tukey's HSD test. Bars with same letters such as "a" and "ab" are not significantly different. Resveratrol and RDHA at same concentration showed significantly different ($P < 0.05$) performed by t-test except for control (C) and *tert*-butylhydroperoxide (t-BOOH). All determinations were repeated three times using the same combined starting materials and mean values and standard deviations reported.

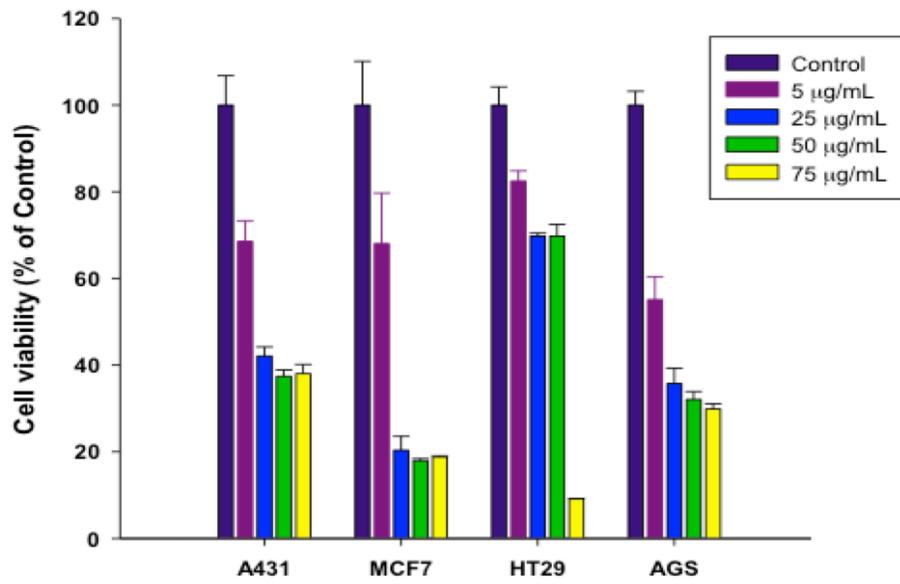
6.4 Effect of resveratrol and its esters on the viability of human epidermoid carcinoma (A431) cells, human breast adenocarcinoma (MCF7) cells, human colorectal adenocarcinoma (HT-29) cells, and human gastric adenocarcinoma (AGS) cells

The effect of resveratrol and its esters at various concentrations on the viability of A431, MCF7, HT-29, and AGS cells was determined by employing the MTT assay. As shown in Figure 6.4, resveratrol and its esters decreased the growth in cultured human cancer cells. Resveratrol at 5 $\mu\text{g/mL}$ was able to decrease cell viability in all tested cancer cell lines except for A431 cells, whereas 5 $\mu\text{g/mL}$ of RC3:0 showed cytotoxicity in A431, MCF7, AGS cells. Although 5-50 $\mu\text{g/mL}$ of RC3:0 showed a lower cytotoxicity than that of resveratrol in HT29 cells, 75 $\mu\text{g/mL}$ of RC3:0 showed a higher cytotoxicity than resveratrol. RDHA at all concentrations showed the lowest cytotoxicity in A431, HT29, and AGS cells compared to resveratrol and RC3:0. However, 50 and 75 $\mu\text{g/mL}$ of RDHA in MCF7 cells showed no cell viability.

a)



b)



c)

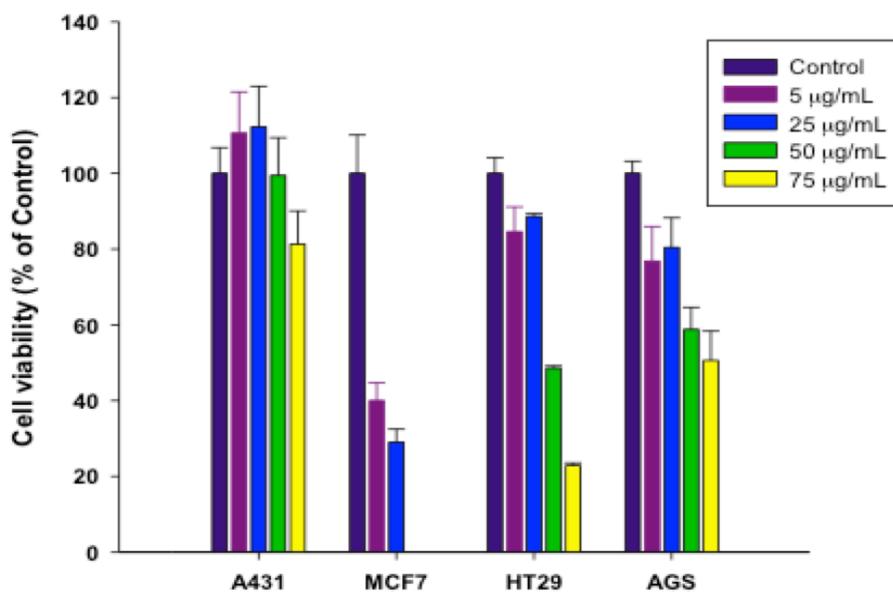


Figure 6.4. Effect of a) resveratrol, b) RC3:0, and c) RDHA on cell viability of various human cancer cells. All determinations were repeated three times and mean values and standard deviations reported.

Several studies have demonstrated the anticancer activity of resveratrol and its derivatives. Jang et al. (1997) studied the anticancer activity of resveratrol in three major stages of carcinogenesis and found its chemopreventive effect due to acting as an antioxidant, antimutagen, anti-inflammatory, antipromotion, and antiproliferation agent. In addition, resveratrol inhibited preneoplastic lesions in a mouse mammary glands culture model of carcinogenesis and inhibited tumorigenesis in a mouse skin cancer model. Pan et al. (2007) demonstrated that resveratrol ester also possessed effects on cancer cells. They studied the inhibition of cell proliferation in pterostilbene treated human cancer cells such

as COLO 205, AGS, HL-60, HepG2, and HT-29. They reported that pterostilbene (60 μM) showed the largest sensitive inhibitory effect on AGS cell-growth compared to the other cell tested. Patel et al. (2010) revealed the concentration of resveratrol and its metabolites in the colorectal tissue of cancer patients and the potential benefit of resveratrol as a chemopreventive agent in colorectal cancer. They found that daily doses of 0.5 or 1.0 g resveratrol could reduce cancer cell proliferation by 5 %. However, some studies have failed to show such anticancer activity. For example, Bove et al. (2002) found resveratrol showed inhibitory effect on the growth of 4T1 breast cancer cell line (*in vitro*) in a dose- and time-dependent manner, however it showed no effect on the growth of 4T1 breast cancer in mice (*in vivo*). Baur and Sinclair (2006) suggested that this failure could be due to an inadequate dose of resveratrol, delivery method, and tumour origin. In pharmacokinetic perspective, resveratrol has extremely low bioavailability, and increasing the dose of resveratrol to overcome its low bioavailability might not be possible due to toxicity. Therefore, lipophilised resveratrols need to be evaluated further *in vivo* for their anticancer potential.

6.5 Summary

Two resveratrol esters demonstrated antioxidant and antiproliferative activities in this study. The test compounds were able to inhibit NO production in murine RAW 264.7 cells, suggesting their potential anti-inflammatory effect, which needs to be further investigated on the generation of iNOS protein, iNOS gene expression, the release of IL-6 and TNF- α . RDHA was studied further in HepG2 cells and showed decreased ROS generation. In addition, all test compounds in this chapter showed decreased cell viability

of A431, MCF7, HT-29, and AGS. The results suggest that resveratrol esters need to be further investigated for their anti-inflammatory and antiproliferative potential.

CHAPTER 7

Preparation of esterified quercetin derivatives and effects on antioxidant activity

7.1 Introduction

Quercetin, another flavonoid, serves as a robust antioxidant with numerous reported health benefits such as cardioprotection, anti-inflammatory, and anticancer activities (Pace-Asciak et al., 1995; Kaidama and Gacche, 2015; Lu et al., 2006). Despite its potential beneficial effects, its low absorption does not allow the body to take full advantage of it. Some 90% of intake of phenolic compounds is transferred to the colon without being absorbed, and is fermented by the gut microflora (Clifford, 2004). In addition, the hydrophilicity of the compound may limit its antioxidant potential in lipophilic systems such as lipid-based food and cosmetic formulations. The hydrophilicity may also affect absorption of quercetin due to the limited permeability of lipophilic cell membranes. Hence, many attempts have been made to maximise its efficiency by different means, including structure modification. Esterification of phenolic compounds has been extensively studied. Zhong and Shahidi (2011, 2012) studied the antioxidant activities of esterified epigallocatechin gallate (EGCG); EGCG derivatives showed a better antioxidant activity than EGCG itself. Moreover, Zhong et al. (2012) reported that EGCG derivatives exhibited anticarcinogenic activity on colon cancer. Zhou et al. (2017) studied the antioxidant activity of tyrosol derivatives and observed an unsaturation-dependent antioxidant trend in a DNA scission assay. Pokorski et al. (2003) found that commercially available ascorbyl palmitate might serve as an ascorbate carrier to brain tissues. Esterification with fatty acids would increase the lipophilicity of phenolic

compounds. Hence, quercetin derivatives may also show an improved application in food, cosmetics, and even biological environments. Besides the potential health benefit of quercetin, fatty acids have their own advantages. For example, short-chain fatty acids such as propionic acid and butyric acid are generally produced via fermentation by gut microbes (Wong et al., 2006). Propionic acid, which can be used for hepatic gluconeogenesis, has been shown to inhibit cholesterol synthesis (Brüssow and Parkinson, 2014; Wong et al., 2006). On the other hand, butyric acid, an energy source for colonic mucosa, can prevent colon cancer (Brüssow and Parkinson, 2014; Wong et al., 2006). In addition, the beneficial effect of polyunsaturated fatty acids (PUFA) towards health has been well documented. PUFA such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been known to have anti-inflammatory, cardioprotective, and anticancer activities (Tapiero et al., 2002). Hence, it is of interest to see the effects in the case of joining two biologically active components such as EPA and DHA with quercetin. For this reason, quercetin was esterified with 12 different acyl chlorides varying in chain length as well as the degree of unsaturation.

7.2 Preparation of quercetin derivatives

The quercetin derivatives were prepared chemically using quercetin and fatty acyl chlorides. The esterification was monitored by thin-layer chromatography (TLC). After completion of the reaction, the selected crude products were subjected to HPLC-MS with methanol/5% acetonitrile in water (90:10, v/v) as the mobile phase. As shown in Table 7.1, the HPLC-MS analysis of quercetin with caproic acid (Q-C6:0) showed that there

were intact quercetin (9.4%), monoesters (35.7%), diesters (38.7%), triesters (14.2%), and tetraester (2%). The yield of crude product of Q-C6:0 was 90.6%. Oh and Shahidi (2017) reported that the yields of resveratryl propionate and resveratryl docosahexaenoate were 74 and 37.7%, respectively. Zhong and Shahidi (2011) prepared EGCG derivatives using stearic acid (C18:0), EPA, and DHA and reported that the yields of EGCG-C18:0, EGCG-EPA, and EGCG-DHA were 56.9, 42.7, 30.7%, respectively. They also reported that esterification by chemical method had less selectivity and specificity. Both studies suggested that structural properties might affect the esterification reaction. Indeed, the resultant crude product of quercetin with propionic acid (Q-C3:0) did not show any remaining intact quercetin by HPLC-MS analysis. On the other hand, the crude resultant product of the reaction with quercetin and DHA (Q-DHA) showed around 56.7% of intact quercetin (triesters and tetraester were not considered). The elongation of the carbon chain and kink characteristics might cause steric hindrance in the case of DHA.

Table 7.1. Composition of quercetin (Q) and Q-C6:0 derivatives in crude products.

	Q	monoester	diester	triester	tetraester
Composition (%)	9.4	35.7	38.7	14.2	2

7.3 Identification of quercetin derivatives

The HPLC-MS analysis was carried out with Q-C6:0, because the isomers of quercetin with shorter chain fatty acids could not be separated in the column due to their similar lipophilicity. Quercetin with longer chain fatty acids, on the other hand, could be separated in the column. However, substitution of more than 3 hydroxyl groups could not be detected due to its extremely increased lipophilicity. Zhong and Shahidi (2011) reported that although the yield of EGCG-C18:0, EGCG-EPA, and EGCG-DHA might be different due to the structural characteristics, the main resultant products remained the same (tetraester). The crude product of Q-C6:0 was subjected to HPLC-MS with methanol/ 5% acetonitrile in water 90:10 (v/v) as the mobile phase (Figure 7.1). Intact quercetin (2.907-3.32 min), monoesters (3.32-4.44 min), diesters (4.44-6.293 min), triesters (8.133-12.113 min), and tetraester (19.26-20.68 min) were detected at their expected m/z of 303.05, 401.12, 499.20, 597.27, and 695.35, respectively (Figure 7.2). After that, the crude product of Q-C6:0 was subjected to HPLC-MS with methanol/ 5% acetonitrile in water 80:20, v/v) as the mobile phase to achieve better separation. Since quercetin has five hydroxyl groups, the number of different esterification products or the products with the same number of esterification products in the different positions could vary. In this study, four monoesters (4.427, 5.067-5.2, and 6.787 min), four diesters (10.873, 13.307, 15.18, and 18.947 min), and three triesters (43.42, 51.5, and 75.527 min) were detected (Table 7.2) by HPLC-MS. The monoesters from 5.067 to 5.2 min were not fully resolved and the peak had a small shoulder, which could be expected as two compounds existed. In this analysis (methanol/ 5% acetonitrile in water, 80:20, v/v),

tetraester was not detected due to increased lipophilicity. Two monoesters (15.8 and 15.5%) and three diesters (10.8, 11.2, and 15.4%) were predominant products.

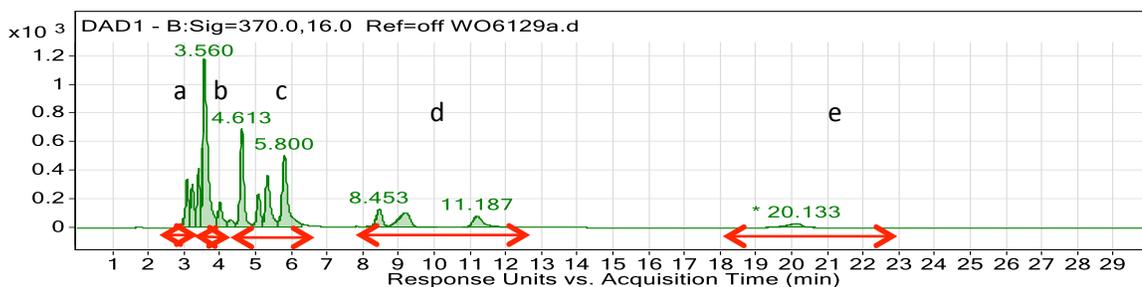


Figure 7.1. HPLC chromatogram of Q-C6:0 with methanol/ 5% acetonitrile in water, 90:10 as the mobile phase (a, quercetin (Q); b, Q-C6:0 monoesters; c, Q-C6:0 diesters; d, Q-C6:0 triesters; and e, Q-C6:0 tetraester).

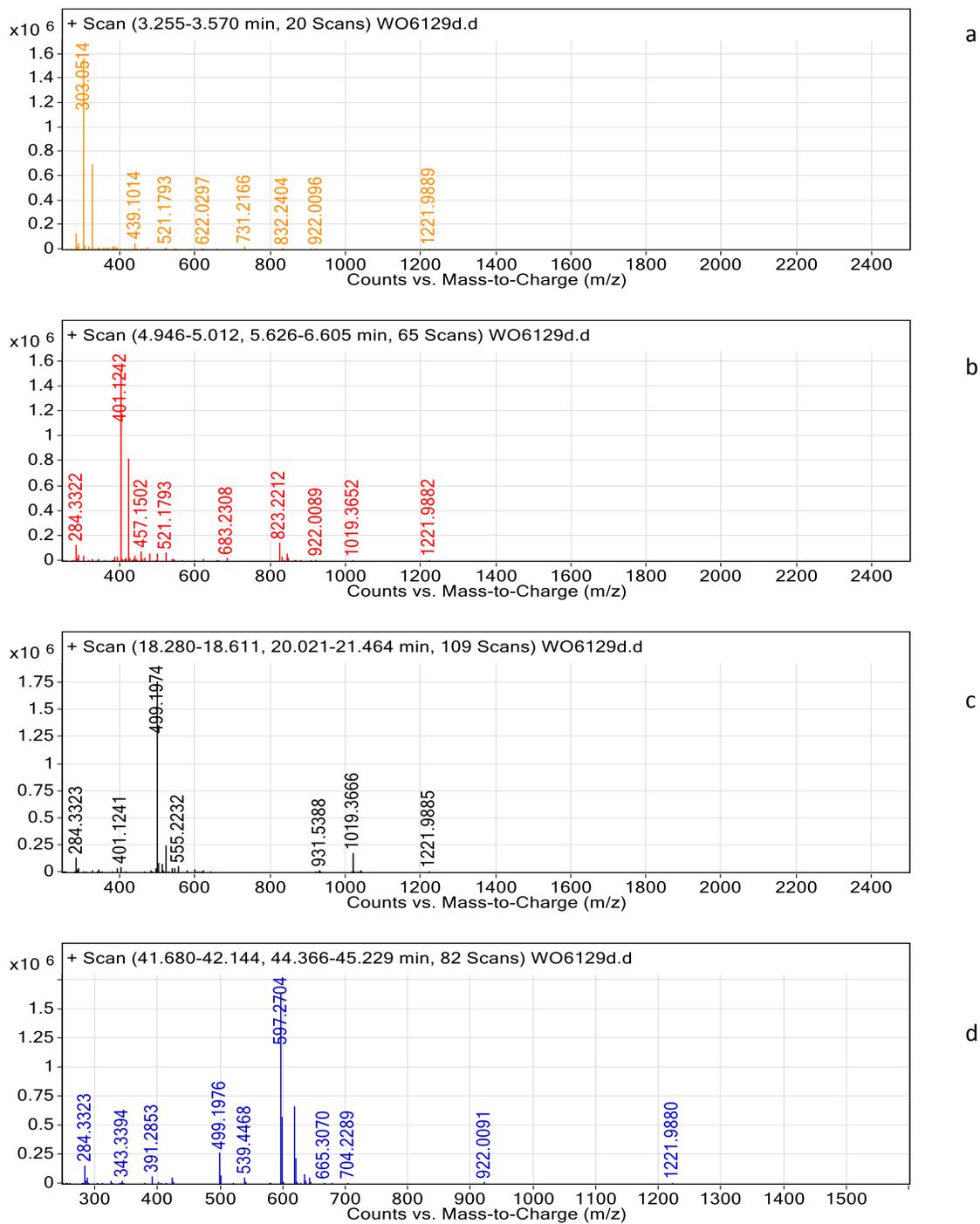


Figure 7.2. Mass spectra of quercetin (Q) and Q-C6:0 derivatives (a, quercetin (Q); b, Q-C6:0 monoesters; c, Q-C6:0 diesters; and d, Q-C6:0 triesters).

