ANTIOXIDANT AND BIOLOGICAL ACTIVITIES OF TYROSOL, HYDROXYTYROSOL AND THEIR ESTERS

by

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ABSTRACT

A series of lipophilic esters of tyrosol and hydroxytyrosol, two naturally occurring phenols in olive oil with interesting biological properties, were prepared by an enzymatic and simplified base extraction procedure. Both tyrosol and hydroxytyrosol are hydrophilic molecules with poor solubility in lipophilic media, resulting in limited usage in foods and limited uptake by the cells and bioavailability *in vivo*. The antioxidant activities of esters so produced were evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH), human low-density lipoprotein (LDL) oxidation, and DNA strand scission assays. The antiviral properties of selected esters were measured using hepatitis C virus (HCV) protease and alpha-glucosidase inhibitory activities. Antiglycation by bovine serum albumin (BSA)-glucose assay, protection effect against oxidative stress, generation anti-inflammatory products by nitrite assay, and cytotoxic properties by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay of selected esters were also evaluated. All methods used for the antioxidant activity evaluation indicated that tyrosol (TY) and its derivatives were less active than the corresponding hydroxytyrosols (HTY), which reflected the influence of the ortho-diphenolic (catechol) structure of the latter on antioxidant capacity. For hydroxytyrosol, the introduction of the lipid moiety decreased its antioxidant activity. We observed the inhibition of HTY saturated fatty acid esters against hydroxyl radical induced DNA oxidation decreased as alkyl chain length increased. Meanwhile, an unsaturation-dependent antioxidant effect was observed for TY and HTY esters in DNA strand scission assay, and for TY esters in DPPH assay. However, in the LDL oxidation assay, the polyunsaturated fatty acid (PUFA) moiety of TY esters may be oxidized. For antiviral properties of selected esters, most of the TY derivatives that showed potent inhibition on α-glucosidase were not active against HCV while HTY esters showed very good HCV protease inhibition, especially HTY caprylate, stearate

and oleate esters which displayed 3-fold stronger inhibition than that of embelin (the positive control). Moreover, it was found that lipophilization by esterification could improve the anti-inflammatory and antiglycation effects of tyrosol and hydroxytyrosol. These results indicate that the lipophilic ester derivatives can served as antioxidant ingredients in food, as well as anti-inflammatory, antiglycation, and antiviral agents, and ingredients in other therapeutic applications, supplements and natural health products.

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LIST OF ABBREVIATION

AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
ABTS	2,2′-Azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
AGEs	Advanced glycation end-products
APCI	Atmospheric pressure chemical ionization
BSA	Bovine serum albumin
CD	Conjugated dienes
CML	Carboxymethyllysine
COX-2	Cyclooxygenase-2
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	E-scan electron paramagnetic resonance

FA	Fatty acid
FBS	Fetal bovine serum
FRAP	Ferric reducing antioxidant power
H_2O_2	Hydrogen peroxide
HAT	Hydrogen atom transfer
HCV	Hepatitis C virus
HDL	High-density lipoprotein
HepG2	Human hepatoma carcinoma cells
HIV	Human immunodeficiency virus
HLB	Hydrophilic-lipophilic balance
$HO^{\bullet}$	Hydroxyl radical
HPLC-MS	High-performance liquid chromatography-mass spectrometry
HTY	2–(3,4-Dihydroxyphenyl)ethanol
iNOS	Inducible nitric oxide synthase
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUFA	Monounsaturated fatty acid
NO	Nitric oxide
$O_2^{\bullet -}$	Superoxide
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate buffer
PUFA	Polyunsaturated fatty acids

RNS	Reactive nitrogen species
$ROO^{\bullet}$	Lipid peroxyl radicals
ROS	Reactive oxygen species
SET	Single electron transfer
SFA	Saturated fatty acids
TAE	Tris−acetic acid−EDTA
t-BuOOH	tert-Butyl hydroperoxide
TLC	Thin layer chromatography
Trolox	6-Hydroxy-2,5,7,8-tetratnethylchroman-2-carboxylic acid
TY	2–(4-Hydroxyphenyl)ethanol
UFA	Unsaturated fatty acid
UV-DAD	UV diode array detector

CHAPTER 1

INTRODUCTION

The Mediterranean diet has always been considered to be very healthy due to a high content of olive oil with a multitude of benefits such as cardioprotective and anti-cancer effects. The olive oil-enriched Mediterranean diet was found to reduce the risk of different types of heart attack or cardiovascular disease complications by 50% (de Lorgeril et al., 1996). Another study enrolled individuals in a randomized way to the Mediterranean diet, supplemented with extra virgin olive oil (EVOO), over a follow-up of about 5 years, and found that adopting the Mediterranean-type diet reduced the risk of CVD complications by 30% (Estruch et al., 2013). Studies have found that the minor components of olive oil exert their anti-cancer effect by inhibiting cell proliferation and promoting apoptosis in colorectal cancer cell lines (Gill et al., 2005; Fini et al., 2008), and can prevent colon carcinomas in rats (Bartoli et al., 2000), possibly by regulating cell division associated with intestinal diamine oxidase (Wollin & Jaques, 1976). Santos-González et al. (2012) studied the effect of dietary oils on the level of plasma proteins during aging in rats using a proteomic approach, and demonstrated that the intake of a diet rich in virgin olive oil had great benefits for improving and maintaining antioxidant status, an anti-inflammatory state and an anti-atherogenic lipid profile during aging. The health benefits of olive oil are mainly due to the presence of the high content of monounsaturated fatty acid (MUFAs) and minor components with biological properties such as tocopherols, squalene and phenolics (Murkovic et al., 2004; Roufs, 2007; Silva et al., 2016).

Phenolics have been demonstrated to have antioxidant property and inhibit oxidation of biomolecules (e.g. membrane lipids, LDL, proteins and DNA), and thus prevent or inhibit pathologies such as inflammation, atherosclerosis and carcinogenesis (Biesalski, 2007). Among

olive oil phenolics, tyrosol (TY) and hydroxytyrosol (HTY) are two characteristic olive oil phenolic compounds with antioxidant properties *in vitro*. Meanwhile, they also exhibit biological benefits such as antibacterial, antiviral, anti-inflammatory, neuroprotective and anticancer effects, among others. As an antioxidant, tyrosol plays a defensive role in cells against injury due to oxidation (Giovannini et al., 1999), and has a cardioprotective effect (Lucas et al., 2010). Hydroxytyrosol has anti-leishmanial activity against promastigotes of Leishmania infantum, L. donovani, and L. major (Kyriazis et al., 2013). However, due to its lack of ortho-diphenolic structure, the *in vitro* antioxidant activity of tyrosol is weak, when compared with hydroxytyrosol.

Generally, the solubility of phenolic compounds in aqueous media is good due to their high polarity, compared to that of the living cell, where the antioxidant activity is required. Therefore, because of the limited solubility of these phenolics in lipid media, the search for new lipophilic derivatives with enhanced properties that could extend their application in oil-based foods and cosmetics, as well as making them more efficient in emulsions, is of great interest. In addition, the food industry is demanding powerful and economical antioxidants with nutritional properties to improve the value and the quality of foods (Moure et al., 2001).

Tyrosol and hydroxytyrosol derivatives have also been synthesized in order to improve the antioxidant and biological properties of the parent compounds. Glycosylated derivatives and lipophilic derivatives in the form of alkyl ether and fatty acid ester have been prepared. Hydroxytyrosol alkyl ether derivatives were found to play a positive role in liver cancer (Pereira-Caro et al., 2011) and colon adenocarcinoma (Pereira-Caro et al., 2013; Mateos et al., 2013), and are more stable under biological conditions than hydroxytyrosol. Hydroxytyrosol fatty acid esters possessed increased antioxidant activity and improved inhibition against oxidation of proteins

and lipids caused by peroxyl radicals (Trujillo et al., 2006), and have better neuroprotective (Munoz-Marin et al., 2012; Guerrero et al., 2012), antiplatelet and anti-inflammatory effects (Reyes et al., 2013). Aissa et al. (2012) synthesized several tyrosyl esters with increasing lipophilicity and found that they exhibit antibacterial and anti-leishmanial activities with a better affinity with lipophilic membrane constituents. Hence, these modified compounds could be important for further application in food and pharmaceutical fields. However, the relationship between the length of alkyl side chain and their antioxidant and biological ability is still unclear. Little information exists on polyunsaturated fatty acid esters of tyrosol or hydroxytyrosol and comparison of the ability of whole series of tyrosol esters with hydroxytyrosol esters.