Table 7.2. Composition of each Q-C6:0 derivatives.^a

Peak number	Monoesters (%)	Diesters (%)	Triesters (%)
1	6.0	10.8	4.5
2	15.8	5.2	7.1
3	15.5	11.2	4.9
4	3.6	15.4	N/A*

^a Intact quercetin and tetraester were not considered. * Not available.

7.4 ¹H-NMR assignment of quercetin derivatives

The predominant products of quercetin derivatives identified by HPLC-MS were two monoesters and three diesters. Since there were many possibilities to form the different degrees of substitution and isomers, information of the specific location of substitution was required. This was conducted using ¹H-NMR by comparing the parent molecule as well as literature values. Although the yields of esterification with different fatty acids were different, the esterification pattern for all fatty acid esters remained the same. Therefore, one compound was selected for further investigation. For this study, Q-DHA was selected due to its complex structure and its high lipophilicity. The crude product of Q-DHA was separated by TLC (hexane/ ethyl acetate/ formic acid, 3:3:0.12, v/v/v). Then each compound was subjected to ¹H-NMR. The chemical shifts of the parent molecule were very similar to those in the literature values (Bukhari et al., 2008; Galati et al., 2001). Since the separation of two monoesters was unsuccessful due to extremely similar lipophilicity, two spectral assignments of two monoesters were carried out as a mixture.

The chemical shifts of two unknown monoesters (QM1 and QM2) and three unknown diesters (QD1, QD2, and QD3) are summarized in Table 7.3. For QM1, H-6 showed a similar chemical shift (δ 6.19 ppm) compared to that of quercetin (δ 6.20 ppm), which indicated esterification did not occur on A ring. While H-2' and H-6' of QM1 showed downfield shifts ($\Delta\delta$ = 0.07 and 0.14 ppm, respectively), H-5' showed similar chemical shifts (δ 6.92 ppm) compared to quercetin (δ 6.89 ppm). There were two possible substitution positions which were either 3'-OH or 4'-OH. Since chemical shift did not occur on H-5', the substitution position could be concluded on 3'-OH. This was similar to that given by Moalin et al. (2011) who reported chemical shifts of 3'-methylated quercetin as 6.21 (H-6), 6.49 (H-8), 6.95 (H-5'), 7.70 (H-6'), and 7.77 (H-2') ppm. For QM2, H-6 slightly shifted to downfield ($\Delta\delta$ = 0.05). Interestingly, H-2' and H-6' of QM2 showed large upfield shifts ($\Delta\delta$ = -0.37 and -0.30 ppm, respectively), whereas H-5' showed similar chemical shifts (δ 6.92 ppm) compared to quercetin (δ 6.89 ppm); this might be explained by anisotropy (Richards and Hollerton, 2011). Therefore, the possible substitution position of QM2 was expected to be on 3-OH. This was also similar to Moalin et al. (2011). Hydrogens in positions 6 and 8 of QD1 showed large downfield chemical shifts ($\Delta\delta$ = 0.38 and 0.56 ppm, respectively). Thus, one of the substitutions could be on 7-OH. The other substitution of QD1 occurred on B ring. The chemical shift of H-2', H-5', and H-6' was similar to QM1, the other substitution could be expected on 3'-OH. For these reasons, QD1 could be Q-7,3'-O-diester. For QD2, the chemical shifts of H-6 (δ 6.20 ppm) and H-8 (δ 6.44 ppm) were similar to those of H-6 (δ 6.20 ppm) and H-8 (δ 6.42 ppm) of quercetin, which meant substitution did not occur on A ring. However, large downfield chemical shifts ($\Delta\delta$ = 0.15 and 0.41 ppm) were observed on H-2' and H-6' of QD2

compared to quercetin. In addition, H-5' of QD2 also showed large downfield shift ($\Delta\delta=0.19$ ppm) compared to quercetin. Hence, the substitution positions of QD2 could occur on 3'-OH and 4'-OH. For QD3, the H-6 and H-8 of QD3 showed downfield shifts ($\Delta\delta$ 0.06 and 0.08, respectively) compared to quercetin. While H-2' of QD3 showed upfield shift ($\Delta\delta$ -0.06), H-5' and H-6' showed downfield shifts ($\Delta\delta=0.21$ and 0.11 ppm, respectively). Therefore, QD3 could be concluded as Q-3,4'-*O*-diester. In summary, QM1, QM2, QD1, QD2, and QD3 were identified as being Q-3'-*O*-monoester, Q-3-*O*-monoester, Q-7,3'-*O*-diester, Q-3',4'-*O*-diester, and Q-3,4'-*O*-diester, respectively (Figure 7.3).

Table 7.3. ^1H chemical shift (δ in ppm) of quercetin and Q-C6:0 derivatives.

^1H position	Quercetin (Q)	QM1	QM2	QD1	QD2	QD3
6	6.20	6.19	6.25	6.58	6.20	6.26
8	6.42	n/a*	n/a*	6.99	6.44	6.50
2'	7.69	7.76	7.32	7.76	7.84	7.54
5'	6.89	6.92	6.92	6.92	7.08	7.10
6'	7.55	7.69	7.25	7.66	7.96	7.66

* Not available. The compounds were identified as Q-3'-*O*-monoester (QM1), Q-3-*O*-monoester (QM2), Q-7,3'-*O*-diester (QD1), Q-3',4'-*O*-diester (QD2), and Q-3,4'-*O*-diester (QD3).

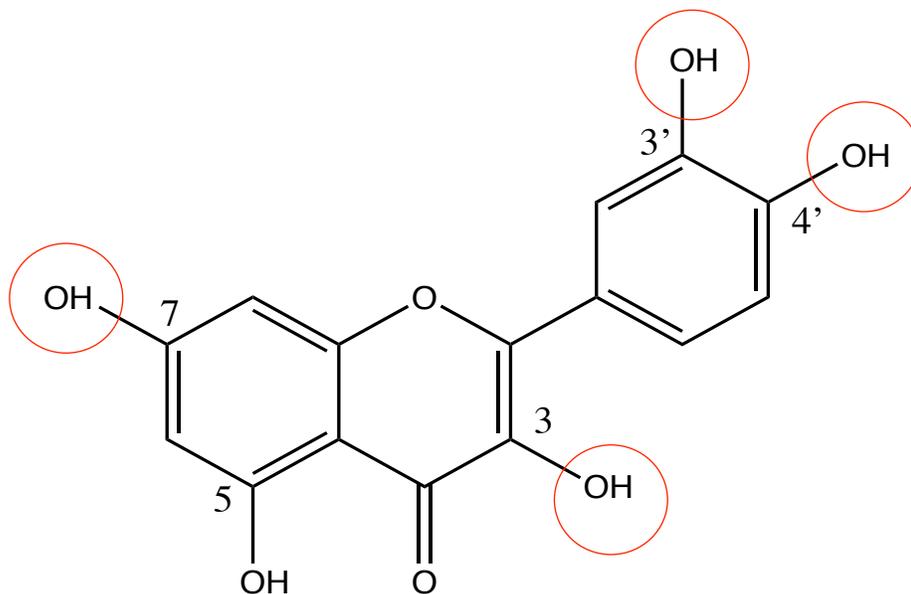


Figure 7.3. Structure of quercetin. Red circles present substitution occurring position.

7.5 Lipophilicity of quercetin derivatives

Esterifications between quercetin and the fatty acyl chlorides were carried out in order to increase the lipophilicity of quercetin. The shake-flask method, a traditional method for measuring lipophilicity, was not employed because it was not suitable for measuring the lipophilicity of highly lipophilic compounds (Ayouni et al., 2008; Giaginis and Tsantili-Kakoulidou, 2008; Finizio et al., 1997). Thus, the lipophilicity was instead calculated using the lipophilicity calculator program, ALOGPS 2.1. The lipophilicity of quercetin was 1.81. As shown in Table 7.4, the lipophilicity of the quercetin derivatives was increased compared to that of quercetin as the chain length and the number of substitution increased. The increased lipophilicity was also observed in the HPLC analysis. However,

unsaturation disturbed the lipophilicity pattern. While the monoester and diester of Q-C18:0 had lipophilicity values of 7.89 and 10.26, respectively, that of Q-C18:1 had lipophilicity values of 7.87 and 10.14, respectively. Moreover, Q-EPA showed a drastic decrease of its lipophilicity compared to Q-C18:0 and Q-C18:1, despite the elongation of chain length. This might be due to the nature of unsaturation. Carbon double bonds have higher density of electrons than carbon single bonds, thus carbon double bonds possess slight polarity (Bruice, 2011). Hence, the large number of unsaturation of EPA (C20:5) could enhance electron density so that the lipophilicity of Q-EPA might decrease, compared to Q-C18:0. The extent of decreased lipophilicity of Q-EPA was increased as the number of substitution increased (diester > monoester). Others also observed similar patterns. Zhong and Shahidi (2011) reported that the lipophilicities of EGCG with stearic acid (SA), EPA, and DHA were in the order EGCG-SA > EGCG-EPA > EGCG-DHA, EGCG-EPA and EGCG-DHA with a nonsignificant difference ($P > 0.05$); this was evaluated by shake flask method. They also reported that the retention time obtained from HPLC analysis was in the order EGCG-SA > EGCG-DHA > EGCG-EPA. A similar sequence of lipophilicity was exhibited when resveratrol was esterified (Oh and Shaidi, 2017). The lipophilicity of resveratrol (R) esterified with SA (9.30) showed the highest value followed by R-DHA (8.59) and R-EPA (8.47).

Table 7.4. Lipophilicity of quercetin and its derivatives.

		Monoester	Diester
Quercetin	1.81		
Q-C3:0		2.88	3.70
Q-C4:0		3.13	4.27
Q-C6:0		3.81	5.51
Q-C8:0		4.65	6.65
Q-C10:0		5.38	7.92
Q-C12:0		6.08	8.97
Q-C14:0		6.78	9.66
Q-C16:0		7.38	10.00
Q-C18:0		7.89	10.26
Q-C18:1		7.87	10.14
Q-EPA		7.23	8.87
Q-DHA		7.48	8.89

The lipophilicity of quercetin and its derivatives was calculated using ALOGPS 2.1. The SMILE structures of the compounds were drawn using ChemDraw Standard 16.0.

7.6 ABTS radical cation scavenging assay

Quercetin is known to be a very powerful antioxidant. Esterification could affect its antioxidant activity either positively or negatively. To evaluate antioxidant activity of quercetin and its derivatives, ABTS radical cation scavenging assay was employed. As shown in Figure 7.4, quercetin showed the highest ABTS radical cation scavenging activity among all tested compounds. Among derivatives, Q-C3:0 and Q-C4:0 showed better ABTS radical cation scavenging activity than other derivatives. Oh and Shahidi (2017) reported a similar behaviour for resveratrol and its derivatives. Resveratrol showed the highest ABTS radical cation scavenging activity among all compounds examined. In addition, resveratrol with short-chain fatty acids showed better antioxidant activity than other derivatives. According to Mainini et al. (2013), quercetin esters showed weaker DPPH and ABTS radical cation scavenging activity compared to the parent molecule. This might be due to their loss of hydroxyl groups by esterification. In addition, the powerful antioxidant activity of quercetin has been known to originate from a combination of the 3',4'-dihydroxyl structure (catechol) in the B ring, 3- and 5-hydroxyl groups in the A and B rings, and the 2,3-double bond and 4-oxo group in the C ring (Bors et al., 1990; Rice-Evans et al., 1996; Pulido et al., 2000). The quercetin derivatives which were tested here might not have the 3',4'-dihydroxy structure in the B ring due to their esterification. Hence this could be another reason why quercetin showed the highest antioxidant activity in this study.

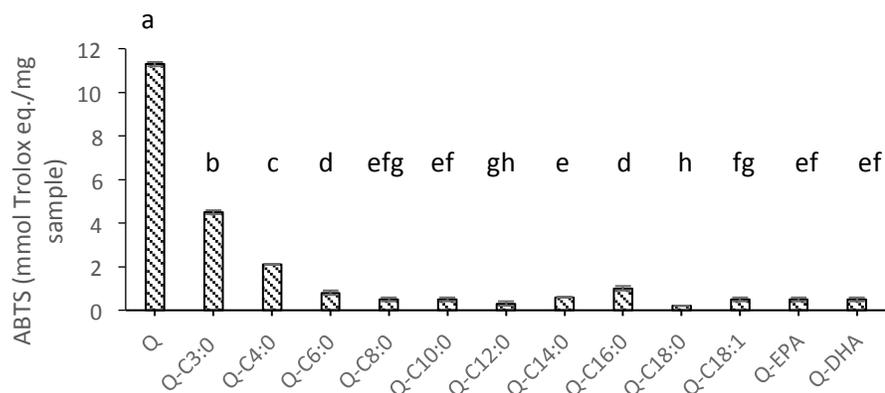


Figure 7.4. ABTS radical cation scavenging activity of quercetin (Q) and its derivatives expressed as trolox equivalents. All determinations were repeated three times using the same combined starting materials and mean values and standard deviations reported. The different letters are significantly different ($P < 0.05$). Columns with same letters such as “a” and “ab” are not significantly different.

7.7 Summary

Esterification of quercetin with the fatty acyl chlorides of different chain length and degree of unsaturation provided derivatives whose structures were confirmed by HPLC-MS and ^1H NMR. These were mainly the Q-3'-*O*-monoester, Q-3-*O*-monoester, Q-7,3'-*O*-diester, Q-3',4'-*O*-diester, and Q-3,4'-*O*-diester. As expected, the lipophilicity of quercetin derivatives was successfully increased. The antioxidant activity of quercetin and its derivatives was evaluated using ABTS radical cation scavenging assay. In this work, quercetin exhibited the highest ABTS radical scavenging activity among all tested sample.

Quercetin with short-chain fatty acids (Q-C3:0 and Q-C4:0) possessed a better antioxidant activity than others. Although ABTS radical cation scavenging assay provided important information about antioxidant activity, this needs to be further extended to include both *in vitro* and *in vivo* studies.

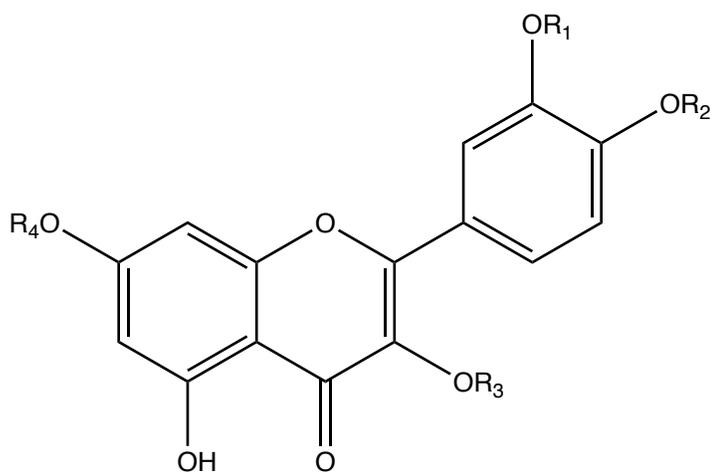
CHAPTER 8

Antioxidant activity of quercetin derivatives in food and biological model systems

8.1 Introduction

Quercetin is widely distributed in the plant kingdom and some commodities such as onions, kale, grapes, and apples contain a high level of it (Formica and Regelson, 1995; Shahidi and Ambigaipalan, 2015). Quercetin is a flavonoid, which is made of a three-ring structure with five hydroxyl groups. This includes a 3',4'-dihydroxyl structure in the B ring, a 2,3-double bond and 4-oxo in heterocycle (C ring), which make it a more powerful antioxidant than other phenolic compounds (Bors et al., 1990; Rice-Evans et al., 1996; Pulido et al., 2000). The antioxidant activity of quercetin has been demonstrated in ferric reducing/antioxidant power (FRAP) assay (Pulido et al., 2000), trolox equivalent antioxidant capacity (TEAC) (Re et al., 1999), reducing activity and metal chelation ability (Mira et al., 2002). Quercetin exhibited antioxidation activity in food model systems such as the oil-in-water emulsion system and bulk fish oil (Huber et al., 2009). It has been documented that quercetin inhibited LDL cholesterol oxidation (Frankel et al., 1993) and reduced NO and ROS production induced by LPS in human acute monocytic leukemia cell (Zhang et al., 2011). Despite its unlimited potential use as an antioxidant, the application of quercetin can be limited due to its hydrophilicity, like other phenolic compounds. It is a well-known fact that phenolic compounds as antioxidants cannot exert their full effects in lipophilic systems which are highly prone to oxidation. Therefore, lipophilicity of phenolic compounds plays an important role with respect to the antioxidant activity in the food industry (Laguerre et al., 2015).

However, this problem is not limited to food and also affects biological systems. Most of the phenolic compounds are excreted without absorption, which may also relate to hydrophilicity. The hydrophilic compounds may be unable to penetrate cell membranes, which are lipid bilayers. In recent studies, structure modification has been carried out to improve lipophilicity of different molecules. One possible way to increase lipophilicity is esterification with fatty acids. In addition, it is of interest to explore the best lipophilicity. For this reason, in this work, quercetin was esterified with 12 different fatty acids as acyl chlorides (propionyl chloride, butyryl chloride, caproyl chloride, capryloyl chloride, capryl chloride, lauroyl chloride, myristoyl chloride, palmitoyl chloride, stearoyl chloride, oleoyl chloride, eicosapentaenoyl chloride, and docosahexaenoyl chloride). Following their preparation, the esters so produced were subjected to the selected antioxidant capacity test as well as being used in food and biological model systems.