Due to the improved biological and antioxidant activity found for several tyrosol and hydroxytyrosol derivatives compared to tyrosol and hydroxytyrosol themselves, we decided to synthesize a series of tyrosol and hydroxytyrisol derivatives using a green enzymatic method. We synthesized tyrosol and hydroxytyrosol with different fatty acids, including saturated fatty acids (from C4:0 to C18:0), MUFA (oleic acid), and Omega-3 polyunsaturated fatty acids (α-linolenic acid, EPA, DHA). Then, we identified the compounds by thin layer chromatography (TLC) and high-performance liquid chromatography-mass spectrometry (HPLC-MS), and purified them using a simplified base extraction method. Later, the antioxidant activities of the compounds so prepared were tested in order to explore the relationships between the activity and structure of compounds including the varying number of phenolic hydroxyl groups, the degree of unsaturation of the fatty acid side chain, and the length of the side chain. The study aimed to provide information about the potential of using tyrosol and hydroxytyrosol fatty acid esters as effective antioxidants by testing their 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging ability. Their antioxidant potential in biological model systems was also investigated in order to

highlight their efficiency by testing cupric ion-induced human low-density lipoprotein (LDL) oxidation and DNA strand scission assay.

The biological activities of tyrosol and hydroxytyrosol esters were also studied by testing the antiviral activity including anti-Hepatitis C virus (HCV) and the anti-human immunodeficiency virus (HIV) effect of selected esters in order to fill the existing gap in the literature in this area. It is well documented that oxidative stress caused by reactive species of oxygen (ROS) damages crucial cellular signaling proteins, and this is recognized as a mediator of inflammation (Mittal et al., 2014). The Human hepatoma cell line, HepG2, is a reliable model for biological studies of intracellular antioxidants (Alia et al., 2005). The protective ability of selected fatty acid esters against oxidative stress induced by tert-butyl hydroperoxide (t-BuOOH) was evaluated in the HepG2 model system. In addition, the cytotoxicity and antiglycation effects of selected compounds were also tested in this study.

CHAPTER 2

LITERATURE REVIEW

2.1 Tyrosol and Hydroxytyrosol

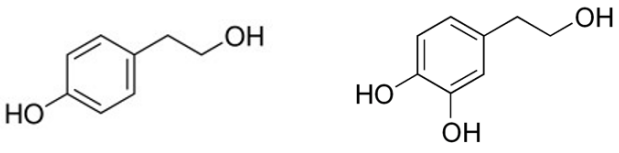
Tyrosol and hydroxytyrosol are phenylethanoids, derivatives of phenethyl alcohol, with antioxidant properties *in vitro.* They are classified as natural phenolic compounds present in a variety of plant sources, especially in olives and olive oil (Ryan & Robards, 1998; Romero & Brenes, 2012). As phenolics, they are expected to have high antioxidant activities (Carrasco-Pancorbo et al., 2005) which are due to the redox potentials (a measure of the electronegativity of a substance compared with hydrogen) of the phenolic hydroxyl groups (Dubey, 2014) and the structural factors in the chemical configuration of the molecules (Cheng et al., 2002). Due to their antioxidant properties, tyrosol and hydroxytyrosol play a defensive role in cells against injury due to oxidation (Manna et al., 1997; Giovannini et al., 1999)

Furthermore, they are revealed to show biological activities *in vivo*, mediated by mechanisms other than just scavenging free radicals (Forman et al., 2014). Tyrosol and hydroxytyrosol exhibit activities such as cardioprotective (Lucas et al., 2010), antibacterial (Capasso et al., 1995; Bisignano et al., 1999), anti-pathogen (Ortega-García & Peragón, 2010; Kyriazis et al., 2013), antiviral (Yamada et al., 2009), anti-inflammatory (de la Puerta et al., 1999; Bitler et al., 2005; Vivancos & Moreno, 2008), neuroprotective (Rodríguez-Morató et al., 2015) and anticancer (Owen et al., 2000; Bernini et al., 2013) effects, inhibition of human LDL oxidation (Visioli et al., 1995) and prevention of platelet aggregation (Petroni et al., 1995), as well as exhibiting activity against *T. brucei* (Belmonte-Reche et al., 2016). They also have positive effects in

metabolic syndrome and other health benefits which are associated with in vitro and in vivo experimental studies (Bulotta et al., 2014; Granados-Principal et al., 2010; Visioli & Bernardini, 2011). Thus, research into tyrosol, hydroxytyrosol and their derivatives has received increasing interest over the last decade, because of their improved biological activities and antioxidant effects.

2.1.1 Structures and Derivatives

Tyrosol (*p*-hydroxyphenethyl alcohol; *p*-hydroxybenzethanol; *p*-tyrosol) can be easily dissolved in water, alcohol, ether, acetone and acetic acid. Needle-like crystals of tyrosol can be obtained from chloroform. Hydroxytyrosol (3,4-dihydroxyphenylethanol) is a water- and fat-soluble bioactive alcoholic ortho-diphenol. Tyrosol and hydroxytyrosol are present in olive oils in the free and conjugated forms as secoroids or aglycones (Miro-Casas et al., 2003). In nature, hydroxytyrosol is rarely present in the free form. Hydroxytyrosol occurs mainly in the esterified form as oleuropein which is hydrolyzed to hydroxytyrosol (Fernandez-Bolanos et al., 2008). The chemical structures of tyrosol and hydroxytyrosol are given in Figure 2-1. In order to improve their fat-solubility and to increase their activities, the molecular structures may be modified to obtain lipophilic derivatives in the form of alkyl ether, lipophilic fatty acid ester, and glycosylated derivatives.



Tyrosol Hydroxytyrosol

Figure 2-1. Chemical structures of tyrosol and hydroxytyrosol.

Madrona et al. (2009) prepared a series of hydroxytyrosol alkyl ether derivatives by a three-step method (Figure 2-2) with good yield ($\geqslant$ 60%) and the derivatives retained the high protective capacity of free hydroxytyrosol (Halaouli et al., 2005), while Procopio et al. (2011) synthesized another type of derivatives- hydroxytyrosol lipophilic fatty acid esters (Figure 2-3) and reported a high free radical-scavenging capacity. In addition, the alkyl ether derivatives were found to play a positive role in liver cancer (Pereira-Caro et al., 2011) and colon adenocarcinoma (Pereira-Caro et al., 2013; Mateos et al., 2013).

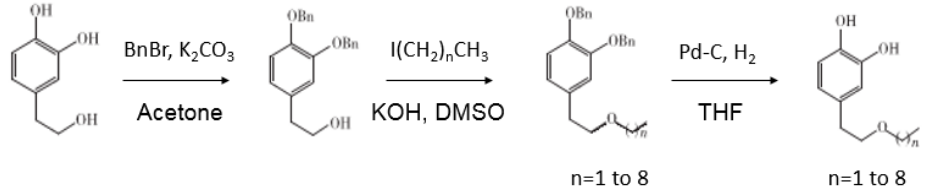


Figure 2-2. Synthesis of hydroxytyrosol alkyl ethers by a three-step method.

Hydroxytyrosol fatty acid esters (Figure 2-4) can also be biosynthesized by enzymatic catalysis via acylation assisted by *Candida antarctica* lipase (Bouallagui et al., 2011). The results of DPPH radical activity showed that the antioxidant activity of esterified derivatives was similar to that of hydroxytyrosol.

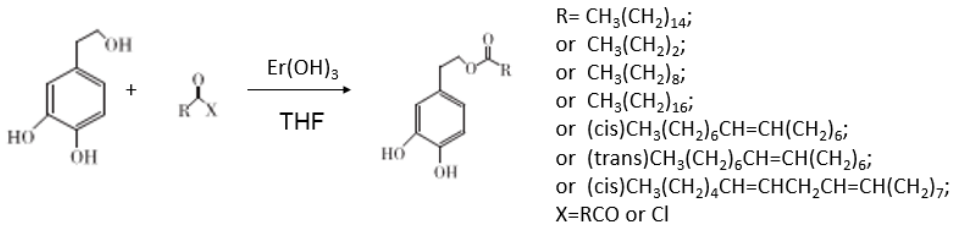


Figure 2-3. Synthesis of hydroxytyrosol lipophilic fatty acid esters by a chemical method.

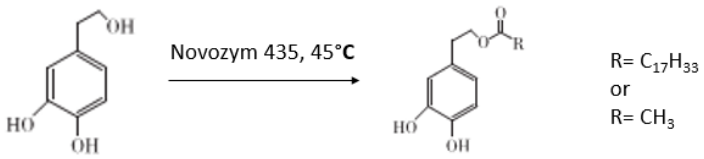


Figure 2-4. Synthesis of hydroxytyrosol lipophilic fatty acid esters by an enzymatic method.

Bernini et al. (2008) reported that hydroxytyrosol and its lipophilic derivatives can be synthesized from tyrosol by acylation or transesterification to protect the alcoholic hydroxyl group in order to curtail its excessive oxidation, then IBX/DMP oxidation and $Na_2S_2O_3$ reduction are used to form a lipophilic ester derivative, and then removal of the protecting group to generate hydroxytyrosol. Bernini et al. (2012) also synthesized a series of catechol derivatives (fatty acid methyl ester and carbonate) and evaluated their antioxidant activity by an *in vitro* ABTS assay and on whole cells by DCF fluorometric assay. Rogaie et al. (2013) showed that tyrosol, hydroxytyrosol and other polyphenols have a high free radical scavenging activity and effective antioxidant properties as evaluated by quantum chemical calculations to establish a structure-activity relationship of antioxidants for tyrosol, hydroxytyrosol, hydroxytyrosol acetate, as well as other derivatives. Trincone et al. (2012) utilized α- glucosidase secreted by the sea hare (*Aplysia fasciata*) viscera for catalytic glycosylation of tyrosol and hydroxytyrosol to form the corresponding glycosides.

Aissa et al. (2012) synthesized several tyrosyl esters with increasing lipophilicity using lipase from *Candida antarctica* (Novozyme 435), and pointed out that tyrosol was esterified via its primary hydroxyl group. Aissa et al. (2007) also reported another enzymatic esterification of

tyrosol and ethyl acetate with lipase from *Staphylococcus xylosus* (SXLi). Five tyrosol derivatives, composed of hypocrol A trichodenol B, 4-hydroxyphenethyl acetate, 4-hydroxyphenethyl tetradecanoate and 1-oleyltyrosol were found by Ding et al. (2016) in sponge-derived fungi.

2.1.2 Sources

Olives are the richest source of tyrosol and hydroxytyrosol. They are present in most parts of the olive tree, including the leaves, fruit, tree wood and, therefore, in olive oil (Ghanbari et al., 2012).

There are health gurus promoting olive oil as the miracle food. Modern science has now discovered that the medicinal effectiveness of foods containg olive tree products is largely due to their content of antioxidants tyrosol and hydroxytyrosol. Although the content of phenolics varies with cultivar and harvest, the total phenolics in virgin olive oil is composed of 30% tyrosol and hydroxytyrosol, and their secoroids derivatives as well as other conjugated forms such as oleuropein and ligstroside aglycones representing almost half of the total phenolic content of the virgin olive oil (Owen et al., 2000). Hydroxytyrosol, tyrosol and their derivatives with elenolic acid, derived from the glycosides ligstroside and oleuropein, are the most abundant phenolic compounds in olive oil (Servili et al., 2004; Tasioula-Margari & Okogeri, 2001; Goulas et al., 2012). In extra virgin olive oil, the most abundant secoiridoids are the dialdehydic form of elenolic acid linked to hydroxytyrosol or tyrosol (142.2 ± 4.7 mg/g) (Incani et al., 2016). During storage of virgin olive oil, secoiridoid derivatives decreased and hydroxytyrosol and tyrosol content increased (Kotsiou & Tasioula-Margari, 2016; Krichene et al., 2015). Early and mid-harvest oils had high hydroxytyrosol and tyrosol (maximum 20.7 mg/kg) while the late harvest had less than 20% of the initial value (Jolayemi et al., 2016). Franco et al. (2014) reported an increase of hydroxytyrosl, tyrosol and their secoiridoid derivatives from green to spotted stage of

maturation, and then a decrease up to the mature stage. The main phenolic compound in fresh olive fruit is oleuropein (Brenes et al., 1995). However, the phenolic content of olive fruit is very complex and depends upon factors such as fruit maturation stage, part of the fruit (e.g., pulp or seed), cultivar, and season (Charoenprasert & Mitchell, 2012). Oleuropein (3-4.5 g/kg) and hydroxytyrosol (0.2-71 g/kg) are the main phenolic compounds in olive pulp (Vinha et al., 2005; Romero et al., 2002; Sivakumar et al., 2005). Olive pulp also has a high content of hydroxytyrosol, tyrosol, and their glycosides (Romero et al., 2002). In pulp, the contents of tyrosol and hydroxytyrosol do not change much during growing stages in different cultivars (Alagna et al., 2012).

Many phenolic compounds present in olive pulp are also found in olive leaf tissue. However, tyrosol and hydroxytyrosol are not as abundant in olive tree leaves as they are in the fruit and oil (Sánchez de Medina et al., 2012). Oleuropein is the major phenolic compound in olive tree leaves, and it represents up to 9% of the dry weight matter (Ryan et al., 2002; Kiritsakis et al., 2010). In addition, leaves contain hydroxytyrosol, hydroxytyrosol glucoside, tyrosol, tyrosol glucoside, oleuroside (Kiritsakis et al., 2010).

Small branches (fibrous softwood) of olive tree have a lesser amount of oleuropein, hydroxytyrosol, tyrosol, α-taxifolin and verbascoside compared to those in the leaves (Japón-Luján & Luque de Castro, 2007). It has been found that microwave-assisted extraction can help the recovery of high levels of phenolics: 19 g/kg oleuropein, 2 g/kg tyrosol, 1 g/kg verbascoside, and 0.7 g/kg hydroxytyrosol were recovered from small branches (Japón-Luján & Luque de Castro, 2007). During ripening of olives, the concentration of hydroxytyrosol was around 1 g/kg (dry weight) in July, and then the amount decreased until October and then increased, whereas

the concentration of tyrosol changed during the ripening period, ranging from 0.51 g/kg (dry weight) in July to 0.13 g/kg (dry weight) in December (Ortega-García & Peragón, 2010).

Tyrosol and hydroxytyrosol can also be found in wine. In wines, tyrosol, which is formed from tyrosine by yeast during alcoholic fermentation (Hazelwood et al. 2008; Piñeiro et al., 2011), had a higher average content when compared to hydroxytyrosol (500-1800 μg/L) produced by hydroxylation of tyrosol, showing values between 20 and 60 mg/L (Bordiga et al., 2016). Hydroxytyrosol and tyrosol concentrations remain relatively constant during wine aging (Ribéreau-Gayon et al. 2000; Barón et al., 1997). The production and accumulation of tyrosol and hydroxytyrosol was influenced by both yeast species involved in the alcoholic fermentation and aeration conditions which could have an effect on the enzymatic conversion of tyrosol to hydroxytyrosol (Romboli et al., 2015).