Quercetin: $R_1=R_2=R_3=R_4=H$

Monoesters: $R_1=X, R_2=R_3=R_4=H$ and $R_3=X, R_1=R_2=R_4=H$

Diesters: $R_1=R_2=X, R_3=R_4=H, R_2=R_3=X, R_1=R_4=H$, and $R_1=R_4=X, R_2=R_3=H$

X : C_3H_5O (C3:0)

C_4H_7O (C4:0)

$C_6H_{11}O$ (C6:0)

$C_8H_{15}O$ (C8:0)

$C_{10}H_{19}O$ (C10:0)

$C_{12}H_{23}O$ (C12:0)

$C_{14}H_{27}O$ (C14:0)

$C_{16}H_{31}O$ (C16:0)

$C_{18}H_{35}O$ (C18:0)

$C_{18}H_{33}O$ (C18:1)

$C_{20}H_{29}O$ (C20:5, EPA)

$C_{22}H_{31}O$ (C22:6, DHA)

Figure 8.1. Structure of quercetin derivatives.

8.2 DPPH radical scavenging assay

The DPPH radical scavenging capacity of quercetin and its derivatives (Figure 8.1) was carried out to determine their electron and/or hydrogen donation (Prior et al., 2005).

DPPH radical is commercially available without any preparation to generate the radical, thus it is generally used as an initial step in the evaluation of antioxidant activity (Shahidi and Zhong, 2015). As shown in Figure 8.2, quercetin showed the highest DPPH radical scavenging activity among all tested samples. This might be due to the decreased number of hydroxyl groups, which can donate their hydrogen and/or electron. Mainini et al. (2013) also observed that quercetin showed the highest DPPH radical scavenging activity, compared to quercetin esters tested. In addition, quercetin with short-chain fatty acids exhibited a better DPPH radical scavenging activity compared to quercetin with long-chain fatty acids except for Q-C16:0. Moreover, Q-C18:0 and Q-C18:1 showed the lowest DPPH radical scavenging activity. On the other hand, resveratrol with C18:0 and C18:1 showed the highest DPPH radical scavenging activity in Chapter 4. DPPH radical is a lipophilic species, thus it was hypothesised that quercetin with long-chain fatty acids would show better radical scavenging activity due to their increased affinity to lipophilic DPPH. Sun et al. (2018) reported that tyrosol with short- and medium-chain fatty acids also showed better DPPH radical scavenging activity than tyrosol with long-chain fatty acids, except for tyrosol with DHA. However, Oh and Shahidi (2017) reported that resveratrol with short- and medium-chain fatty acids exhibited lower DPPH radical scavenging activity than resveratrol with long-chain fatty acids. Furthermore, Zhong and Shahidi (2011) observed that DPPH radical scavenging activity of epigallocatechin

gallate (EGCG) derivatives was enhanced dramatically due to the esterification and EGCG derivatives, which showed better DPPH radical scavenging activity compared to EGCG itself. The effect of esterification on antioxidant activity was compound-dependent, which requires further evaluation.

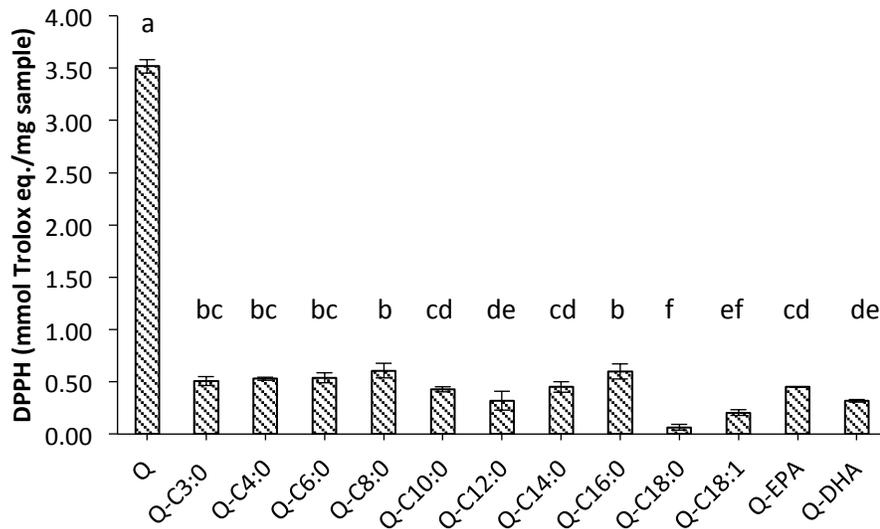


Figure 8.2. DPPH radical scavenging activity of quercetin (Q) and its derivatives expressed as trolox equivalents. All determinations were repeated three times using the same combined starting materials and mean values and standard deviations reported. The different letters are significantly different ($P < 0.05$). Bars with the same letters such as “a” and “ab” are not significantly different.

8.3 Hydroxyl radical scavenging assay

Hydroxyl radical is one of most reactive radicals and a powerful oxidant that can be

produced during lipid oxidation when hydroperoxide breaks down to alkoxy radical (RO•). It can also be generated in the body via both Fenton and Haber-Weiss reactions (Yu, 1994). Thus hydroxyl radical scavenging activity was employed in this study. As shown in Figure 8.3, Q-C3:0 showed the highest hydroxyl radical scavenging activity among the tested compounds. In addition, Q-C4:0, Q-C8:0, Q-C10:0, Q-C12:0, and Q-C18:0 showed an insignificant difference compared to quercetin itself. Hydroxyl radical scavenging activity was measured using EPR. Ambigaipalan et al. (2015) reported that the signals of EPR spectrum with intensities at a ratio of 1:2:2:1. Indeed, blank (only PBS and hydroxyl radical) showed the same spectrum (result not shown). However, another blank (only ethanol and hydroxyl radical) did not show any signal. Since quercetin derivatives could not be fully dissolved in PBS, possibly due to varied lipophilicity, PBS was used to dilute the samples in ethanol. The blank (ethanol without sample) was also diluted with PBS. The EPR spectrum of diluted samples showed signals, however, the number of signals and the intensity ratio was different compared to PBS with hydroxyl radical (Figure 8.4). The formation of hydroxyl radical might be affected by the solvent used. Hiramoto et al. (1996) studied the effect of phenolic compounds on DMPO-OH adduct and DNA scission.

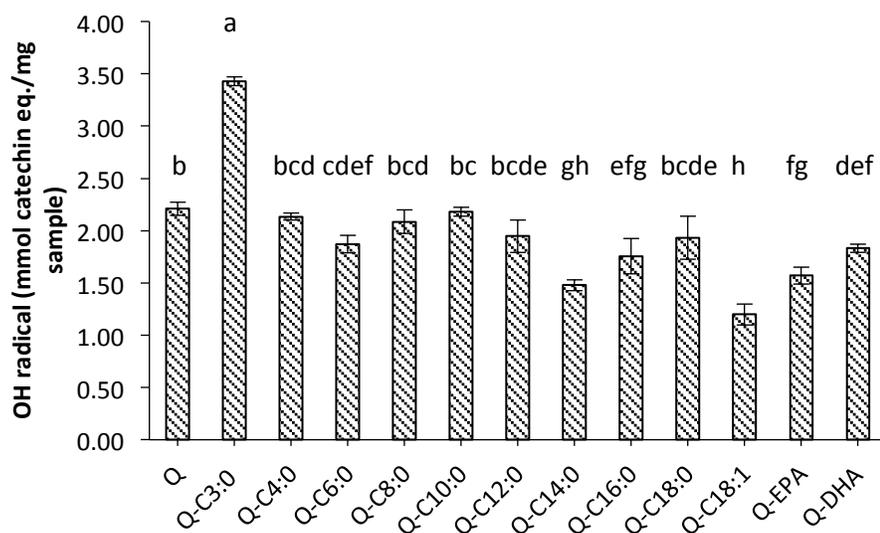


Figure 8.3. Hydroxyl radical scavenging activity of quercetin (Q) and its derivatives expressed as trolox equivalents. All determinations were repeated three times using the same combined starting materials and mean values and standard deviations reported. The different letters are significantly different ($P < 0.05$). Bars with the same letters such as “a” and “ab” are not significantly different.

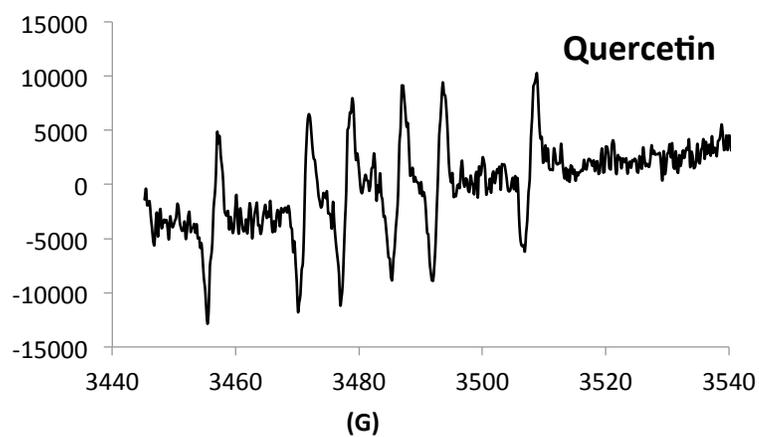
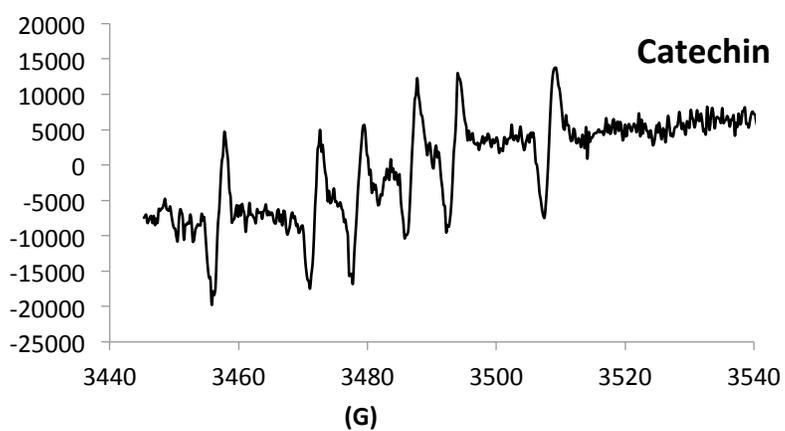
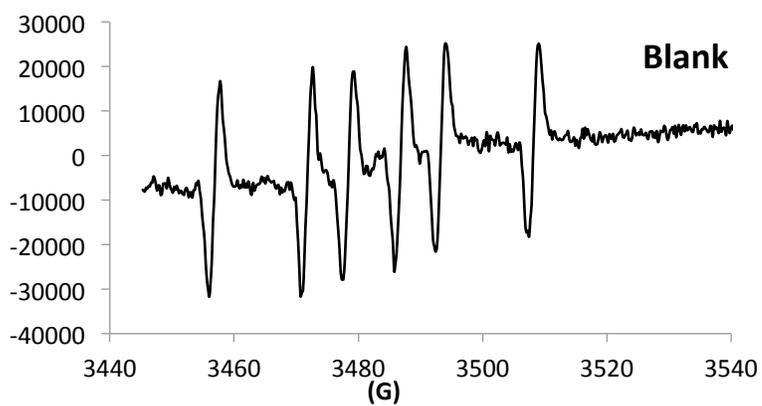


Figure 8.4. DMPO-OH adduct of blank, catechin (standard, 400 μM), and quercetin.

In addition, they compared the electron spin resonance (ESR) signals between DMPO-OH produced in the presence of the phenolic compounds (result A) and production of DMPO-OH then the addition of phenolic compounds (result B). They reported that the hydroxyl radical scavenging activity of result A showed relatively concentration-dependent manner, on the other hand, result B showed a lack of consistency. Moreover, some of the phenolic compounds from result B enhanced the intensity of signals. They suggested that this method might not be reliable and DNA scission induced by hydroxyl radical might be a better method to evaluate the antioxidant activity of phenolics towards hydroxyl radical. In this study, DNA scission induced by hydroxyl radical also studied, as it will be discussed later.

8.4 Reducing power

The antioxidant activities of quercetin and its derivatives were determined according to their reducing power. An antioxidant can retard the oxidation via donating its hydrogen atom and/or transferring an electron. Unlike DPPH radical scavenging activity, this assay is for determining its electron transfer ability because this is measuring from ferric ion (Fe^{3+})-ligand complex to ferrous ion (Fe^{2+}) complex, which is blue colour (Shahidi and Zhong, 2015). As shown in Figure 8.5, quercetin showed the highest reducing power among tested samples. Among derivatives, Q-C3:0, Q-C4:0, and Q-C8:0, in the order shown, had higher reducing power than other derivatives in this study. The reducing power assay was highly correlated to ABTS radical cation scavenging activity (correlation coefficient: 0.97) (see results in Chapter 7, section 7.6). According to Mira et al. (2002), the reducing ability of quercetin might come from the presence of catechol

structure in the B ring and 3-OH, 2,3-double bond, and 4-oxo group in the C ring. Thus, the esterification might compromise the reducing power of quercetin derivatives, compared to quercetin itself. Danihelová et al. (2013) reported that quercetin ($IC_{50} 12.5 \pm 0.9$) and monochloropivaloyl quercetin (substitution on 3-OH; $IC_{50} 12.5 \pm 0.3$) showed a similar reducing power, however tri(monochloropivaloyl) quercetin and monoacetylferuloyl quercetin (substitution on 3-OH) was more than IC_{50} of 200. Zhong and Shahidi (2012) reported that reducing power of EGCG dramatically decreased compared to EGCG derivatives, although free radical scavenging activity of EGCG derivatives was better than EGCG itself. This disagreement was thought to be possibly due to the different mechanisms of action.

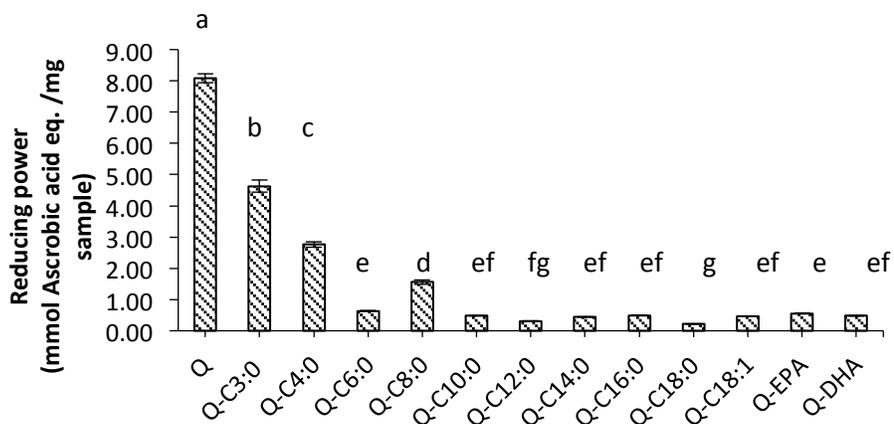


Figure 8.5. Reducing power of quercetin (Q) and its derivatives as ascorbic acid equivalents. All determinations were repeated three times using the same starting materials and mean values and standard deviations reported. The different letters are significantly different ($P < 0.05$). Bars with the same letters such as “a” and “ab” are not significantly different.

8.5 Antioxidant activity of quercetin derivatives in oil-in-water emulsion (β -carotene bleaching assay)

Emulsion, a mixture of two immiscible liquids, is commonly encountered in food. In order to evaluate the possible application in an oil-in-water emulsion system, a β -carotene bleaching assay was employed to determine antioxidant activity of quercetin and its derivatives. Tween 40 was used for stabilising the emulsions. Linoleic acid in the surface of the micelle is first oxidized by induced heat, which is then producing oxidation products. These will attack β -carotene, which is inside the micelle. When β -carotene is oxidized, it will lose its colour and this can be monitored by monitoring the absorbance at 470 nm (Ambigaipalan and Shahidi, 2015). The antioxidant activity of quercetin and its derivatives in the oil-in-water emulsion over a 105-minute period is shown in Figure 8.6A. Quercetin showed the highest antioxidant activity over a 105-minute period for quercetin and its esters. Among quercetin derivatives, Q-C3:0 and Q-C4:0 exhibited a better antioxidant activity than other derivatives, whereas Q-C16:0, Q-C18:0, and Q-EPA showed a lower antioxidant activity than other tested compounds over a 105-minute period. Inhibition against β -carotene bleaching after 105 min incubation is shown in Figure 8.6B. While quercetin was the most potent in the inhibition of β -carotene bleaching, Q-C16:0 was the least effective among all after 105 min incubation. Quercetin with short-chain fatty acids showed a better inhibition compared to other derivatives; the inhibition then slowly decreased as alkyl chain increased. This result was well correlated with ABTS radical cation scavenging activity (see results in Chapter 7, section 7.6) and reducing power (the correlation coefficient was 0.82 and 0.87, respectively). This might be due to their increased lipophilicity. Oh and Shahidi (2018) reported that resveratrol

with short-chain fatty acids was more effective in the oil-in-water emulsion systems than other derivatives. In addition, resveratrol derivatives showed a better antioxidant activity than the parent compound in a bulk oil system. Those results, both quercetin derivatives and resveratrol derivatives, contradicted polar paradox. The polar paradox stated that the nonpolar antioxidants possessed better antioxidant potent than polar antioxidants in the oil-in-water emulsions, whereas polar antioxidants were more effective than nonpolar antioxidants in water-in-oil emulsions and/or bulk oil system (Shahidi and Zhong, 2011). For example, Zhong and Shahidi (2012) reported that EGCG derivatives showed better inhibition in β -carotene bleaching assays than EGCG itself. However, many studies have provided evidence that not all antioxidants correspond with polar paradox and researchers suggested that polar paradox might need to be revisited (Torres de Pinedo et al., 2007; Stöckmann et al., 2000; Laguerre et al., 2015; Shahidi and Zhong, 2011).

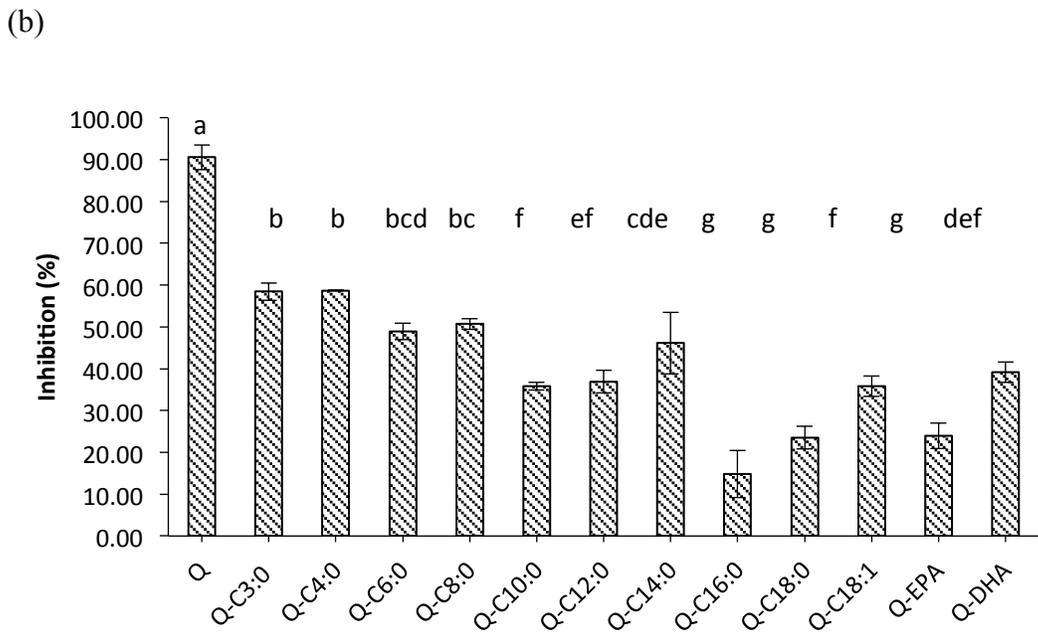
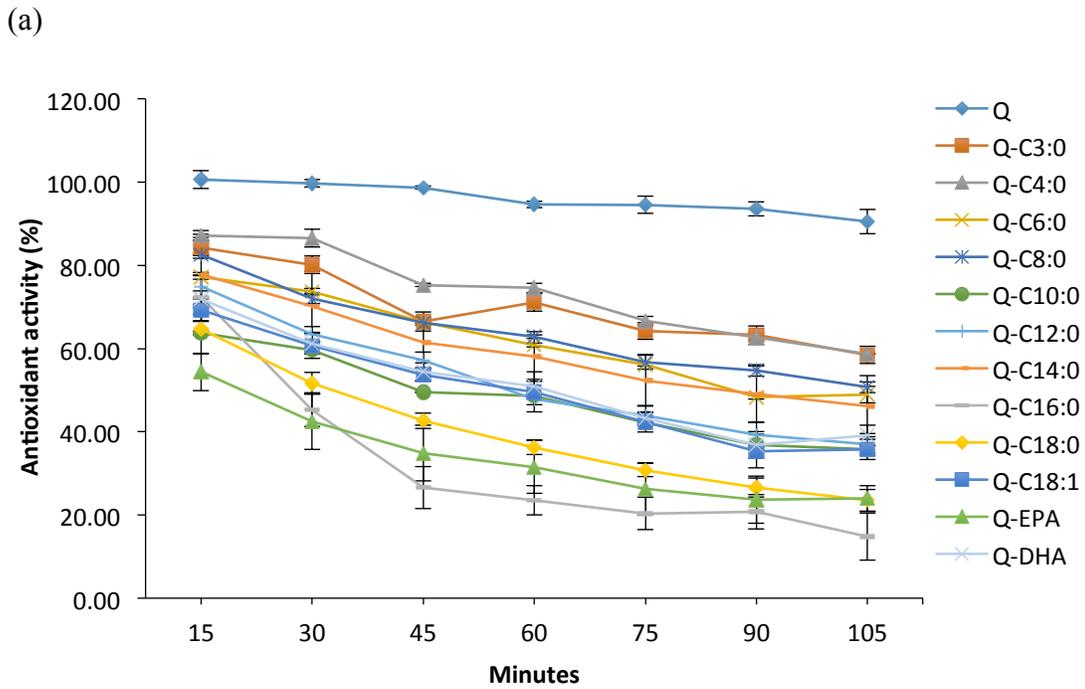


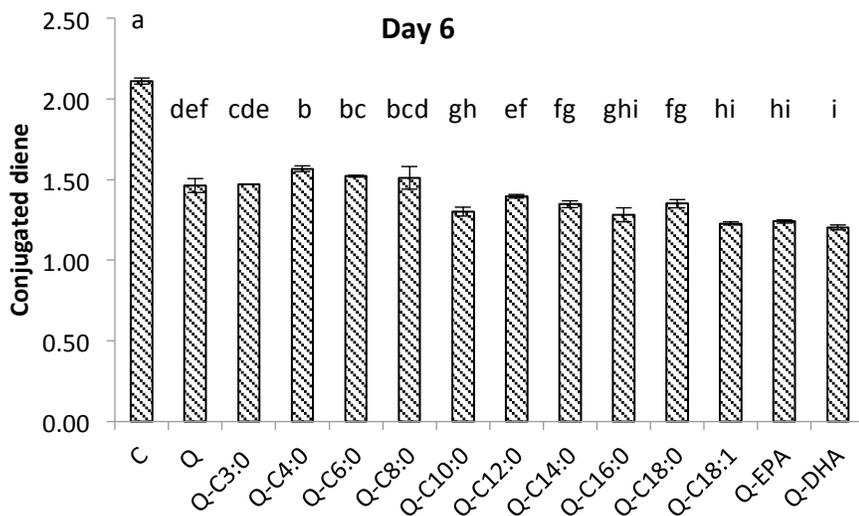
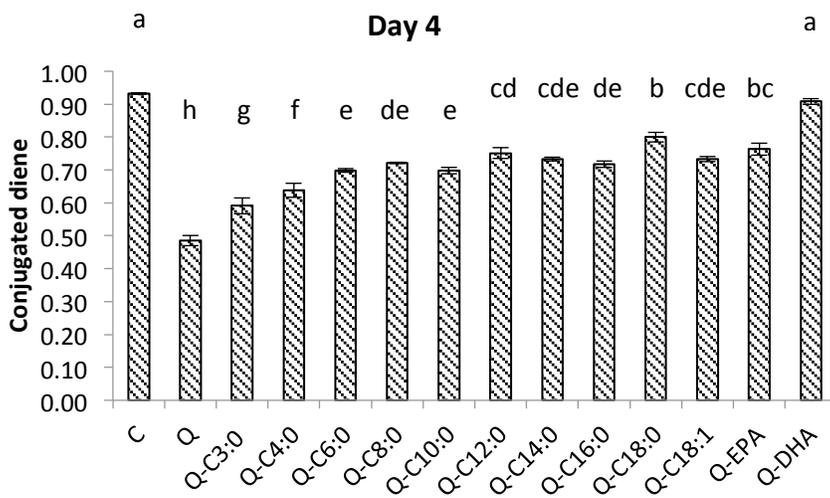
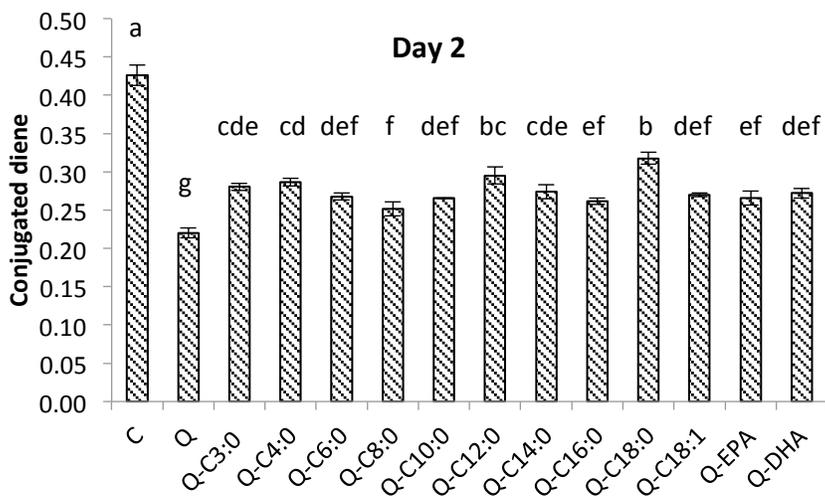
Figure 8.6. Antioxidant activities of quercetin (Q) and its derivatives against β -carotene bleaching over a 105 min period (a) and inhibition against β -carotene bleaching after 105 min incubation (b) by Q and its derivatives. All determinations were repeated three times using the same starting materials and mean values and standard deviations reported. The

different letters are significantly different ($P < 0.05$). Bars with the same letters such as “a” and “ab” are not significantly different.

8.6 Antioxidant activity of quercetin derivatives in bulk oil

Corn oil is generally used for cooking purposes and its composition is well known. In addition, linoleic acid (C18:2, 47.9 %), one of the major fatty acids in corn oil, accounted for almost half of the fatty acids composition and this could generate sufficient amount of conjugated dienes (Ferrari et al., 1996). In such context, corn oil was selected in this study. The minor components in corn oil were removed by a stripping process to minimise their effect on oil oxidation. Conjugated dienes could be considered as primary oxidation products, like hydroperoxides, and these compounds could be broken down to form secondary oxidation products. Therefore, conjugated dienes and *p*-anisidine value were measured for both primary and secondary oxidative products, respectively. Figure 8.7 indicates the conjugated dienes formation of stripped corn oil over an 8-day period in the presence of quercetin and its derivatives. All test compounds were able to inhibit the formation of conjugated dienes throughout the entire 8-day period compared to the control. On day 2, while quercetin showed the highest inhibition among all, Q-C18:0 exhibited the least inhibition. On day 4, quercetin was still most effective in inhibiting the formation of conjugated dienes. In contrast, Q-DHA showed an insignificant difference compared to the control. However, quercetin with medium- and long-chain fatty acids showed a better inhibition of the conjugated dienes formation compared to the parent molecule on day 6. On day 8, Q-C18:1 and Q-DHA showed a similar inhibition compared

to quercetin. This might be due to the increased lipophilicity, which could improve the affinity to the oil. Although the quercetin derivatives had lower concentration due to the heavier molecular weight compared to quercetin, the enhanced affinity might overcome the concentration gap. In addition, quercetin with long-chain fatty acids displayed a better inhibition compared to quercetin with short-chain fatty acids on days 6 and 8. This was an opposite trend compared to the antioxidant activity in the oil-in-water emulsion system. Furthermore, this result also contradicted the polar paradox.



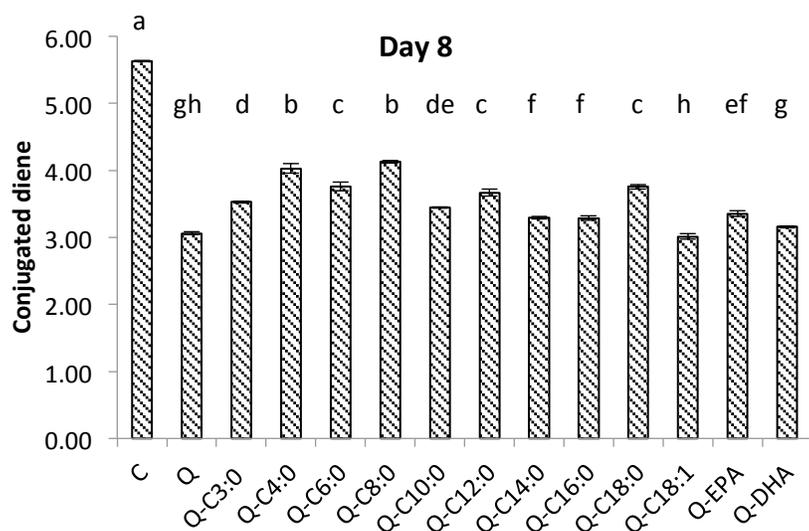


Figure 8.7. Conjugated dienes formation of stripped corn oil over an 8-day period in the presence of quercetin and its derivatives. All determinations were repeated three times using the same starting materials and mean values and standard deviations reported. The different letters are significantly different ($P < 0.05$). Bars with the same letters such as “a” and “ab” are not significantly different.

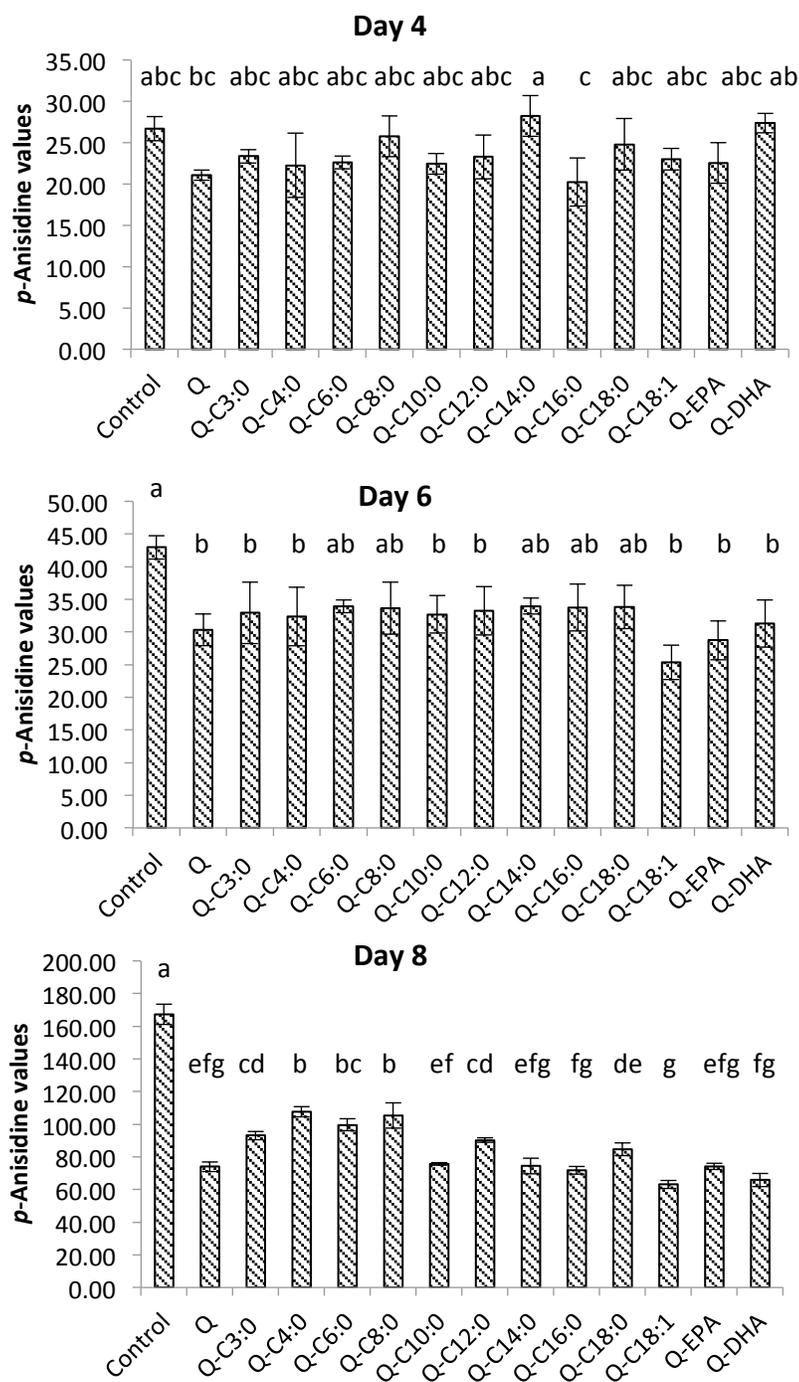


Figure 8.8. p-Anisidine values of stripped corn oil over an 8-day period in the presence of quercetin and its derivatives. All determinations were repeated three times and mean

values and standard deviations reported. The different letters are significantly different ($P < 0.05$). Bars with the same letters such as “a” and “ab” are not significantly different.

Meanwhile, Figure 8.8 indicates *p*-anisidine values of stripped corn oil over an 8-day period in the presence of quercetin and its derivatives. The *p*-anisidine values exhibited a lag phase of 2 days (data not shown). On day 4, all test compounds showed an insignificant difference compared to the control. On day 6, the test compounds showed lower *p*-anisidine values than the control. Among the test compounds, Q-C18:1 had the lowest *p*-anisidine value. On day 8, quercetin with long-chain fatty acids showed lower *p*-anisidine values than quercetin with short-chain fatty acids and this was as effective as the quercetin itself. The correlation coefficients between the formation of conjugated dienes and *p*-anisidine values on days 4, 6, and 8 were 0.63, 0.82, and 0.98, respectively.