Tyrosol, hydroxytyrosol and 3,4-dihydroxyphenyl acetic acid could also be found in the activated charcoal used during the fermentation process of the Japanese rice wine "sake" (Mizushina et al., 2014). Pre-fermentative cold maceration Tannat red wines showed the highest concentration of tyrosol (37.85 ± 5.02 mg/L) among traditional maceration, pre-fermentative cold maceration, maceration enzyme and grapeseed tannins addition (Favre et al., 2014). Tyrosol exhibited an unusual high value (143 mg/l, as average) in the wines elaborated with the recombinant *Saccharomyces cerevisiae* EKD13 strain (Juega et al., 2014). Among the red wines analyzed, Piñeiro et al. (2011) found tyrosol concentrations ranging from 20.51 to 44.46 mg/L, whereas hydroxytyrosol ranged from zero to 5.02 mg/L, and the Cabernet Sauvignon B and Tempranillo F varieties contained the highest amounts of hydroxytyrosol while Merlot variety showed the highest tyrosol content. There are different red wines containing much higher tyrosol concentrations than Pinot noir Champagnes (18 mg/L; Chamkha et al.,

2003), Mazuelo (20–30 mg/L; Garde-Cerdán & Ancín-Azpilicueta, 2008), Graciano, Tempranillo, or Cabernet Sauvignon, 7–26 mg/L; Monagas et al., 2005), autochthonous Italian wines (17–62 mg/L; Bevilacqua et al., 2004), and autochthonous Hungarian and Canadian wines (38–82 mg/L; Nikfardjam & Pickering, 2008).

2.2 Fatty Acids

Fatty acids can be classified into saturated and unsaturated fatty acids, the latter being further divided into monounsaturated and polyunsaturated. Fatty acids can form more complex molecules, including triacylglycerols, phospholipids, sterols and their esters, among others. In this study, we selected different types of fatty acids, saturated (butyric acid to stearic acid), monounsaturated (MUFA, oleic acid) and polyunsaturated (α-linolenic acid, EPA, DHA) fatty acids.

2.2.1 Saturated fatty acids

Saturated fatty acids (SFA), such as stearic acid, contain no unsaturated linkages between carbon atoms and cannot incorporate any more hydrogen atoms. Fatty acids can be classified according to their chain length: short chain fatty acids (SCFA), medium chain fatty acids (MCFA), and long chain fatty acids (LCFA).

SCFA, also called the volatile fatty acids (VFA), range from C2:0 to C4:0, such as acetic acid (C2:0), propionic acid (C3:0) and butyric acid (C4:0). Among their various properties, SCFA are readily absorbed by intestinal mucosa (Cummings et al., 1987), and can be more quickly absorbed in the stomach than MCFA because of their higher solubility in water, smaller molecular size, and shorter chain length (Bezard & Bugaut, 1986). MCFA have six to twelve carbon atoms while LCFA contain 14 or more carbon atoms (Shahidi, 2006). MCFA can be

rapidly cleared from the blood and get absorbed and metabolized much like glucose (Babayan, 1987). MCFA can be absorbed more quickly into the intestinal lumen than LCFA (Bach & Babayan, 1982). LCFA have low water solubility because water solubility and oxidation susceptibility decrease as chain length increases. Triacylglycerols containing high amounts of long chain SFAs, especially stearic acid, have low absorption in the human body (Hashim & Babayan, 1978). General recommendations frequently focus on reducing SFA intake for improving cardiometabolic health.

2.2.2 Monounsaturated fatty acids

MUFAs are fatty acids that have only one double bond in their carbon chain and exist in cis and trans configurations. The high-MUFA diets can lower total cholesterol and LDL cholesterol and hence decrease the cardiovascular disease (CVD) risk (Kris-Etherton et al., 1999). MUFAs can also decrease risk for cancer, age-related cognitive decline and Alzheimer's disease (López-Miranda et al., 2010). In the experiments carried out in this work, oleic acid was selected. Oleic acid is an 18 carbon monounsaturated omega-9 fatty acid. Olive oil is predominantly composed of oleic acid. Oleic acid shows multiple benefits such as anti-inflammatory, reducing cardiovascular risk, and anticancer (Reardon et al., 2012; Gonçalves-de-Albuquerque et al., 2016; Guzmán et al., 2016; Fonolla-Joya et al., 2015; Perdomo et al., 2015; Menendez et al., 2006).

2.2.3 Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) contain two or more double bonds in their backbone. The essential fatty acids (EFA) are the PUFA that must be taken by humans and other animals through their diet as they cannot be synthesized in the body, including omega-3 (ω-3) and

omega-6 (ω-6) fatty acids. Omega-3 fatty acids have a final carbon–carbon double bond in the 3rd carbon from the methyl end group whereas ω-6 fatty acids have it in the 6th position. Omega-3 fatty acids (Figure 2-5), α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have various properties for which they can be classified as functional food ingredients. The omega-3 fatty acids play an important role in cardiovascular health, relieving arthritis and contributing to the brain and central nervous system as well as healthy eyes and healthy cholesterol levels (Sargent et al, 1995). Many studies have shown that higher consumption of n ω -3 PUFA, especially ALA, EPA, and DHA, is associated with lower incidences of heart failure and other cardiovascular events (Yashodhara et al., 2009; Lee et al., 2009; Saremi et al., 2009). The efficacy of omega -3 fatty acids in primary and secondary prevention of CHD (Coronary Heart Disease) has been demonstrated (Kochar et al., 2014). Omega-3 PUFAs have shown cancer preventive effects in some rodent models of mammary carcinogenesis by impacting gene expression, reducing angiogenic signals, and promoting anti-inflammatory mechanisms via alterations in the biosynthesis of lipid mediator molecules (Signori et al., 2011).

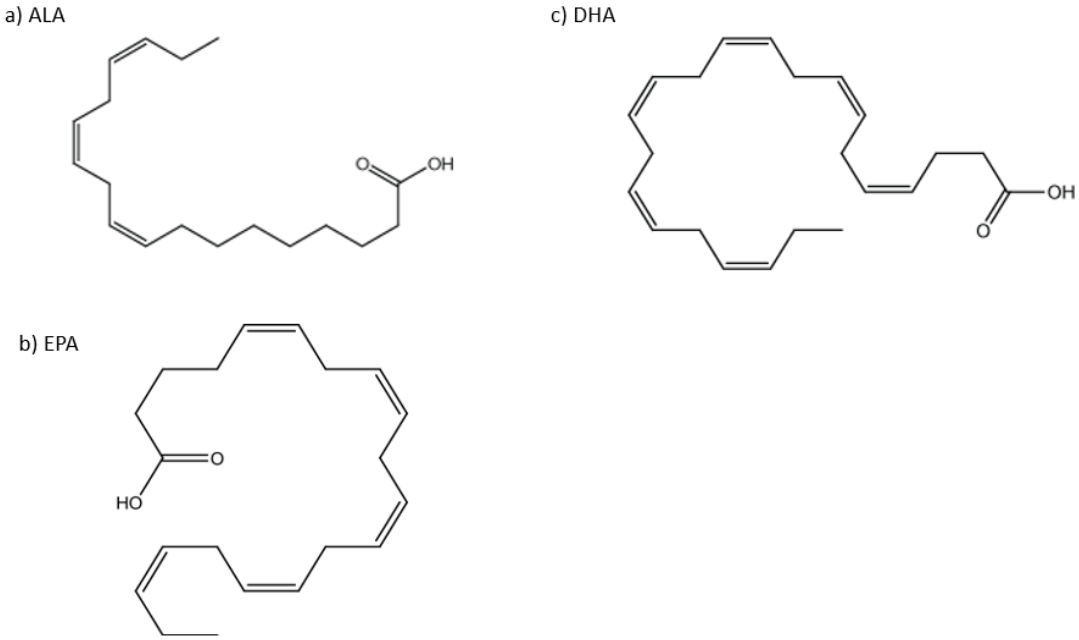


Figure 2-5. Chemical structure of α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

2.3 Lipid oxidation

The unsaturated fatty acids, especially PUFAs, are mainly responsible for lipid oxidation. The risk of oxidation increases with the number of double bonds present in the fatty acids. For instance, DHA (C22:6) with six double bonds, is oxidized easier than linolenic acid (C18:3) with only three double bonds. During lipid oxidation, various primary and secondary oxidation products are formed that influence food quality. Oxidized foods can cause oxidative stress in biological systems and thus initiate numerous diseases.