8.7 Inhibition of copper-induced low-density lipoprotein (LDL) oxidation

The oxidized LDL has been in the spotlight due to the association with coronary heart disease. It can be involved in different steps of atherosclerosis such as accumulating in the endothelium cells and forming foam cells (Young and McEneny, 2001). In addition, circulating oxidized LDL has been proposed as a marker for coronary artery disease (Holvoet et al., 2001) and prediction of the risk of heart attack (Holvoet, 2004). Hence, controlling the oxidation of LDL has been very important to decrease the incidence of heart disease. The antioxidant activity of quercetin towards LDL oxidation has well been reported (Frankel et al., 1993; Frémont et al., 1999; Aviram and Fuhrman, 2002). Hence, it was of interest to evaluate how the esterification affects the inhibition of quercetin

against LDL oxidation. In this study, copper was used to induce the LDL oxidation. As shown in Figure 8.9, quercetin showed the highest inhibition among all tested samples ($P > 0.05$). Other studies have been demonstrated that quercetin showed radical scavenging activity as well as metal chelating activity (Rice-Evans et al., 1996; Mira et al., 2002, Frémont et al., 1999). Therefore, the inhibition of quercetin against LDL oxidation might be through a combination of radical scavenging and metal chelating activities. Among quercetin derivatives, quercetin with short-chain fatty acids exhibited lower inhibition than other quercetin derivatives. In addition, although there were big drops (Q-C12:0 and Q-C18:0), quercetin derivatives showed increased inhibition of LDL oxidation as carbon chain length increased. Moreover, quercetin with long-chain fatty acids (Q-C14:0, Q-C16:0, Q-C18:1, Q-EPA, and Q-DHA) showed an insignificant difference compared to quercetin itself ($P > 0.05$). In fact, inhibition of quercetin against LDL oxidation was expected to be higher than quercetin derivatives because quercetin derivatives lost their hydroxyl groups and hence favourable structure for metal chelation, due to esterification. In addition, the samples were prepared by weight (5 $\mu\text{g/mL}$), therefore quercetin derivatives, which have heavier molecular weight than quercetin itself due to the esterification, had lower molar concentration and this could lead to a lower inhibition compared to that of the parent molecule. Despite the lower number of the hydroxyl groups and lower concentration of quercetin derivatives, the improved inhibition might be due to the increased lipophilicity. Phenolic compounds esterified with fatty acids showed better inhibition of LDL oxidation compared to that of the parent molecule (Zhong and Shahidi, 2012; Oh and Shahidi, 2018). According to Zhong and Shahidi (2012), the increased lipophilicity might improve the affinity to the surface of LDL, which are

phospholipids. It seemed the improved lipophilicity was strong enough to compromise the factors, namely number of hydroxyl groups and different concentration, which could lower the antioxidant activity of quercetin derivatives in this study.

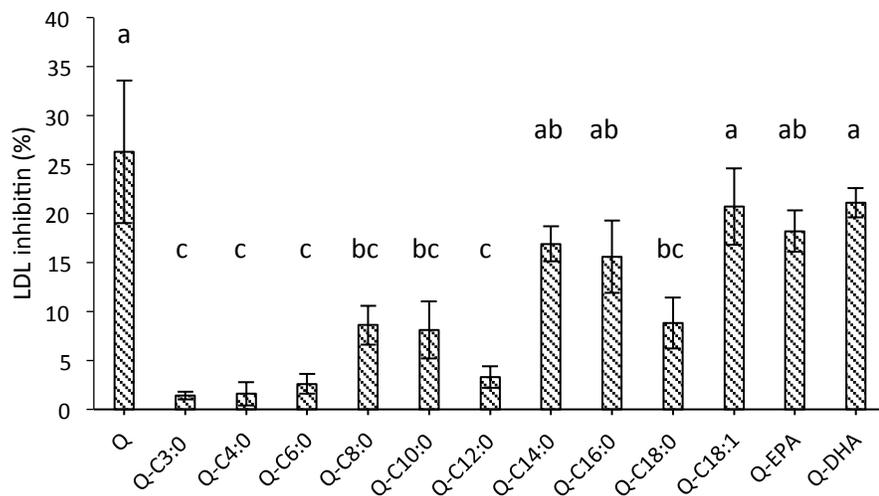


Figure 8.9. Inhibition (%) of quercetin and its derivatives against LDL oxidation induced by copper. All determinations were repeated three times and mean values and standard deviations reported. The different letters are significantly different ($P < 0.05$). Bars with the same letters such as “a” and “ab” are not significantly different.

8.8 Inhibition of hydroxyl radical-induced DNA scission

Damaged DNA is a well-known cause of mutations which can lead to cancer. There are several ways to cause damaged DNA, one of which is because of the reactive oxygen species (ROS). Among ROS, hydroxyl radical, which is constantly produced in the body, is the most powerful oxidant (Yu, 1994). Thus, there is an interest to prevent oxidation of DNA. Inhibition against hydroxyl radical-induced DNA scission was carried out to evaluate the inhibitory capacity of quercetin and its derivatives. As shown in Figure 8.10, all test compounds showed 68.5-88.3% inhibition of DNA oxidation, among which Q-C3:0 exhibited the highest inhibition. However, there was an insignificant difference of this compared with quercetin, Q-C4:0, Q-C6:0, and Q-C16:0 ($p > 0.05$). Interestingly, Q-C3:0 showed the highest radical scavenging activity among all tested samples for hydroxyl radical scavenging effect with significant difference (see section 8.3). The correlation coefficient between the hydroxyl radical scavenging activity and inhibition of hydroxyl radical-induced DNA scission was 0.695. According to Mukaka (2012), a correlation coefficient from 0.7 to 0.9 could be interpreted as being highly positive. Although hydroxyl radical scavenging activity of quercetin and its derivatives showed a different type of signals from EPR spectrum in hydroxyl radical scavenging assay and the method was deemed unreliable by Hiramoto et al. (1996) (see section 8.3); the results between hydroxyl radical scavenging activity and inhibition of DNA scission induced by hydroxyl radical showed a positive correlation. In addition, the inhibition against hydroxyl radical-induced DNA scission showed a decreasing pattern as chain length increased. This could be due to the varying concentration, which was already mentioned above. A similar pattern was observed by Oh and Shahidi (2018) who studied the

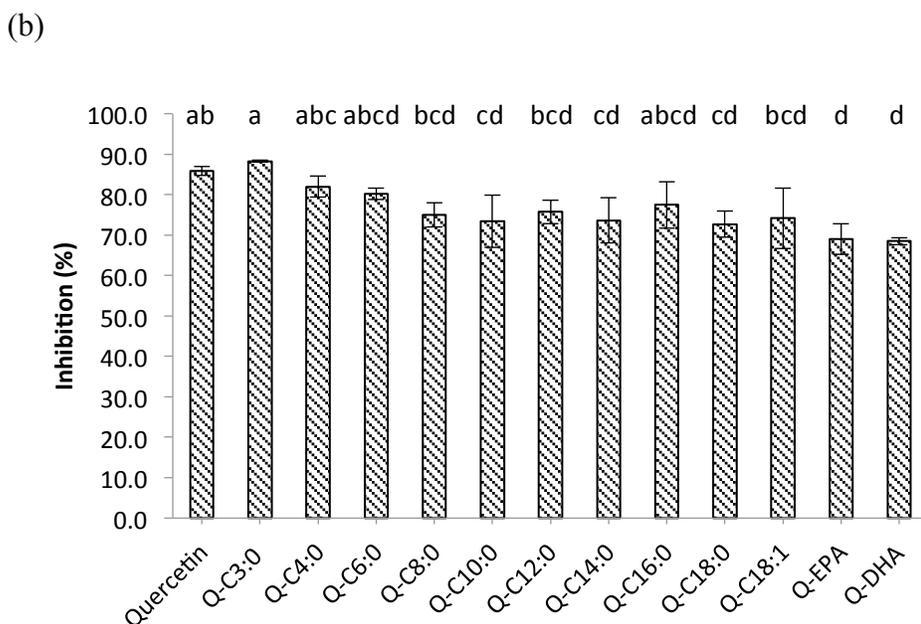
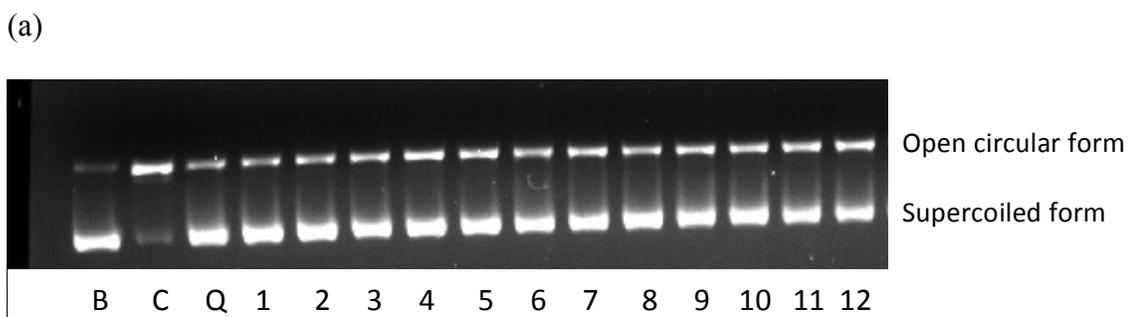


Figure 8.10. (a) DNA scission induced by hydroxyl radical (B, DNA only; C, DNA + $\text{FeSO}_4 + \text{H}_2\text{O}_2$; Q, quercetin; 1, Q-C3:0; 2, Q-C4:0; 3, Q-C6:0; 4, Q-C8:0; 5, Q-C10:0; 6, Q-C12:0; 7, Q-C14:0; 8, Q-C16:0; 9, Q-C18:0; 10, Q-C18:1; 11, Q-EPA; 12, Q-DHA) and (b) their inhibition (%). All determinations were repeated three times using the same combined starting materials and mean values and standard deviations reported. The different letters are significantly different ($P < 0.05$). Bars with the same letters such as “a” and “ab” are not significantly different. The visualisation of DNA bands was obtained using the Alpha-Imager gel documentation system (Cell Biosciences, Santa Clara, CA,

USA) and the data processing was performed with the Chemi-Imager 4400 software (Cell Biosciences).

inhibition of resveratrol and its derivatives against DNA scission induced by hydroxyl radical and resveratrol with short-chain fatty acids were better than resveratrol with long-chain fatty acids. Sun et al. (2018) also reported that hydroxytyrosol with short-chain fatty acids showed better inhibition of DNA scission than other derivatives except for that of hydroxytyrosol with stearic acid. However, they reported that the inhibition of tyrosol with medium-chain fatty acids was higher than the other derivatives.

8.9 Summary

The application of quercetin and its derivatives was evaluated in selected food and biological model systems. The test compounds showed the antioxidant activity in a system-dependent manner. Quercetin showed the highest antioxidant activity in DPPH radical scavenging activity, reducing power, and an oil-in-water emulsion system. Moreover, quercetin with short-chain fatty acids exhibited a better antioxidant activity than other derivatives in those assays. On the other hand, some quercetin derivatives displayed a similar or better antioxidant activity than the parent molecule in hydroxyl radical scavenging activity, bulk oil system, inhibition of LDL oxidation, and inhibition of DNA scission. In this study, both oil-in-water emulsion and bulk oil systems contradicted the polar paradox theory. These results demonstrate that quercetin and its derivatives may be used as possible antioxidants in food and biological systems.

CHAPTER 9

Summary and recommendations

This study evaluated the effect of lipophilisation of two selected phenolic compounds, namely resveratrol and quercetin, for their potential extended application in food, nutraceuticals, cosmetics, and possibly pharmaceuticals.

The resveratrol derivatives were prepared via esterification with 12 different fatty acids with the corresponding acyl chlorides. The main products were resveratrol-3- and resveratrol-4'-monoesters and this was confirmed by HPLC-MS and NMR spectroscopy ($^1\text{H-NMR}$, $^{13}\text{C-NMR}$, COSY, HSQC, NOESY, and HMBC). The lipophilicity of resveratrol derivatives was better than the parent molecule which also exhibited the highest DPPH and ABTS radical scavenging activities among all derivatives. However, the radical scavenging activities of resveratrol with long-chain fatty acids (RC18:0 and RC18:1) in the assay, using the lipophilic DPPH, were 87 and 95%, respectively, compared to resveratrol. Thus, resveratrol derivatives could be used as powerful antioxidants in lipophilic systems. Therefore, we expanded our investigation to food and biological model systems. The results were varied depending on the test system employed. Some resveratrol derivatives showed a similar and/or better antioxidant activity than resveratrol in hydrogen peroxide scavenging activity, bulk oil, cooked ground pork model system, inhibition of LDL oxidation, and inhibition of DNA scission. This work demonstrated that resveratrol derivatives could serve as possible antioxidants in food and biological systems. Two selected resveratrol derivatives, namely RC3:0 and RDHA were

evaluated further for their potential antioxidant, anti-inflammatory, and antiproliferative activities in A431, MCF7, HT-29, and AGS cell lines.

Resveratrol, RC3:0, and RDHA were able to attenuate NO production in murine RAW 264.7 cells. Moreover, these compounds could decrease the cell viability of A431, MCF7, HT-29, and AGS. Resveratrol and RDHA were investigated further, and they were found to decrease ROS generation in HepG2 cells. These findings implied that some of the resveratrol derivatives might be used as antioxidants, possibly anti-inflammatory and anticancer agents but this needs to be further confirmed in future studies.

Meanwhile, quercetin derivatives were also prepared with the fatty acyl chlorides of different chain lengths and degrees of unsaturation. The quercetin derivatives produced were mainly Q-3'-O-monoester, Q-3-O-monoester, Q-7,3'-O-diester, Q-3',4'-O-diester, and Q-3,4'-O-diester and this was confirmed by HPLC-MS and ¹H NMR. The lipophilicity of quercetin derivatives was superior to that of quercetin itself. In terms of antioxidant activity, quercetin showed the highest antioxidant activity among all tested samples in ABTS radical scavenging activity, DPPH radical scavenging activity, reducing power, and an oil-in-water emulsion system. In addition, quercetin with short-chain fatty acids showed a better antioxidant activity than other quercetin derivatives in those assays. On the other hand, some quercetin derivatives exhibited a similar and/or better antioxidant activity than quercetin itself in hydroxyl radical scavenging activity, bulk oil system, inhibition of LDL oxidation, and inhibition of DNA scission. The results suggested that quercetin derivatives might serve as potential antioxidants in food and biological systems.

Taking all together, the findings of this work suggest that lipophilisation of resveratrol and quercetin could potentially enhance their application in lipophilic media of food and biological systems. It is also expected that the lipophilisation can also improve their bioavailability because it can increase the affinity to the cell membrane. In such context, they may be used as functional food ingredients and nutraceuticals for health promotion and disease risk reduction. To that end, more studies should be carried out on 1) bioactivities both *ex vivo* and *in vivo*; 2) toxicities; 3) bioavailabilities, including absorption and metabolism. Moreover, practical aspects such as the economy of the process need further evaluation.

This study will provide a better understanding of the antioxidant behaviour of lipophilised resveratrol and quercetin in different systems. Moreover, to the best of our knowledge, this is the first work incorporating short-, medium-, and long-chain fatty acids, especially the omega-3 PUFA into the resveratrol and quercetin molecules. This can also fill the existing gap in the knowledge about the effect of fatty acid chain length and degree of unsaturation of lipophilised resveratrol and quercetin on their antioxidant properties. Moreover, this study provides an opportunity for the expanded use of polyphenols tested which will add value to food and nutraceutical industry.

Publications included in this thesis

1. **Won Young Oh**, Fereidoon Shahidi. (2017). Lipophilization of resveratrol and effects on antioxidant activities. *Journal of Agricultural and Food Chemistry*, 65, 8617-8625 (Chapter 4).
2. **Won Young Oh**, Fereidoon Shahidi. (2018). Antioxidant activity of resveratrol ester derivatives in food and biological model systems. *Food Chemistry*, 261, 267-273 (Chapter 5).

Permission to use copyrighted material



RightsLink®

Home

Create Account

Help



ACS Publications
Most Trusted. Most Cited. Most Read.

Title: Lipophilization of Resveratrol and Effects on Antioxidant Activities
Author: Won Young Oh, Fereidoon Shahidi
Publication: Journal of Agricultural and Food Chemistry
Publisher: American Chemical Society
Date: Oct 1, 2017
Copyright © 2017, American Chemical Society

LOGIN

If you're a **copyright.com user**, you can login to RightsLink using your copyright.com credentials. Already a **RightsLink user** or want to [learn more?](#)

PERMISSION/LICENSE IS GRANTED FOR YOUR ORDER AT NO CHARGE

This type of permission/license, instead of the standard Terms & Conditions, is sent to you because no fee is being charged for your order. Please note the following:

- Permission is granted for your request in both print and electronic formats, and translations.
- If figures and/or tables were requested, they may be adapted or used in part.
- Please print this page for your records and send a copy of it to your publisher/graduate school.
- Appropriate credit for the requested material should be given as follows: "Reprinted (adapted) with permission from (COMPLETE REFERENCE CITATION). Copyright (YEAR) American Chemical Society." Insert appropriate information in place of the capitalized words.
- One-time permission is granted only for the use specified in your request. No additional uses are granted (such as derivative works or other editions). For any other uses, please submit a new request.

BACK

CLOSE WINDOW

Copyright © 2018 [Copyright Clearance Center, Inc.](#) All Rights Reserved. [Privacy statement.](#) [Terms and Conditions.](#) Comments? We would like to hear from you. E-mail us at customer@copyright.com



Title: Antioxidant activity of resveratrol ester derivatives in food and biological model systems

Author: Won Young Oh, Fereidoon Shahidi

Publication: Food Chemistry

Publisher: Elsevier

Date: Available online 21 March 2018

© 2018 Published by Elsevier Ltd.