2.3.1 Mechanism of lipid oxidation

Lipids are prone to oxidation in the presence of catalytic systems such as light, heat, enzymes, metals, metalloproteins and microorganisms (Shahidi & Zhong, 2010). Four different

mechanisms are able to induce lipid peroxidation, namely autoxidation, photooxidation, thermal oxidation and enzymatic oxidation; most of which involve reactive species such as free radicals as the intermediate. Autoxidation is the most frequently encountered peroxidation and involves a free radical mechanism as shown in Figure 2-6. The simplified pathways of lipid autoxidation consists of three phases: initiation, propagation, and termination.

In the initiation stage, unsaturated lipid molecules (RH) lose a hydrogen atom and produce a lipid free radical (R·) and this oxidation normally proceeds very slowly until it reaches a sudden increase after an induction period. This reaction requires the presence of initiators such as heat, light/ionizing radiation and metal ions/metalloproteins. The hydrogen at the carbon next to the double bond of unsaturated fatty acids is the easiest one to be donated because of its lower C-H bond energy.

During the propagation stage, the lipid radicals (R·) react with oxygen to form peroxyl radicals (ROO·) and get a hydrogen atom to form hydroperoxides (ROOH) which is the primary products of oxidation. This reaction may be repeated for thousands of times during propagation until no hydrogen source is available or the chain is interrupted, for instance, by antioxidants (DeMan, 1999). The hydroperoxides can later break down to form alkoxyl (RO·), peroxyl (ROO·), hydroxyl (HO·) and new lipid radicals (R·) under light, heat, or metals.

During the termination step, the accumulated lipid radicals (R·) and peroxyl radicals (ROO·) react with each other to from non-radical products. The unstable accumulated hydroperoxides will break down to a wide range of secondary oxidation products, including aldehydes, ketones, alcohols, hydrocarbons, volatile organic acids and furans, among others, some of which possess off odours. The possible pathways of secondary oxidation of oleic acid are shown in Figure 2-7 (Leray, 2016). The C8-hydroperoxide, formed from oleic acid in primary oxidation, produces the

alkoxyl radical which is then cleaved by the homolytic beta-scission of a carbon-carbon bond on either side of the oxygen-containing carbon atom, and later produces the alkene (1-decene) and the aldehydes (decanal and 2-undecenal), among others. Most of the unsaturated aldehydes may undergo further oxidation to produce other volatile compounds. Among these compounds, malonidialdehyde (MDA) is one of the most cited product originating from PUFAs. The possible generation pathways of MDA are shown in Figure 2-8 (adapted from Esterbauer et al., 1991).

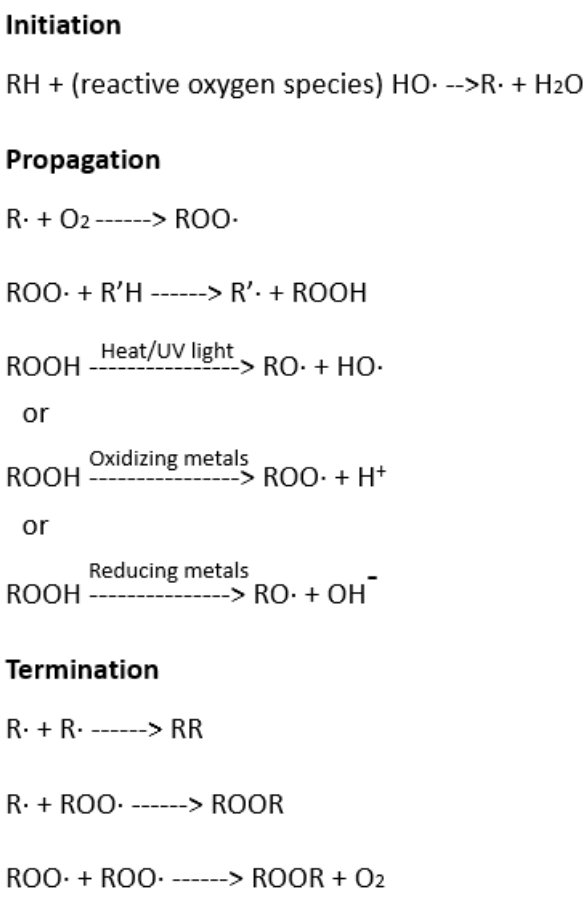


Figure 2-6. Lipid autoxidation pathways.

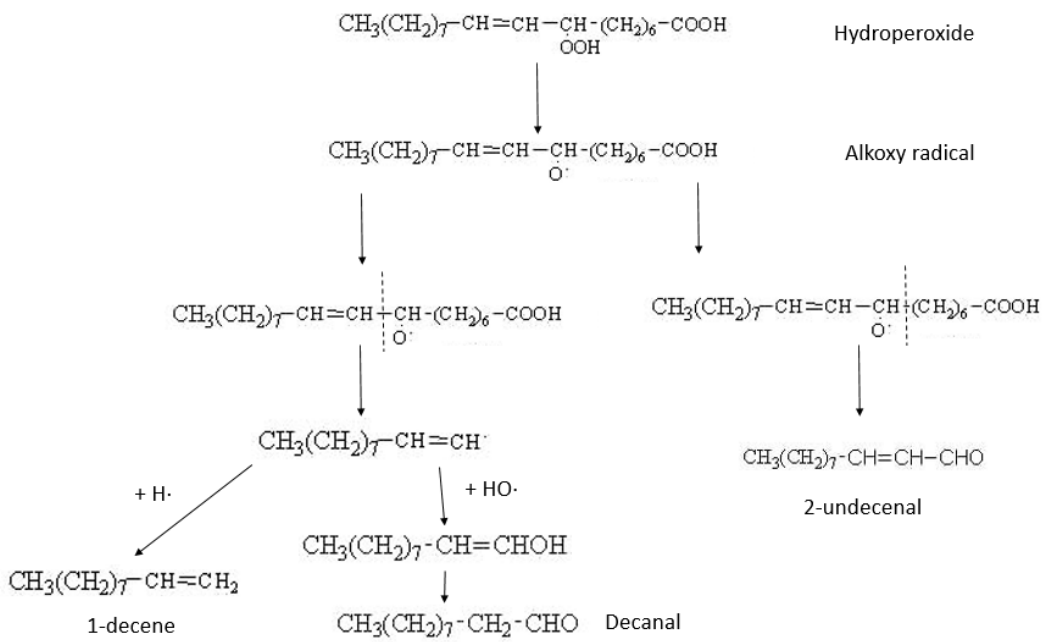


Figure 2-7. The possible pathways of secondary oxidation of oleic acid.

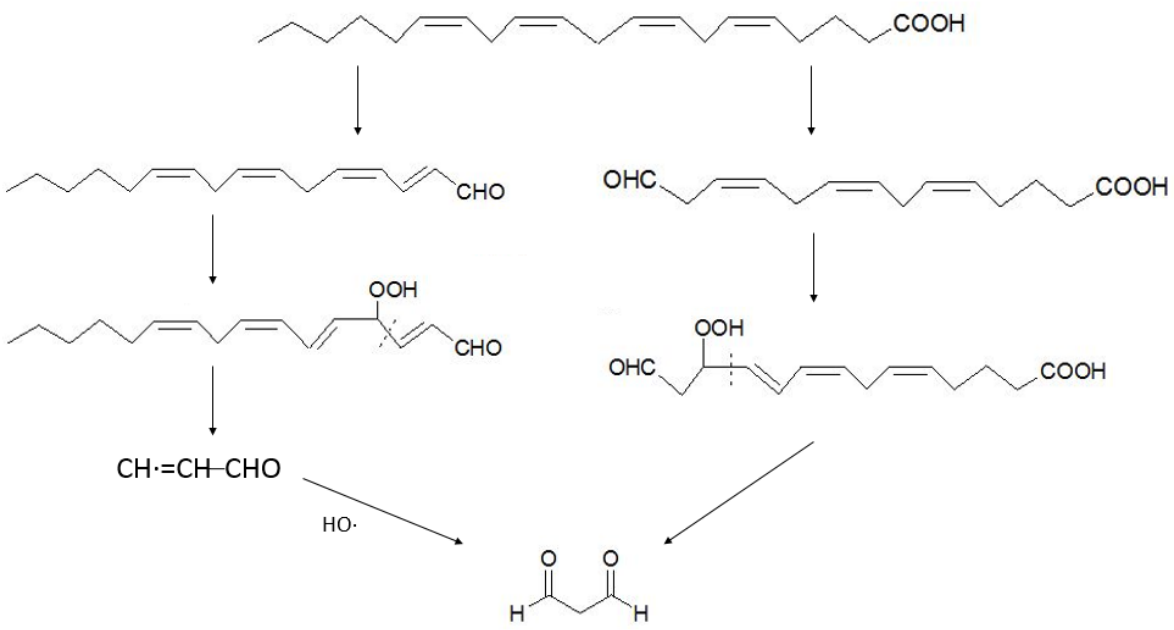


Figure 2-8. The possible generation pathways of malondialdehyde (MDA).

2.3.2 Factors affecting lipid oxidation

Lipid oxidation is influenced by various internal and external factors. The main factors are fatty acid profile and processing method, the energy input such as light or heat, the concentration of oxygen, minor components in the oil such as free fatty acids, monoacylglycerols, diacylglycerols, metal, phospholipids, pigments, peroxides and antioxidants, among others.

Both saturated and unsaturated fatty acids can undergo oxidation, but the oxidation of saturated fatty acids requires special conditions, such as the propagation of mold and the presence of an enzyme, to allow saturated fatty acids to undergo β-oxidation in order to form acid and methyl ketone (Nelson et al., 2008). However, the oxidation rate of saturated fatty acids is much slower than that of unsaturated fatty acids. The rate of oxidation of unsaturated fatty acids is related to the number and the position of double bonds, and the geometric shape of the molecule. The fatty acids with more double bonds are most susceptible to oxidation. The oxidation rate of stearic, oleic, linoleic and linolenic acid (C18 series) can be in the ratio of 1: 100: 1200: 2500 (DeMan, 1999). In addition, the fatty acids with cis configuration or conjugated double bonds are easier to be oxidized than those with trans configuration or non-conjugated double bonds (Rustan & Drevon, 2005). In general, the oxidation rate of free fatty acids is higher compared with their esterified form, possibly due to their greater ability to pick up trace metals from the environment (Taub & Singh, 1997).

In general, the oxidative rate is accelerated with increasing temperature. The high temperature can promote not only the disappearance and the production of free radicals, but also the decomposition and polymerization of peroxides. In addition, temperature can affect the rate of oxidation, and the mechanism of the reaction. At room temperature, oxidation occurs mostly on the methylene adjacent to a double bond to generate peroxides. However, when the temperature

exceeds 200°C, oxidation will also occur on double bonds of the unsaturated fatty acid to generate cyclic compound by the Diels-Alder reaction (Figure 2-9; Choe & Min, 2006).

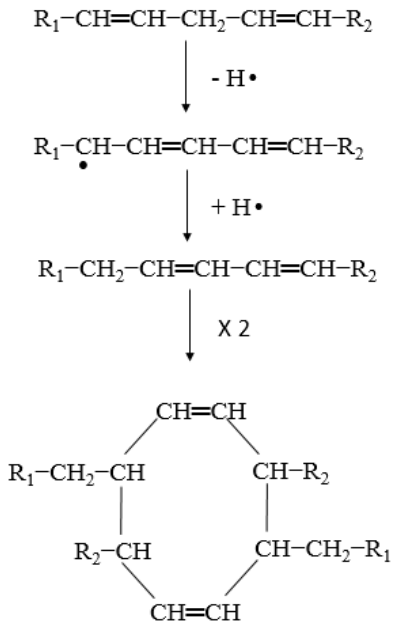


Figure 2-9. Cyclic compound formation from linoleic acid by Diels-Alder reaction at high temperature.

Some minor components in the oil, such as metal, phospholipids, pigments, and antioxidants will also affect the oxidative stability. The presence of metal ions such as copper and iron will accelerate the oxidation of the oil, as metal ions can reduce the activation energy of the initial reaction of autoxidation. Some pigments, such as carotenoids, are good antioxidants that can reduce the oxidation rate, if they are not removed by deodourization and bleaching during the refining process.

2.3.3 Influence of lipid oxidation

Lipid oxidation compromises the sensory quality of food products and limits the shelf-life of others. The foods containing lipids are susceptible to oxidation which leads to their quality

deterioration and rancidity development. Rancidity of foods caused by lipid oxidation causes not only loss of flavour, but also loss of colour, nutrient value, and the accumulation of compounds which may have detrimental effects on the health of consumers. Lipid oxidation leads to the decomposition of fatty acids, resulting in the formation of volatile compounds. Many of these compounds have an unpleasant odour, and are responsible for flavour problems in food products (Grosch, 1982). The oils that are most susceptible to oxidation are those oils which are rich in PUFAs such as fish oil and algae oil. The oxidation of PUFA will reduce the amount of essential fatty acids of edible lipids, and has a detrimental effect on other components, such as vitamins and proteins. The toxicity of oxidized cholesterols has been demonstrated with their powerful atherogenic effect *in vivo* and *in vitro* and cytotoxic and mutagenic properties (Addis & Warner, 1991; Osada et al., 1998).

The harmful radicals produced as a result of lipid oxidation will accelerate human aging, especially at high levels in the body. In addition, they will also produce toxins and carcinogens that seriously affect human health. Free radicals can lead to cancer by damaging DNA (Dreher & Junod, 1996). The DNA damage, caused by reactive oxygen metabolites such as hydroxyl radical and hydroperoxides which are generated through lipid oxidation, has been classified into the form of base damage, single-strand and double-strand breaks, crosslinking between DNA, chromosomal aberrations, and sister chromatid exchanges (Ray et al., 2000). Malondialdehyde (MDA), a secondary oxidation product of oxidation of PUFA with 3 or more double bonds, can cause cross-linking in lipids, proteins and nucleic acids (Freeman & Crapo, 1982; Flohe et al., 1985).

2.4 Mechanisms of action of antioxidants

According to the diverse mechanisms of action of antioxidants, they can be classified into free radical terminators, chelators of metal ions that catalyze lipid oxidation, or as oxygen scavengers that react with oxygen in closed systems. Antioxidants that can break the chain reaction of autoxidation by hydrogen (or electron) donation, and then generate more stable radicals, can be called primary antioxidants. Others are considered as secondary antioxidants. Phenolic antioxidants are classified as free radical terminators, and are regarded as primary antioxidants. The emphasis of this study is on phenolic antioxidants, and their action mechanism is discussed below.