LOGIN

If you're a **copyright.com user**, you can login to RightsLink using your copyright.com credentials. Already a **RightsLink user** or want to [learn more?](#)

Please note that, as the author of this Elsevier article, you retain the right to include it in a thesis or dissertation, provided it is not published commercially. Permission is not required, but please ensure that you reference the journal as the original source. For more information on this and on your other retained rights, please visit: <https://www.elsevier.com/about/our-business/policies/copyright#Author-rights>

BACK

CLOSE WINDOW

Copyright © 2018 [Copyright Clearance Center, Inc.](#) All Rights Reserved. [Privacy statement](#). [Terms and Conditions](#).
Comments? We would like to hear from you. E-mail us at customercare@copyright.com

Reference

- Agullo, G., Garnet, L., Besson, C., Demigné, C. and Rémésy, C. (1994). Quercetin exerts a preferential cytotoxic effect on active dividing colon carcinoma HT29 and Caco-2 cells. *Cancer Letters*, 87, 55-63.
- Ahmad, K. A., Clement, M. -V. and Pervaiz, S. (2003). Pro-oxidant activity of low doses of resveratrol inhibits hydrogen peroxide induced apoptosis. *Annals of the New York Academy of Sciences*, 1010, 365-373.
- Aires, V., Limagne, E., Cotte, A. K., Latruffe, N., Ghiringhelli, F. and Delmas, D. (2013). Resveratrol metabolites inhibit human metastatic colon cancer cells progression and synergize with chemotherapeutic drugs to induce cell death. *Molecular Nutrition & Food Research*, 57, 1170-1181.
- Alemán, M., Bou, R., Guardiola, F., Durand, E., Villeneuve, P., Jacobsen, C. and Sørensen, A. -D. M. (2015) Antioxidative effect of lipophilized caffeic acid in fish oil enriched mayonnaise and milk. *Food Chemistry*, 167, 236-244.
- Ambigaipalan, P., Al-Khalifa, A. S. and Shahidi, F. (2015). Antioxidant and angiotensin I converting enzyme (ACE) inhibitory activities of date seed protein hydrolysates prepared using alcalase, flavourzyme and thermolysin. *Journal of Functional Foods*, 18, 1125-1137.
- Ambigaipalan, P. and Shahidi, F. (2015) Antioxidant potential of date (*Phoenix dactylifera L.*) seed protein hydrolysates and carnosine in food and biological systems. *Journal of Agricultural and Food Chemistry*, 63, 864-871.

- AOAC. (1980). Official and Tentative Method of the AOAC. 4th ed. Method Ti 1a-64. American Oil Chemists' Society Press: Champaign ,IL, USA.
- AOAC. (1990). Official and Tentative Method of the AOAC. 4th ed. Method Cd 18-90. American Oil Chemists' Society Press: Champaign ,IL, USA.
- Aviram, M. and Fuhrman, B. (2002). Wine flavonoids protect against LDL oxidation and atherosclerosis. *Annals of the New York Academy of Sciences*, 957, 146-161.
- Ayouni, L., Cazorla, G., Chaillou, D., Herbreteau, B., Rudaz, S., Lantéri, P. and Carrupt, P. A. (2005). Fast determination of lipophilicity by HPLC. *Chromatographia*, 62, 251-255.
- Bach, A. C. and Babayan, V. K. (1982). Medium-chain triglycerides: an update. *The American Journal of Clinical Nutrition*, 36, 950-962.
- Baur, J. A., Pearson, K. J., Price, N. L., Jamieson, H. A., Lerin, C., Kalra, A., Prabhu, V. V., Allard, J. S., Lopez-Lluch, G., Lewis, K., Pistell, P. J., Poosala, S., Becker, K. G., Boss, O., Gwinn, D., Wang, M., Ramaswamy, S., Fishbein, K. W., Spencer, R. G., Lakatta, E. G., Couteur, D. L., Shaw, R. J., Navas, P., Puigserver, P., Ingram, D. K., de Cabo, R. and Sinclair, D. A. (2006). Resveratrol improves health and survival of mice on a high-calorie diet. *Nature*, 444, 337-342.
- Baur, J. A. and Sinclair, D. A. (2006). Therapeutic potential of resveratrol: the *in vivo* evidence. *Nature Reviews Drug Discovery*, 5, 493-506.
- Belguendouz, L., Frémont, L. and Gozzelino, M. -T. (1998). Interaction of trans-

- resveratrol with plasma lipoproteins. *Biochemical Pharmacology*, 55, 811-816.
- Bhandari, M. R., Nilubon, J. A., Gao, H. and Kawabata, J. (2008). α -Glucosidase and α -amylase inhibitory activities of Nepalese medicinal herb Pakhanbhed (*Bergenia ciliata* Haw.). *Food Chemistry*, 106, 247-252.
- Bi, X. L., Yang, J. Y., Dong, Y. X., Wang, J. M., Cui, Y. H., Ikeshima, T., Zhao, Y. Q. and Wu, C. F. (2005). Resveratrol inhibits nitric oxide and TNF- α production by lipopolysaccharide-activated microglia. *International Immunopharmacology*, 5, 185-193.
- Biasutto, L., Marotta, E., Marchi, U. D., Zoratti, M. and Paradisi, C. (2007). Ester-based precursors to increase the bioavailability of quercetin. *Journal of Medicinal Chemistry*, 50, 241-253.
- Biasutto, L., Mattarei, A., Marotta, E., Bradaschia, A., Sassi, N., Garbisa, S., Zoratti, M. and Paradisi, C. (2008). Development of mitochondria-targeted derivatives of resveratrol. *Bioorganic & Medicinal Chemistry Letters*, 18, 5594-5597.
- Bors, W., Heller, W. and Saran, H. (1990). Flavonoids as antioxidants: determination of radical scavenging efficiencies. *Methods in Enzymology*, 186, 343-355.
- Bove, K., Lincoln, D. W. and Tsan, M. -F. (2002). Effect of resveratrol on growth of 4T1 breast cancer cells *in vitro* and *in vivo*. *Biochemical and Biophysical Research Communications*, 291, 1001-1005.
- Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews*, 56, 317-333.

- Bruice, P. Y. (2011). Electronic structure and bonding • acids and bases. In Organic chemistry (pp. 25-33). Prentice hall: Glenview, IL, USA.
- Brüssow, H. and Parkinson, S. J. (2014). You are what you eat. *Nature Biotechnology*, 32, 243-245.
- Bukhari, S. B., Memon, S., Tahir, M. M. and Bhanger, M. I. (2008). Synthesis, characterization and investigation of antioxidant activity of cobalt-quercetin complex. *Journal of Molecular Structure*, 892, 39-46.
- Bureau, G., Longpré, F. and Martinoli, M. -G. (2008). Resveratrol and quercetin, two natural polyphenols, reduce apoptotic neuronal cell death induced by neuroinflammation. *Journal of Neuroscience Research*, 86, 403-410.
- Burns, J., Yokota, T., Ashihara, H., Lean, M. E. J. and Crozier, A. (2002). Plant foods and herbal sources of resveratrol. *Journal of Agricultural and Food Chemistry*, 50, 3337-3340.
- Cai, Y., Luo, Q., Sun, M. and Corke, H. (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sciences*, 74, 2157-2184.
- Cardile, V., Lombardo, L., Spatafora, C. and Tringali, C. (2005). Chemo-enzymatic synthesis and cell-growth inhibition activity of resveratrol analogues. *Bioorganic Chemistry*, 33, 22-33.
- Caruso, F., Tanski, J., Villegas-Estrada, A. and Rossi, M. (2004). Structural basis for antioxidant activity of trans-resveratrol: ab initio calculations and crystal and

- molecular structure. *Journal of Agricultural and Food Chemistry*, 52, 7279-7285.
- Cesa, S. (2004). Malondialdehyde contents in infant milk formulas. *Journal of Agricultural and Food Chemistry*, 52, 2119-2122.
- Chandrasekara, A. and Shahidi, F. (2010). Content of insoluble bound phenolics in millets and their contribution to antioxidant capacity. *Journal of Agricultural and Food Chemistry*, 58, 6706-6714.
- Chandrasekara, A. and Shahidi, F. (2011). Inhibitory activities of soluble and bound millet seed phenolics on free radicals and reactive oxygen species. *Journal of Agricultural and Food Chemistry*, 59, 428-436.
- Chandrasekara, A. and Shahidi, F. (2012). Antioxidant phenolics of millet control lipid peroxidation in human LDL cholesterol and food systems. *Journal of the American Oil Chemists' Society*, 89, 275-285.
- Chao, J., Li, H., Cheng, K. W., Yu, M. S., Chang, R. C. C. and Wang, M. (2010). Protective effects of pinostilbene, a resveratrol methylated derivative, against 6-hydroxydopamine-induced neurotoxicity in SH-SY5Y cells. *Journal of Nutritional Biochemistry*, 21, 482-489.
- Chiou, Y. -S., Wu, J. -C., Huang, Q., Shahidi, F., Wang, Y. -J., Ho, C. -T. and Pan, M. -H. (2014). Metabolic and colonic microbiota transformation may enhance the bioactivities of dietary polyphenols. *Journal of Functional Food*, 7, 3-25.
- Choe, E. and Min, D. B. (2006). Mechanisms and factors for edible oil oxidation. *Comprehensive Reviews in Food Science and Food Safety*, 5, 169-186.

- Cichocki, M., Paluszczak, J., Szafer, H., Piechowiak, A., Rimando, A. M. and Baer-Dubowska, W. (2008). Pterostilbene is equally potent as resveratrol in inhibiting 12-O-tetradecanoylphorbol-13-acetate activated NF κ B, AP-1, COX-2, and iNOS in mouse epidermis. *Molecular Nutrition and Food Research*, 52, 62-70.
- Clifford, M. N. (2004). Diet-derived phenols in plasma and tissues and their implications for health. *Planta Medica*, 70, 1103-1114.
- Coleman, J. W. (2001). Nitric oxide in immunity and inflammation. *International Immunopharmacology*, 1, 1397-1406.
- Coskun, O., Kanter, M., Korkmaz, A. and Oter, S. (2005). Quercetin, a flavonoid antioxidant, prevents and protects streptozotocin-induced oxidative stress and β -cell damage in rat pancreas. *Pharmacological Research*, 51, 117-123.
- Dai, J. and Mumper, R. J. (2010). Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules*, 15, 7313-7352.
- Danihelová, M., Veverka, M., Šturdík, E. and Jantová, S. (2013). Antioxidant action and cytotoxicity on HeLa and NIH-3T3 cells of new quercetin derivatives. *Interdisciplinary Toxicology*, 6, 209-216.
- Dercks, W. and Creasy, L. L. (1989). Influence of fosetyl-A1 on phytoalexin accumulation in the *Plasmopara viticola*-grapevine interaction. *Physiological Molecular Plant Pathology*, 34, 203-213.
- Davidov-Pardo, G. and McClements, D. J. (2014). Resveratrol encapsulation: Designing delivery systems to overcome solubility, stability and bioavailability issues. *Trends*

in Food Science & Technology, 38, 88-103.

Day, A. J., Canada, F. J., Diaz, J. C., Kroon, P. A., Mclauchlan, R., Faulds, C. B., Plumb, G. W., Morgan, M. R. A. and Williamson, G. (2000). Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. *Federation of European Biochemical Societies Letters*, 468, 166-170.

Devínský, F., Kopecka-Leimanová, A., Šeršeň, F. and Balgavý, P. (1990). Cutoff effect in antimicrobial activity and in membrane perturbation efficiency of the homologous series of N,N-dimethylamine oxides. *Journal of Pharmacy and Pharmacology*, 42, 790-794.

Donnelly, L. E., Newton, R., Kennedy, G. E., Fenwick, P. S., Leung, R. H. F., Ito, K., Russell, R. E. K. and Barnes, P. J. (2004). Anti-inflammatory effects of resveratrol in lung epithelial cells: molecular mechanisms. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 287, 774 -783.

Egert, S., Bosy-Westphal, A., Seiberl, J., Kürbitz, C., Settler, U., Plachta-Danielzik, S., Wagner, A. E., Frank, J., Schrezenmeir, J., Rimbach, G., Wolfram, S. and Müller, M. J. (2009). Quercetin reduces systolic blood pressure and plasma oxidised low-density lipoprotein concentrations in overweight subjects with a high-cardiovascular disease risk phenotype: a double-blinded, placebo-controlled cross-over study. *British Journal of Nutrition*, 102, 1065-1074.

Ferrari, R. A., Schulte, E., Esteves, W., Brühl, L. and Mukherjee, K. D. (1996). Minor

- constituents of vegetable oils during industrial processing. *Journal of the American Oil Chemists' Society*, 73, 587-592.
- Finizio, A., Vighi, M. and Sandroni, D. (1997). Determination of n-octanol/water partition coefficient (K_{ow}) of critical review and comparison of methods. *Chemosphere*, 34, 131-161.
- Fontana, L., Meyer, T. E., Klein, S. and Holloszy, J. O. (2004). Long-term calorie restriction is highly effective in reducing the risk for atherosclerosis in humans. *Proceedings of the National Academy of Sciences*, 101, 6659-6663.
- Formica, J. V. and Regelson, W. (1995). Review of the biology of quercetin and related bioflavonoids. *Food and Chemical Toxicology*, 33, 1061-1080.
- Fragopoulou, E., Nomikos, T., Karantonis, H. C., Apostolakis, C., Pliakis, E., Samiotaki, M., Panayotou, G. and Antonopoulou, S. (2007). Biological activity of acetylated phenolic compounds. *Journal of Agricultural and Food Chemistry*, 55, 80-89.
- Frankel, E. N. (1980). Lipid oxidation. *Progress in Lipid Research*. 19, 1-22.
- Frankel, E. N. (1984). Lipid oxidation: mechanisms, products and biological significance. *Journal of the American Oil Chemists' Society*, 61, 1908-1917.
- Frankel, E. N., Waterhouse, A. L. and Kinsella, J. E. (1993). Inhibition of human LDL oxidation by resveratrol. *The lancet*, 341, 1103-1104.
- Fransen, M., Nordgren, M., Wang, B. and Apanasets, O. (2012). Role of peroxisomes in ROS/RNS-metabolism: Implications for human disease. *Biochimica et Biophysica Acta*, 1822, 1363-1373.

- Frémont, L., Belguendouz, L. and Delpal, S. (1999). Antioxidant activity of resveratrol and alcohol-free wine polyphenols related to LDL oxidation and polyunsaturated fatty acids. *Life Sciences*, 64, 2511-2521.
- Fukuhara, K. and Miyata, N. (1998). Resveratrol as a new type of DNA-cleaving agent. *Bioorganic & Medicinal Chemistry Letters*, 8, 3187-3192.
- Fukuhara, K., Nagakawa, M., Nakanishi, I., Ohkubo, K., Imai, K., Urano, S., Fukuzumi, S., Ozawa, T., Ikota, N., Mochizuki, M., Miyata, N. and Okuda, H. (2006). Structural basis for DNA-cleaving activity of resveratrol in the presence of Cu (II). *Bioorganic & Medicinal Chemistry*, 14, 1437-1443.
- Fulda, S. (2010). Resveratrol and derivatives for the prevention and treatment of cancer. *Drug Discovery Today*, 15, 757-765.
- Galati, G., Moridani, M. Y., Chan, T. S. and O'Brien, P. J. (2001). Peroxidative metabolism of apigenin and naringenin versus luteolin and quercetin: glutathione oxidation and conjugation. *Free Radical Biology & Medicine*, 30, 370-382.
- Galati, G. and O'Brien, P. J. (2004). Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. *Free Radical Biology & Medicine*, 37, 287-303.
- García-Villalba, R., Carrasco-Pancorbo, A., Oliveras-Ferraros, C., Vázquez-Martín, A., Menéndez, J. A., Segura-Carretero, A. and Fernández-Gutiérrez, A. (2010). Characterization and quantification of phenolic compounds of extra-virgin olive oils with anticancer properties by a rapid and resolute LC-ESI-TOF MS method. *Journal of Pharmaceutical and Biomedical Analysis*, 51, 416-429.
- Giaginis, C. and Tsantili-Kakoulidou, A. (2008). Current state of the art in HPLC

- methodology for lipophilicity assessment of basic drugs. A Review. *Journal of Liquid Chromatography Related Technologies*, 31, 79-96.
- Gordon, M. H. (1990). The mechanism of antioxidant action *in vitro*. In Hudson, B. J. F. (Ed.), *Food Antioxidants*. Elsevier: Amsterdam.
- Gordon, M. H. and Roedig-Penman, A. (1998). Antioxidant activity of quercetin and myricetin in liposomes. *Chemistry and Physics of Lipids*. 97, 79-85.
- Guardia, T., Rotelli, A. E., Juarez, A. O. and Pelzer, L. E. (2001). Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. *Farmaco*, 56, 683-687.
- Guarente, L. and Picard, F. (2005). Calorie restriction-the SIR2 connection. *Cell*, 120, 473-482.
- Gülçin, İ. (2010). Antioxidant properties of resveratrol: A structure–activity insight. *Innovative Food Science and Emerging Technologies*, 11, 210-218.
- Hadi, S. M., Asad, S. F., Singh, S. and Ahmad, A. (2000). Putative mechanism for anticancer and apoptosis-inducing properties of plantderived polyphenolic compounds. *International Union of Biochemistry and Molecular Biology Life*, 50, 167-171.
- Halliwell, B. (1991). Reactive oxygen species in living systems: source, biochemistry, and role in human disease. *The American Journal of Medicine*, 91, 3C-14S-22S.
- Halliwell, B. (1996). Free radicals, proteins and DNA: oxidative damage versus redox regulation. *Biochemical Society Transactions*. 24, 1023-1027.