The first detailed kinetic study of phenolic antioxidant activity was reported by Boland and ten Have (1947). Phenolic antioxidants (AH), interfere with lipid oxidation at both of the initiation stage (Reactions 1 and 2) and propagation stage (Reactions 4 and 5), and can donate hydrogen atoms (Reactions 1 and 5) or transfer electrons to lipid radicals, alkoxyl or peroxyl radicals and produce more stable antioxidant radicals. The antioxidant reactions are shown below.

$$ROO^{\cdot} / RO^{\cdot} + AH \longrightarrow ROOH/RH + A^{\cdot} \quad (1)$$

$$ROO^{\cdot} / RO^{\cdot} + A^{\cdot} \longrightarrow ROOA/ROA \quad (2)$$

$$A^{\cdot} + A^{\cdot} \longrightarrow A\text{-}A \quad (3)$$

$$ROO^{\cdot} + RH \longrightarrow ROOH + R^{\cdot} \quad (4)$$

$$R^{\cdot} + AH \longrightarrow RH + A^{\cdot} \quad (5)$$

As the carbon-hydrogen bond energy of the free radical scavenger decreases, the transfer of hydrogen to the free radical is more energetically favourable (Akoh & Min, 2008). And during reaction 1 and 2, molecules were not in balance. Therefore, as a good antioxidant, Reaction 1

should be faster than Reaction 3 and the antioxidant radical produced via Reactions 1 and 5 should be more stable and more difficult to promote autoxidation. The antioxidant radicals ($A^{\cdot}$) produced by Reaction 1 can react with alkoxyl radical to form non-radical products (ROA) or with peroxyl radical to form ROOA or with another antioxidant radical to form A-A. These reactions are thermodynamically favourable.

Any compound that has a reduction potential lower than the reduction potential of a free radical is capable of donating its hydrogen atom to that of the free radical unless the reaction is not kinetically feasible. The efficiency of an antioxidant depends mainly on its speed of releasing hydrogen atom which is influenced by mainly three factors: shielding effect of phenolic hydroxyl groups, inductive effect of substituent groups, and conjugation effect (Silva et al., 2000; Craft et al., 2012). The effects of these factors can be demonstrated by O-H bond dissociation energy (BDE) of the phenolic hydroxyl group.

Figure 2-10 provides an explanation of the conjugated resonance stabilization of phenoxyl radicals by delocalization of its unpaired electron around the aromatic ring (Craft et al., 2012). The weaker the O-H bond of the antioxidant, the more likely and faster it will react with free radicals. In other words, the BDE of an antioxidant is a parameter of the capacity of a phenolic compound as a free-radical terminator (Wright et al., 2001). The greater the BDE required, the less the efficiency of a phenolic compound in participating in free-radical scavenging.

When hydrogens at *para* and *ortho* positions of the phenolic are replaced by other groups, such as hydroxy, alkoxy, and amino groups, BDE decreases and the antioxidant activity increases (Table 2-1; Lucarini et al., 1996). This explains the increase of the number of methyl groups on benzene ring that leads to increased antioxidant activity. Gordon (1990) reported that the presence of chain or branched alkyl groups in the para position decreases the antioxidant activity.

The efficiency of phenolic antioxidants on autoxidation is also influenced by oxidation conditions, and the nature of the sample being oxidized (Naczk & Shahidi, 2004). Often phenolic compounds lose their activity as an antioxidant at very high concentrations and are involved in initiation reactions as prooxidants (Reactions 6, 7) (Gordon, 1990).

$$AH + O_2 \longrightarrow HOO^{\cdot} + A^{\cdot} \qquad (6)$$

$$AH + ROOH \longrightarrow H_2O + RO^{\cdot} + A^{\cdot} \qquad (7)$$

In the oil in which the deterioration is not serious, phenolic antioxidants can effectively prolong the induction period, while they do not work well in retarding decomposition of already deteriorated lipids (Mabrouk & Dugan, 1961). This is the reason why the phenolic antioxidants should be added to foodstuffs as early as possible, better at the beginning, during processing and storage in order to obtain maximum protection effect against oxidation (Shahidi et al, 1992).

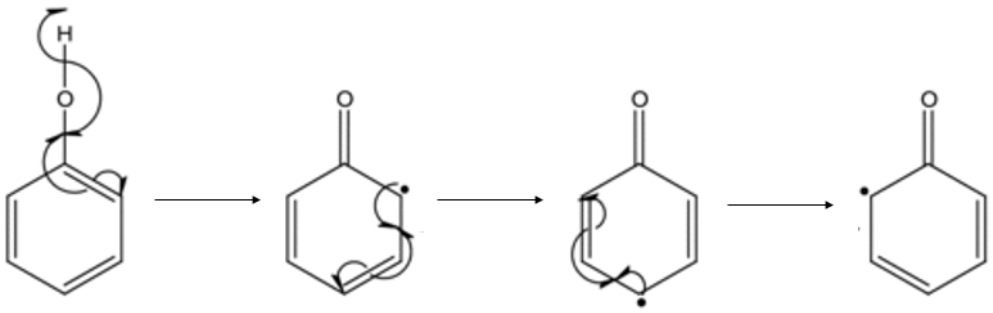


Figure 2-10. Conjugative resonance stabilization of phenoxyl radical.

Table 2-1. Bond dissociation energies (BDE) of substituted phenols.

Substituted Phenol	BDE (kcal/mol)
Phenol	87.6
***o*-cresol**	84.1
***p*-cresol**	85.9
Tri-tert-butylphenol	80.9
4-tert-butylphenol	85.7
3,5-ditert-butylphenol	86.6
3,5-dimethoxyphenol	86.7
Butylated hydroxytoluene (BHT)	81.1
2,3,6-trimethyl-4-methoxyphenol	79.2
2,4,6-Trimethoxyphenol	79.3
2,6-di-tert-butyl-4-methoxyphenol	78.4
6-hydroxy-2,2,5,7,8-pentamethylchroman (HPMC)	78.7
α-tocopherol	78.7

2.5 Measurement of antioxidant activity

Protection mechanisms against the detrimental effects of oxidations are provided by the action of antioxidants, and the measurement of antioxidant activity is well documented. Researchers have traditionally measured the antioxidant activity by identifying and quantifying the exact species of oxidation products and by lipid oxidation measurements such as acid value (AV), peroxide value (PV), thiobarbituric acid reactive substances (TBARS), conjugated dienes (CD), and by assessing volatile compounds (Kristinová et al., 2009). More recently, various chemical and

biological methods have been reported, for instance, scavenging activity against certain types of free radicals, reducing power, metal chelation, and LDL-cholesterol oxidation inhibition assay, among others. Methods of assessing antioxidant activity fall into two broad categories: radical scavenging assays and measuring the ability of antioxidants in inhibiting oxidation reactions in a model system. The two types of methods used for measuring antioxidant activity are discussed in the sections below.

2.5.1 Radical scavenging assays

Radical scavenging assays are simple, quick, and usually automated, and widely used in initial screening and evaluation of various antioxidant compounds or extracts of natural products/by-products. They can be classified into hydrogen atom transfer (HAT) reaction-based and single electron transfer (SET) reaction-based methods. Antioxidants can scavenge free radicals or other oxidation products such as hydroperoxides by HAT or/and SET (Prior et al., 2003), leading to the same end results, although the mechanism involved is different (Prior, Wu, & Schaich, 2005). HAT-based methods measure the efficiency of an antioxidant to scavenge free radicals by hydrogen donation while SET-based methods are dependent on transferring one electron to reduce any compound (reduce higher valent elements to their lower valence state), mainly metals (iron, copper, among others), carbonyls and radicals (Shahidi & Zhong, 2005, 2007, 2015). Antioxidant activities can be expressed not only as inhibition against ROS-mediated oxidation of the probe, but also equivalent to a selected reference antioxidant such as trolox, ascorbic acid or other compounds. Oxidation of the probe can be easily measured by various up-to-date detection instrumentation such as spectrophotometric, fluorometric, EPR (electron paramagnetic resonance), FT-IR (Fourier transform-infrared), and amperometric methods, among others. Oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter

(TRAP) and crocin bleaching assays are the example for HAT-based methods, while trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assays are major measurement for SET (Shahidi & Ho, 2007).