- Halliwell, B. and Aruoma, O. I. (1991). DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. *Federation of European Biochemical Societies letters*, 281, 9-19.
- Harborne, J. B. and Williams, C. A. (2000). Advances in flavonoid research since 1992. *Phytochemistry*, 55, 481-504.
- Hempel, J. and Böhm, H. (1996). Quality and quantity of prevailing flavonoid glycosides of yellow and green French beans (*Phaseolus vulgaris L.*). *Journal of Agricultural and Food Chemistry*, 44, 2114-2116.
- Herderich, M., Richling, E., Roscher, R., Schneider, C., Schwab, W., Humpf, H. -U. and Schreier, P. (1997). Application of atmospheric pressure ionization HPLC-MS-MS for the analysis of natural products. *Chromatographia*, 45, 127-132.
- Hertog, M. G. L., Feskens, E. J. M., Hollman, P. C. H., Katan, M. B. and Kromhout, D. (1993). Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *The Lancet*, 342, 1007-1011.
- Hertog M. G. L., Hollman P. C. H. and Katan M. B. (1992). Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. *Journal of Agricultural and Food Chemistry*, 40, 2379-2383.
- Hertog M. G. L., Hollman P. C. H. and van de Putte, B. (1993). Content of potentially anticarcinogenic flavonoids of tea infusions, wines and fruit juices. *Journal of Agricultural and Food Chemistry*, 41, 1242-1246.
- Hiramoto, K., Ojima, N., Sako, K. and Kukugawa, K. (1996). Effect of plant phenolics on

- the formation of the spin-adduct of hydroxyl radical and the DNA strand breaking by hydroxyl radical. *Biological & Pharmaceutical Bulletin*, 19, 558-563.
- Hollman, P. C. H., Buijsman, M. N. C. P., van Gameren, Y., Cnossen, E. P. J., de Vries, J. H. M. and Katan, M. B. (1999). The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man. *Free Radical Research*, 31, 569-573.
- Holvoet, P. (2004). Oxidized LDL and coronary heart disease. *Acta Cardiologica*, 59, 479-484.
- Holvoet, P., Mertens, A., Verhamme, P., Bogaerts, K., Beyens, G., Verhaeghe, R., Collen, D., Muls, E. and Werf, F. V. D. (2001). Circulating oxidized LDL is a useful marker for identifying patients with coronary artery disease. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 21, 844-848.
- Hollman P. C. H, Devries J. H. M, Vanleeuwen S. D, Mengelers M. J. B. and Katan, M. B. (1995). Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *The American Journal of Clinical Nutrition*, 62, 1276-1282.
- Howitz, K. T., Bitterman, K. J., Cohen, H. Y., Lamming, D. W., Lavu, S., Wood, J. G., Zipkin, R. E., Chung, P., Kisielewski, A., Zhang, L. -L., Scherer, B. and Sinclair, D. A. (2003). Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature*, 425, 191-196.
- Huber, G. M., Rupasinghe, H. V. and Shahidi, F. (2009). Inhibition of oxidation of omega-3 polyunsaturated fatty acids and fish oil by quercetin glycosides. *Food Chemistry*, 117, 290-295.
- Hussein, M. A. (2011). A convenient mechanism for the free radical scavenging activity

- of resveratrol. *International Journal of Phytomedicine*, 3, 459-469.
- Ito, N., Hirose, M., Fukushima, S., Tsuda, H., Shirai, T. and Tatematsu, M. (1986). Studies on antioxidants: their carcinogenic and modifying effects on chemical carcinogenesis. *Food and Chemical Toxicology*, 24, 1071-1082.
- Jang, J. H. and Surh, Y. J. (2001). Protective effects of resveratrol on hydrogen peroxide-induced apoptosis in rat pheochromocytoma (PC12) cells. *Mutation Research*, 496, 181-190.
- Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W. W., Fong, H. H. S., Farnsworth, N. R., Kinghorn, A. D., Mehta, R. G., Moon, R. C. and Pezzuto, J. M. (1997). Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science*, 275, 218-220.
- Jayatilake, G. S., Jayasuriya, H., Lee, E. -S., Koonchanok, N. M., Geahlen, R. L., Ashendel, C. L., McLaughlin, J. L. and Chang, C. -J. (1993). Kinase inhibitors from *Polygonum cuspidatum*. *Journal of Natural Products*, 56, 1805-1810.
- Johnson, L. A. (2002). Recovery, refining, converting, and stabilizing edible fats and oils. In Akoh, K. and Min, D. B. (Eds.), *Food lipids* (pp. 223-274). Marcel Dekker Inc.: New York, NY, USA.
- Juan, M. E., Wenzel, U., Daniel, H. and Planas, J. M. (2008). Resveratrol induces apoptosis through ROS-dependent mitochondria pathway in HT-29 human colorectal carcinoma cells. *Journal of Agricultural and Food Chemistry*, 56, 4813-4818.

- Jung, J. S. and Woo, J. S. (2016). Resveratrol induces cell death through ROS-dependent MAPK activation in A172 human glioma cells. *Journal of Life Science*, 26, 212-219.
- Jung, M. Y. and Min, D. B. (1990). Effects of α -, γ -, and δ - tocopherols on oxidative stability of soybean oil. *Journal of Food Science*, 55, 1464-1465.
- Kaidama, W. M. and Gacche, R. N. (2015). Anti-inflammatory activity of quercetin in acute and chronic phases of inflammation in guinea pigs. *American Journal of Phytomedicine and Clinical Therapeutics*, 3, 129-136.
- Kellett, M. E., Greenspan, P. and Pegg, R. B. (2018). Modification of the cellular antioxidant activity (CAA) assay to study phenolic antioxidants in a Caco-2 cell line. *Food Chemistry*, 244, 359-363.
- Khanduja, K. L., Bhardwaj, A. and Kaushik, G. (2004). Resveratrol inhibits N-nitrosodiethylamine-induced ornithine decarboxylase and cyclooxygenase in mice. *Journal of Nutrition Science Vitaminology*, 50, 61-65.
- King, R. E., Bomser, J. A. and Min, D. B. (2006). Bioactivity of resveratrol. *Comprehensive Reviews in Food Science and Food Safety*, 5, 65-70.
- Konyalioglu, S., Armagan, G., Yalcin, A., Atalayin, C. and Dagci, T. (2013). Effects of resveratrol on hydrogen peroxide-induced oxidative stress in embryonic neural stem cells. *Neural Regeneration Research*, 8, 485-495.
- Kopp, P. (1998). Resveratrol, a phytoestrogen found in red wine. A possible explanation for the conundrum of the 'French paradox'? *European Journal of Endocrinology*,

138, 619-620.

Kruidenier, L. and Verspaget, H. W. (2002). Review article: oxidative stress as a pathogenic factor in inflammatory bowel disease- radicals or ridiculous? *Alimentary Pharmacology & Therapeutics*, 16, 1997-2015.

Kumar, S. and Pandey, A. K. (2013). Chemistry and biological activities of flavonoids: an overview. *The scientific World Journal*, 2013, 1-16.

Laguerre, M., Bayrasy, C., Panya, A., Weiss, J., McClements, D. J., Lecomte, J., Decker, E. A. and Villeneuve P. (2015). What makes good antioxidants in lipid-based systems? The next theories beyond the polar paradox. *Critical Reviews in Food Science and Nutrition*, 55, 183-201.

Laguerre, M., Bayrasy, C., Lecomte, J., Chabi, B., Decker, E. A., Wrutniak-Cabello, C., Cabello, G. and Villeneuve, P. (2013). How to boost antioxidants by lipophilization? *Biochimie*, 95, 20-26.

Laguerre, M., Chen, B., Lecomte, J., Villeneuve, P., McClements, D. J. and Decker, E. A. (2011). Antioxidant properties of chlorogenic acid and its alkyl esters in stripped corn oil in combination with phospholipids and/or water. *Journal of Agricultural and Food Chemistry*, 59, 10361-10366.

Laguerre, M., Giraldo, L. J. L., Lecomte, J., Figueroa-Espinoza, M. -C., Baréa, B., Weiss, J., Decker, E. A. and Villeneuve, P. (2009). Chain length affects antioxidant properties of chlorogenate esters in emulsion: The cutoff theory behind the polar paradox. *Journal of Agricultural and Food Chemistry*, 57, 11335-11342.

Laguerre, M., Giraldo, L. J. L., Lecomte, J., Figueroa-Espinoza, M. -C. Baréa, B., Weiss,

- J., Decker, E. A. and Villeneuve, P. (2010). Relationship between hydrophobicity and antioxidant ability of “Phenolipids” in emulsion: a parabolic effect of the chain length of rosmarinate esters. *Journal of Agricultural and Food Chemistry*, 58, 2869-2876.
- Langcake, P. and Pryce, R. J. (1977). A new class of phytoalexins from grapevines. *Experientia*, 33, 151-152.
- Le Bras, M., Clément, M. -V., Pervaiz, S. and Brenner, C. (2005). Reactive oxygen species and the mitochondrial signaling pathway of cell death. *Histology Histopathology*, 20, 205-220.
- Lee, J. P., Min, B. S., An, R. B., Na, M. K., Lee, S. M., Lee, H. K., Kim, J. G., Ki, H. B. and Kang, S. S. (2003). Stilbenes from the roots of *Pleuropterus ciliinervis* and their antioxidant activities. *Phytochemistry*, 64, 759-763.
- Leopoldini, M., Russo, N., Chiodo, S. and Toscano, M. (2006). Iron chelation by the powerful antioxidant flavonoid quercetin. *Journal of Agricultural and Food Chemistry*, 54, 6343-6351.
- Locatelli, C., Rosso, R., Santos-Silva, M. C., de Souza, C. A., Licínio, M. A., Leal, P., Bazzo, M. L., Yunes, R. A. and Creczynski-Pasa, T. B. (2008). Ester derivatives of gallic acid with potential toxicity toward L1210 leukemia cells. *Bioorganic & Medicinal Chemistry*, 16, 3791-3799.
- Loft, S. and Poulsen, H. E. (1996). Cancer risk and oxidative DNA damage in man. *Journal of Molecular Medicine*, 74, 297-312.

- Lu, J., Papp, L. V., Fang, J., Rodriguez-Nieto, S., Zhivotovsky, B. and Holmgren, A. (2006). Inhibition of mammalian thioredoxin reductase by some flavonoids: implications for myricetin and quercetin anticancer activity. *Cancer Research*, 66, 4410-4418.
- Ma, C. M., Wei, Y., Wang, Z. G. and Hattori, M. (2009). Triterpenes from *Cynomorium songaricum*- Analysis of HCV protease inhibitory activity, quantification, and content change under the influence of heating. *Journal of Natural Medicines*, 63, 9-14.
- Madhujith, T. and Shahidi, F. (2006). Optimization of the extraction of antioxidative constituents of six barley cultivars and their antioxidant properties. *Journal of Agricultural and Food Chemistry*, 54, 8048-8057.
- Magalhães, L. M., Segundo, M. A., Reis, S. and Lima, J. L. F. C. (2008). Methodological aspects about *in vitro* evaluation of antioxidant properties. *Analytica chimica acta*, 613, 1-19.
- Mainini, F., Contini, A., Nava, D., Corsetto, P. A., Rizzo, A. M., Agradi, E. and Pini, E. (2013). Synthesis, molecular characterization and preliminary antioxidant activity evaluation of quercetin fatty esters. *Journal of American Oil Chemists' Society*, 90, 1751-1759.
- Manach, C., Scalbert, A., Morand, C., Rémésy, C. and Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition*, 79, 727-747.
- Manach, C., Williamson, G., Morand, C., Scalbert, A. and Rémésy, C. (2005).

- Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *The American Journal of Clinical Nutrition*, 81, 230S-242S.
- Manna, C., Galletti, P., Maisto, G., Cucciolla, V., D'Angelo, S. and Zappia, V. (2000). Transport mechanism and metabolism of olive oil hydroxytyrosol in Caco-2 cells. *Federation of European Biochemical Societies Letters*. 470, 341-344.
- Mannila, E., Talvitie, A. and Kolehmainen, E. (1993). Anti-leukaemic compounds derived from stilbenes in *Picea abies* bark. *Phytochemistry*, 33, 813-816.
- Martín, A. R., Villegas, I., Casa, C. L. and de la Lastra, C. A. (2004). Resveratrol, a polyphenol found in grapes, suppresses oxidative damage and stimulates apoptosis during early colonic inflammation in rats. *Biochemical Pharmacology*, 67, 1399-1410.
- Mattivi, F., Reniero, F. and Korhammer, S. (1995). Isolation, characterization, and evolution in red wine vinification of resveratrol monomers. *Journal of Agricultural and Food Chemistry*, 43, 1820-1823.
- Mazué, F., Colin, D., Gobbo, J., Wegner, M., Rescifina, A., Spatafora, C., Fasseur, D., Delmas, D., Meunier, P., Tringali, C. and Latruffe, N. (2010). Structural determinants of resveratrol for cell proliferation inhibition potency: experimental and docking studies of new analogs. *European Journal of Medicinal Chemistry*, 45, 2972-2980.
- McClements, D. J. (2002). Lipid-based emulsions and emulsifiers. In Akoh, K. & Min, D. B. (Eds.), *Food lipids* (pp. 63-102). Marcel Dekker Inc.: New York, NY, USA.

- McClements, D. J. (2007). Critical review of techniques and methodologies for characterization of emulsion stability. *Critical Reviews in Food Science and Nutrition*, 47, 611-649.
- McClements, D. J. and Decker, E. A. (2007). Lipids. In Damodaran, S., Parkin, K., L. and Fennema, O., R. (Eds.), *Fennema's Food Chemistry* (pp. 155-216). CRC Press: Boca Raton, FL, USA.
- Medina, I., Alcántara, D., González, M. J., Torres, P., Lucas, R., Roque, J., Plou, F. J. and Morales, J. C. (2010). Antioxidant activity of resveratrol in several fish lipid matrices: effect of acylation and glucosylation. *Journal of Agricultural and Food Chemistry*, 58, 9778-9786.
- Meyer, A. S., Heinonen, M. and Frankel E. N. (1998). Antioxidant interactions of catechin, cyanidin, caffeic acid, quercetin, and ellagic acid on human LDL oxidation. *Food Chemistry*, 61, 71-75.
- Michaelis, L., Schubert, M. P. and Smythe, C. V. (1936). Potentiometric study of the flavins. *Journal of Biological Chemistry*, 116, 587-607.
- Miller, N., Rice-Evans, C., Davies, M., Gopinathan, V. and Milner, A. (1993). A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical Science*, 84, 407-412.
- Mira, L., Fernandez, M. T., Santos, M., Rocha, R., Florêncio, M. H. and Jennings, K. R. (2002). Interactions of flavonoids with iron and copper ions: A mechanism for their antioxidant activity. *Free Radical Research*, 36, 1199-1208.

- Moalin, M., van Strijdonck, G. P. F., Beckers, M., Hagemen, G. J., Borm, P. J., Bast, A. and Haenen, G. R. M. M. (2011). A planar conformation and the hydroxyl groups in the b and c rings play a pivotal role in the antioxidant capacity of quercetin and quercetin derivatives. *Molecules*, 16, 9636-9650.
- Montenegro, L., Carbone, C., Maniscalco, C., Lambusta, D., Nicolosi, G., Ventura, C. A. and Puglisi, G. (2007). *In vitro* evaluation of quercetin-3-O-acyl esters as topical prodrugs. *International Journal of Pharmaceutics*, 336, 257-262.
- Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65, 55-63.
- Mozaffarian, D., Katan, M. B., Ascherio, A., Stampfer, M. J. and Willett, W. C. (2006). Trans fatty acids and cardiovascular disease. *The New England Journal of Medicine*, 354, 1601-1613.
- Mukaka, M. M. (2012). Statistics corner: a guide to appropriate use of correlation coefficient in medical research. *Malawi Medical Journal*, 24, 69-71.
- Murota, K., Shimizu, S., Miyamoto, S., Izumi, T., Obata, A., Kikuchi, M. and Terao, J. (2002). Unique uptake and transport of isoflavone aglycones by human intestinal caco-2 cells: comparison of isoflavonoids and flavonoids. *The Journal of Nutrition*, 132, 1956-1961.
- Oh, W. Y. and Shahidi, F. (2017). Lipophilization of resveratrol and effects on antioxidant activities. *Journal of Agricultural and Food Chemistry*, 65, 8617-8625 (Chapter 4).
- Oh, W. Y. and Shahidi, F. (2018). Antioxidant activities of resveratrol ester derivatives in

food and biological model systems. *Food Chemistry*, 261, 267-273 (Chapter 5).

Owen, R. W., Giacosa, A. Hull, W. E., Haubner, R., Spiegelhalder, B. and Bartsch, H. (2000). The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. *European Journal of Cancer*, 36, 1235-1247.

Pace-Asciak, C. R., Hahn, S., Diamandis, E. P., Soleas, G. and Goldberg, D. M. (1995). The red wine phenolics trans-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: implications for protection against coronary heart disease. *Clinica Chimica Acta*, 235, 207-219.

Pan, M. -H., Chang, Y. -H., Badmaev, V., Nagabhushanam, K. and Ho, C. -T. (2007). Pterostilbene induces apoptosis and cell cycle arrest in human gastric carcinoma cells. *Journal of Agricultural and Food Chemistry*, 55, 7777-7785.

Pan, M. -H., Sin, Y. -H., Lai, C. -S., Wang, Y. -J., Lin, J. -K., Wang, M. and Ho, C. -T. (2005). Induction of apoptosis by 1-(2-hydroxy-5-methylphenyl)-3-phenyl-1,3-propanedione through reactive oxygen species production, GADD153 expression, and caspases activation in human epidermoid carcinoma cells. *Journal of Agricultural and Food Chemistry*, 53, 9039-9049.

Panya, A., Laguerre, M., Bayrasy, C., Lecomte, J., Villeneuve, P., McClements, D. J. and Decker, E. A. (2012). An investigation of the versatile antioxidant mechanisms of action of rosmarinate alkyl esters in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, 60, 2692-2700.

Patel, K. R., Brown, V. A., Jones, D. J. L., Britton, R. G., Hemingway, D., Miller, A. S., West, K. P., Booth, T. D., Perloff, M., Crowell, J. A., Brenner, D. E., Steward, W.

- P., Gescher, A. J. and Brown, K. (2010). Clinical pharmacology of resveratrol and its metabolites in colorectal cancer patients. *Cancer Research*, 70, 7392-7399.
- Percival, M. (1998). Antioxidants. *Clinical Nutrition Insights*, 1, 1-4.
- Pereira, D. M., Calentão, P., Pereira, J. A. and Andrade, P. B. (2009). Phenolics: from chemistry to biology. *Molecules*, 14, 2202-2211.
- Pokorski, M., Marczak, M., Dymecka, A. and Suchocki, P. (2003). Ascorbyl palmitate as a carrier of ascorbate into neural tissues. *Journal of Biomedical Science*, 10, 193-198.
- Prior, R. L., Wu, X. and Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, 53, 4290-4302.
- Pulido, R., Bravo, L. and Saura-Calixto, F. (2000). Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *Journal of Agricultural and Food Chemistry*, 48, 3396-3402.
- Puupponen-Pimiä, R., Nohynek, L., Meier, C., Kähkönen, M., Heinonen, M., Hopia, A. and Oksman-Caldentey, K. -M. (2001). Antimicrobial properties of phenolic compounds from berries. *Journal of Applied Microbiology*, 90, 494-507.
- Rajalakshmi, D. and Narasimhan, S. (1996). Food antioxidants: sources and methods of evaluation. In Madhavi, D. L., Desphande, S. S. and D. K. Salunkhe (Eds.), Food

antioxidants: technological, toxicological, and health perspectives (pp. 65). Marcel Dekker Inc.: New York, NY, USA.

Rauha, J. -P., Remes, S., Heinonen, M., Hopia, A., Kähkönen, M., Kujala, T., Pihlaja, K., Vuorela, H. and Vuorela, P. (2000). Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *International Journal of Food Microbiology*, 56, 3-12.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology Medicine*, 26, 1231-1237.

Reische, D. W., Lillard, D. A. and Eitenmiller, R. R. (2002). Antioxidants. In Akoh, K. and Min, D. B. (Eds.), *Food lipids* (pp. 489–516). Marcel Dekker Inc.: New York, NY, USA.

Reyes-Duarte, D., Lopez-Cortes, N., Torres, P., Comelles, F., Parra, J. L., Peña, S., Ugidos, A. V., Ballesteros, A. and Plou, F. J. (2011). Synthesis and properties of ascorbyl esters catalyzed by lipozyme TL IM using triglycerides as acyl donors. *Journal of the American Oil Chemists' Society*, 88, 57-64.

Rice-Evans, C. A., Miller, N. J. and Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine*, 20, 933-956.

Richards, S. A. and Hollerton, J. C. (2011). 2-D proton-carbon (multiple bond) correlated spectroscopy. In *Essential practical NMR for organic chemistry* (pp. 74-135). John Wiley & Sons, Ltd.: West Sussex, United Kingdom.

- Robbins, R. J. (2003). Phenolic acids in foods: an overview of analytical methodology. *Journal of Agricultural and Food Chemistry*, 51, 2866-2887.
- Saito, S., Okamoto, Y. and Kawabata, J. (2004). Effect of alcoholic solvents on antiradical abilities of protocatechuic acid and its alkyl esters. *Bioscience, Biotechnology, and Biochemistry*, 68, 1221-1227.
- Salah, N., Miller, N. J., Paganga, G., Tijburg, L., Bolwell, G. P. and Rice-Evans, C. (1995). Polyphenolic flavonols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Archives of Biochemistry and Biophysics*, 322, 339-346.
- Sanders, T. H., McMichael, R. W. and Hendrix, K. W. (2000). Occurrence of resveratrol in edible peanuts. *Journal of Agricultural and Food Chemistry*, 48, 1243-1246.
- Sgambato, A., Ardito, R., Faraglia, B., Boninsegna, A., Wolf, F. I. and Cittadini, A. (2001). Resveratrol, a natural phenolic compound, inhibits cell proliferation and prevents oxidative DNA damage. *Mutation Research*, 496, 171-180.
- Shahidi, F. (2002, a). Phytochemicals in oilseeds. In Meskin, M. S., Bidlack, W. R., Davies, A. J. and Omaye S. T. (Eds.), *Phytochemicals in nutrition and health* (pp. 139-156). CRC PRESS: Boca Raton, FL, USA.
- Shahidi, F. (2002, b). Antioxidants in food and food antioxidants. *Molecular Nutrition & Food Research*, 44, 158-163.
- Shahidi, F. and Ambigaipalan, P. (2015). Phenolics and polyphenolics in foods, beverages and spices: antioxidant activity and health effects- a review. *Journal of Functional Foods*, 18, 820-897.

- Shahidi, F. and Hong, C. (1991). Evaluation of malonaldehyde as a marker of oxidative rancidity in meat products. *Journal of Food Biochemistry*, 15, 97-105.
- Shahidi, F. and Naczk, M. (2004). Phenolics in food and nutraceuticals (pp. 1-558). CRC Press: Boca Raton, FL, USA.
- Shahidi, F. and Wanasundara, P. K. J. (1992). Phenolic antioxidants. *Critical Reviews in Food Science and Nutrition*, 32, 7-103.
- Shahidi, F. and Wanasundara, U. N. (2002). Methods for measuring oxidative rancidity in fats and oils. In Akoh, K. and Min, D. B. (Eds.), *Food lipids* (pp. 223-274). Marcel Dekker Inc.: New York, NY, USA.
- Shahidi, F. and Yeo, J. D-. (2016). Insoluble-bound phenolics in food. *Molecules*, 21, 1-22.
- Shahidi, F. and Zhong, Y. (2010). Lipid oxidation and improving the oxidative stability. *Chemical Society Reviews*, 39, 4067-4079.
- Shahidi, F. and Zhong, Y. (2011). Revisiting the polar paradox theory: a critical overview. *Journal of Agricultural and Food Chemistry*, 59, 3499-3504.
- Shahidi, F. and Zhong, Y. (2015). Measurement of antioxidant activity. *Journal of Functional Foods*, 18, 757-781.
- Siemann, E. H. and Creasy, L. L. (1992). Concentration of the phytoalexin resveratrol in wine. *American Society for Enology and Viticulture*, 43, 49-52.
- Simmons, D. L., Botting, R. M. and Hla, T. (2004). Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. *Pharmacological Reviews*, 56,

387-437.

- Soares, D. G., Andreatza, A. C. and Salvador, M. (2003). Sequestering ability of butylated hydroxytoluene, propyl gallate, resveratrol, and vitamin C and E against ABTS, DPPH, and hydroxyl free radicals in chemical and biological systems. *Journal of Agricultural and Food Chemistry*, 51, 1077-1080.
- Sobolev, V. S. and Cole, R. J. (1999). *Trans*-Resveratrol contents in commercial peanuts and peanut products. *Journal of Agricultural and Food Chemistry*, 47, 1435-1439.
- Soleas, G. J., Diamandis, E. P. and Goldberg, D. M. (1997). Resveratrol: a molecule whose time has come? And gone? *Clinical Biochemistry*, 30, 91-113.
- Sun, Y., Zhou, D. and Shahidi, F. (2018). Antioxidant properties of tyrosol and hydroxytyrosol saturated fatty acid esters. *Food Chemistry*, 245, 1262-1268.
- Steinberg, D. (2002). Atherogenesis in perspective: hypercholesterolemia and inflammation as partners in crime. *Nature Medicine*, 8, 1211-1217.
- Stewart, A. J., Bozonnet, S., Mullen, W., Jenkins, G. I., Lean, M. E. J. and Crozier, A. (2000). Occurrence of flavonols in tomatoes and tomato-based products. *Journal of Agricultural and Food Chemistry*, 48, 2663-2669.
- Stivala, L. A., Savio, M., Carafoli, F., Perucca, P., Bianchi, L., Maga, G., Forti, L., Pagnoni, U. M., Albini, A., Prosperi, E. and Vannini, V. (2001). Specific structural determinants are responsible for the antioxidant activity and the cell cycle effects of resveratrol. *Journal of Biological Chemistry*, 276, 22586-22594.
- Stöckmann, H., Schwarz, K. and Huynh-Ba, T. (2000). The influence of various

- emulsifiers on the partitioning and antioxidant activity of hydroxybenzoic acids and their derivatives in oil-in-water emulsions. *Journal of the American Oil Chemists' Society*, 77, 535-542.
- Sugandhi, E. W., Macri, R. V., Williams, A. A., Kite, B. L., Slebodnick, C., Falkinham, J. O., Esker, A. R. and Gandour, R. D. (2007). Synthesis, critical micelle concentrations, and antimycobacterial properties of homologous, dendritic amphiphiles. Probing intrinsic activity and the “cutoff” effect. *Journal of Medicinal Chemistry*, 50, 1645-1650.
- Tapiero, H., Ba, G. N., Couvreur, P. and Tew, K. D. (2002). Polyunsaturated fatty acids (PUFA) and eicosanoids in human health and pathologies. *Biomedicine & Pharmacotherapy*, 56, 215-222.
- Tan, Z., Le, K., Moghadasian, M. and Shahidi, F. (2012). Enzymatic synthesis of phytosteryl docosahexaneates and evaluation of their anti-atherogenic effects in apo-E deficient mice. *Food Chemistry*, 134, 2097-2104.
- Tetko, I. V. and Bruneau, P. (2004). Application of ALOGPS to predict 1-octanol/water distribution coefficients, logP, and logD, of AstraZeneca in-house database. *Journal of Pharmaceutical Sciences*, 93, 3101-3110.
- Ting, Y., Jiang, Y., Ho, C. -T., Huang, Q. (2014). Common delivery systems for enhancing in vivo bioavailability and biological efficacy of nutraceuticals. *Journal of Functional Foods*. 7, 112-128.
- Torres de Pinedo, A., Peñalver, P. and Morales, J. C. (2007). Synthesis and evaluation of new phenolic-based antioxidants: structure-activity relationship. *Food Chemistry*,

103, 55-61.

Torres, P., Poveda, A., Jimenez-Barbero, J., Ballesteros, A. and Plou, F. J. (2010). Regioselective lipase-catalyzed synthesis of 3-O-acyl derivatives of resveratrol and study of their antioxidant properties. *Journal of Agricultural and Food Chemistry*, 58, 807-813.

Tresserra-Rimbau, A., Rimm, E. B., Medina-Remón, A., Martínez-González, M. A., de la Torre, R., Corella, D., Salas-Salvadó, J., Gómez-Gracia, E., Lapetra, J., Arós, F., Fiol, M., Ros, E., Serra-Majem, L., Pintó, X., Saez, G. T., Basora, J., Sorlí, J. V., Martínez, J. A., Vinyoles, E., Ruiz-Gutiérrez, V., Estruch, R., Lamuela-Raventós, R. M., on behalf of the PREDIMED Study Investigators (2014). Inverse association between habitual polyphenol intake and incidence of cardiovascular events in the PREDIMED study. *Nutrition, Metabolism & Cardiovascular Diseases*, 24, 639-647.

Tsai, S. -H., Lin-Shiau, S. -Y. and Lin, J. -K. (1999). Suppression of nitric oxide synthase and the down-regulation of the activation of NFκB in macrophages by resveratrol. *British Journal of Pharmacology*, 126, 673-680.

van den Berg, R., Haenen, G. R. M. M., van den Berg, H. and Bast, A. (1999). Applicability of an improved tolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurement of mixtures. *Food Chemistry*, 66, 511-517.

- van Duyn, M. A. S. and Pivonka, E. (2000). Overview of the health benefits of fruit and vegetable consumption for the dietetics professional: selected literature. *Journal of the American Dietetic Association*, 100, 1511-1521.
- van Duynhoven. J., Vaughan, E. E., Jacobs, D. M., Kemperman, R. A., van Velzen, E. J., Gross, G., Roger, L. C., Possemiers, S., Smilde, A. K., Dore, J., Westerhuis, J. A. and Van de, W. T. (2011). Metabolic fate of polyphenols in the human superorganism. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 4531-4538.
- Vlachogianni, I. C., Fragopoulou, E., Kostakis, I., K. and Antonopoulou, S. (2015). *In vitro* assessment of antioxidant activity of tyrosol, resveratrol and their acetylated derivatives. *Food Chemistry*, 177, 165-173.
- Vermerris, W. and Nicholson, R. (2008). Families of phenolic compounds and means of classification. In phenolic compound biochemistry (pp. 1-135). Springer: The Netherland.
- Vessal, M., Hemmati, M. and Vasei, M. (2003). Antidiabetic effects of quercetin in streptozocin-induced diabetic rats. *Comparative Biochemistry and Physiology Part C*, 135, 357-364.
- Walle, T., Hsieh, F., Delegge, M. H., Oatis, J. E. and Walle, U. K. (2004). High absorption but very low bioavailability of oral resveratrol in humans. *Drug Metabolism and Disposition*, 32, 1377-1382.

- Wanasundara, U. N. and Shahidi, F. (1999). Concentration of omega 3-polyunsaturated fatty acids of seal blubber oil by urea complexation: optimization of reaction conditions. *Food Chemistry*, 65, 41-49.
- Wang, L. N., Wang, W., Hattori, M., Daneshtalab, M. and Ma, C. M. (2016). Synthesis, anti-HCV, antioxidant and reduction of intracellular reactive oxygen species generation of a chlorogenic acid analogue with an amide bond replacing the ester bond. *Molecules*, 21, 1-9.
- Wang, M., Zhang, X., Zhong, Y. Z., Perera, N. and Shahidi, F. (2016). Antiglycation activity of lipophilized epigallocatechin gallate (EGCG) derivatives. *Food Chemistry*, 190, 1022-1026.
- Wang, Y., Catana, F., Yang, Y., Roderick, R. and Van Breemen, R. B. (2002). An LC-MS method for analyzing total resveratrol in grape juice, cranberry juice, and in wine. *Journal of Agricultural and Food Chemistry*, 50, 431-435.
- Wang, Z., Huang, Y., Zou, J., Cao, K., Xu, Y. and Wu, J. M. (2002). Effects of red wine and wine polyphenol resveratrol on platelet aggregation *in vivo* and *in vitro*. *International Journal of Molecular Medicine*, 9, 77-79.
- Watkins, S. M. and German, J. B. (2002). Unsaturated fatty acids. In Akoh, K. and Min, D. B. (Eds.), *Food lipids* (pp. 559-588). Marcel Dekker Inc.: New York, NY, USA.
- Wenzel, U., Nickel, A. and Daniel, H. (2005). Alpha-lipoic acid induces apoptosis in

- human colon cancer cells by increasing mitochondrial respiration with a concomitant O₂^{•-} generation. *Apoptosis*, 10, 359-368.
- Wettasinghe, M. and Shahidi, F. (1999). Antioxidant and free radical-scavenging properties of ethanolic extracts of defatted borage (*Borago officinalis* L.) seeds. *Food Chemistry*, 67, 399-414.
- Wiseman, H. and Halliwell, B. (1996). Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochemistry Journal*, 313, 17-29.
- Wong, J. M. W., de Souza, R., Kendall, C. W. C., Emam, A. and Jenkins, D. J. A. (2006). Colonic health: fermentation and short chain fatty acids. *Journal of Clinical Gastroenterology*, 40, 235-243.
- Witztum, J. L. (1994). The oxidation hypothesis of atherosclerosis. *The Lancet*, 344, 793-795.
- You, Q., Chen, F., Wang, X., Jiang, Y. and Lin, S. (2012). Anti-diabetic activities of phenolic compounds in muscadine against alpha-glucosidase and pancreatic lipase. *Lebensmittel-Wissenschaft & Technologie - Food Science and Technology*, 46, 164-168.
- Young, I. S. and McEneny, J. (2001). Lipoprotein oxidation and atherosclerosis. *Biochemical Society Transactions*, 29, 358-362.
- Yu, B. P. (1994). Cellular defenses against damage from reactive oxygen species. *Physiological Reviews*, 74, 139-162.
- Yu, C. W., Shin, Y. G., Chow, A., Li, Y. M., Kosmeder, J. W., Lee, Y. S., Hirschelman,

- W. H., Pezzuto, J. M., Mehta, R. G. and van Breemen, R. B. (2002). Human, rat, and mouse metabolism of resveratrol. *Pharmaceutical Research*, 19, 1907-1914.
- Zhang, M., Swarts, S. G., Yin, L., Liu, C., Tian, Y., Cao, Y., Swarts, M., Yang, S., Zhang, S. B., Zhang, K., Ju, S., Olek, D. J., Schwartz, L., Keng, P. C., Howell, R., Zhang, L. and Okunieff, P. (2011). Antioxidant properties of quercetin. In LaManna, J., Puchowicz, M., Xu, K., Harrison, D. and Bruley, D. (Eds.), Oxygen transport to tissue XXXII. Advances in experimental medicine and biology. Springer: Boston, MA, USA.
- Zhang, Y., Seeram, N. P., Lee, R., Feng, L. and Heber, D. (2008). Isolation and identification of strawberry phenolics with antioxidant and human cancer cell antiproliferative properties. *Journal of Agricultural and Food Chemistry*, 56, 670-675.
- Zheng, L. -F., Wei, Q. -Y., Cai, Y. -J., Fang, J. -G., Zhou, B., Yang, L. and Liu, Z. -L. (2006). DNA damage induced by resveratrol and its synthetic analogues in the presence of Cu (II) ions: mechanism and structure-activity relationship. *Free Radical Biology & Medicine*, 41, 1807-1816.
- Zhong, M., Cheng, G. F., Wang, W. J., Guo, Y., Zhu, X. Y. and Zhang, J. T. (1999). Inhibitory effect of resveratrol on interleukin 6 release by stimulated peritoneal macrophages of mice. *Phytomedicine*, 6, 79-84.
- Zhong, Y., Chiou, Y. -S., Pan, M. -H. and Shahidi, F. (2012). Anti-inflammatory activity of lipophilic epigallocatechin gallate (EGCG) derivatives in LPS-stimulated murine macrophages. *Food Chemistry*, 134, 742-748.

- Zhong, Y., Chiou, Y., -S., Pan, M. -H., Ho, C. -T. and Shahidi, F. (2012). Protective effects of epigallocatechin gallate (EGCG) derivatives on azoxymethane-induced colonic carcinogenesis in mice. *Journal of Functional Foods*, 4, 323-330.
- Zhong, Y., Ma, C. and Shahidi, F. (2012). Antioxidant and antiviral activities of lipophilic epigallocatechin gallate (EGCG) derivatives. *Journal of Functional Foods*, 4, 87-93.
- Zhong, Y. and Shahidi, F. (2009). Lipid oxidation and improving the oxidative stability. *Chemical Society Reviews*, 39, 4067-4079.
- Zhong, Y. and Shahidi, F. (2011). Lipophilized epigallocatechin gallate (EGCG) derivatives as novel antioxidants. *Journal of Agricultural and Food Chemistry*, 59, 6526-6533.
- Zhong, Y. and Shahidi, F. (2012). Lipophilised epigallocatechin gallate (EGCG) derivatives and their antioxidant potential in food and biological systems. *Food Chemistry*, 131, 22-30.
- Zhou, D. -Y., Sun, Y. -X. and Shahidi, F. (2017). Preparation and antioxidant activity of tyrosol and hydroxytyrosol esters. *Journal of Functional Foods*, 37, 66-73.
- Zielinska, D., Wiczkowski, W. and Piskula, M. K. (2008). Determination of the relative contribution of quercetin and its glucosides to the antioxidant capacity of onion by cyclic voltammetry and spectrophotometric methods. *Journal of Agricultural and Food Chemistry*, 56, 3524-3531.