The ORAC assay measures the antioxidant scavenging activity against the peroxyl radicals induced by generators, and thus it reflects classical radical chain breaking antioxidant activity by hydrogen atom transfer (Ou et al., 2001). In the early time, the ORAC involved β-phycoerythrin (β-PE, a fluorescent protein isolated from *Porphyridium cruentum)* as the fluorescent probe (Cao et al., 1993) which was replaced by fluorescein (Figure 2-11) because of the limitations of β-PE (Cao & Prior, 1999). Fluorescein reacts with peroxyl radicals leading to the loss of fluorescence that can be the indicator of the extent of the decomposition. A set of fluorescence decompositon curves can be built in the absence or presence of antioxidants, and the net integrated area under the curves (area obtain in the presence of antioxidants compared to that of a blank run without antioxidants) can be calculated as an indicator of the peroxyl radical scavenging capacity of the antioxidants. Standard antioxidants such as trolox are used as reference, and activity results of the tested antioxidants are often reported as trolox equivalents. Most of the peroxyl radical generators used in ORAC assays are azo compounds such as the lipophilic AIBN (α,α-azobisisobutyronitrile), ABAP (2,2-azobis(2-amidinopropane) hydrochloride) and AMVN (2,2'-azobis(2,4-dimethylnaleronitrile)) and the hydrophilic AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) (Becker et al., 2004). Reaction of peroxyl radical generation is given below:

$$\text{R-N=N-R} \xrightarrow{O_2} N_2 + 2\text{ROO}^{\cdot} \quad (8)$$

The original version of the ORAC assay is limited to measurement of hydrophilic chain breaking antioxidant capacity against only peroxyl radicals; lipophilic antioxidants with particular

importance against lipid oxidation are not included. Later studies adapted the assay to measuring either hydrophilic or lipophilic antioxidants using a solution of acetone/ water (50:50, v/v) containing 7% randomly methylated β-cyclodextrin (RMCD) to solubilize the antioxidants (Huang et al., 2002; Wu et al., 2004). The ORAC assay is automated and excellent results have been obtained using a multichannel liquid handling system coupled with a fluorescence microplate reader in either a 96- or 48-well format (Ou et al., 2001; Huang et al., 2002). As generation of peroxyl radical is sensitive to temperature, the control of temperature throughout the reaction is important. Incubation of the reaction buffer at 37 °C prior to the dissolution of AAPH is recommended in order to decrease the intra-assay variability (Prior et al., 2003). Small temperature differences in the external wells of the microplate can reduce the reproducibility of the assay (Lussignoli et al., 1999). The long time required for the analysis (⩾1 h) has also been a major disadvantage of the ORAC assay, but this limitation has been partially overcome by development of high-throughput assays (Huang et al., 2002).

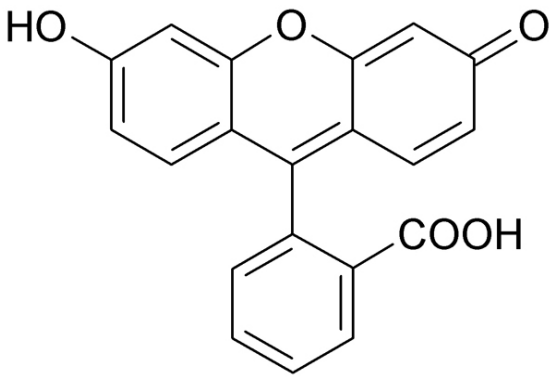


Figure 2-11. The chemical structure of fluorescein.

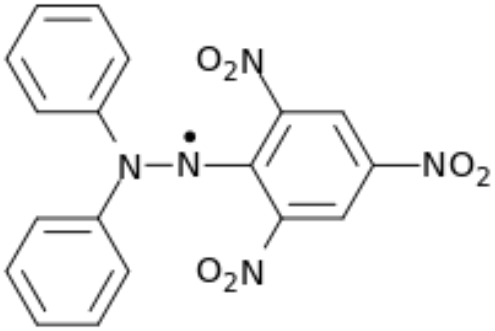


Figure 2-12. The chemical structure of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical.

Other similar HAT-based methods such as TRAP and crocin bleaching assays have the same principle and common features of ORAC assay as they involve azo compounds as peroxyl radical generators, fluorescent probe or UV–vis probe and an antioxidant (Shahidi & Zhong, 2007).

DPPH radical scavenging assay is an SET-based method with HAT mechanism being only a marginal reaction pathway in the assay (Prior et al., 2005). The DPPH (Figure 2-12) radical is one of the few stable organic nitrogen radicals with a deep purple colour due to the delocalization of the spare electron on the whole molecule. The DPPH radical absorbs at 517 nm and is a substrate-free system. This antioxidant assay is based on measurement of the reducing ability of antioxidants toward DPPH radical. The efficiency can be measured by electron paramagnetic resonance (EPR) or by UV spectrophotometry by measuring the decrease of its absorbance due to the loss of DPPH colour at 517 nm.

The DPPH radical and antioxidants are diluted in an alcoholic solutions such as methanol or ethanol. When DPPH radical reacts with a hydrogen donor, the reduced form (DPPH) is

generated and the colour of the DPPH mixture fades. The decrease of absorbance depends linearly on the antioxidant concentration. Trolox is often used as a standard antioxidant (Thaipong et al., 2006; Pisoschi et al., 2009). The widely used DPPH assay was first reported by Brand-Williams et al. (1995). The percentage of the DPPH remaining is calculated as:

$$\% \ DPPH^{\cdot}_{remaining} = 100 * [DPPH^{\cdot}_{remaining}] / [DPPH^{\cdot}_{initial}]$$

Results are reported as the EC_{50} that is defined as the percentage of remaining DPPH radical ($DPPH^{\cdot}_{remaining}$) being proportional to the antioxidant concentration and the concentration of the antioxidant necessary to decrease the initial DPPH radical concentration ($DPPH^{\cdot}_{initial}$) by 50%. The time taken to reach the steady state with EC50 is defined as T_{EC50} that is also calculated. Occasionally, antiradical efficiency (AE) is reported as was proposed by Sánchez‐Moreno et al. (1998) which combines EC_{50} and T_{EC50} into one parameter according to the following equation.

$$AE = (1/EC_{50}) \ T_{EC50}$$

DPPH scavenging abilities of fruit (guava) extracts with the spectrophotometric method have been studied (Thaipong et al., 2006) and results expressed in trolox equivalents (μM trolox equivalents/g fresh mass). Citrus oils were measured by HPLC using DPPH (Choi et al., 2000). Phenolic compounds generally exhibited significant scavenging effects against the DPPH radical (Antolovich et al., 2002). The DPPH assay can been compared with other methods including the ABTS assay, superoxide-anion scavenging and lipid oxidation (Lu & Foo, 2000; Gil et al., 2000). The DPPH assay can also be combined with online HPLC for rapid screening and identification of various antioxidant samples which reduces the loss of antioxidants during purification processes. For instance, Qiu et al. (2012) screened and identified natural antioxidants in peanut shell using the DPPH-HPLC-DAD-TOF/MS method.

The DPPH test is simple and rapid, and does not require special sample treatment, which explains its widespread use in testing antioxidant activity. However, its sensitivity may be affected by a number of factors, such as the type and amount of solvent used, presence and concentration of hydrogen and metal ion and freshness of DPPH reagent (Shahidi & Zhong, 2015). For instance, the result may be complicated when the test compounds have spectra that overlap with that of DPPH at 517 nm. Anthocyanins have strong absorbance at 517 nm, and interfere with the results and their interpretation. DPPH discoloration is related with radical reaction, reduction, and steric accessibility. Therefore, small molecules that have better access to DPPH radical show better antioxidant capacity with this test. The DPPH radical is a stable nitrogen radical, which bears no similarity to the highly reactive peroxyl radicals involved in lipid oxidation. Thus, many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH.

The EPR detection method can perform better when measuring highly coloured and cloudy samples compared with the classic spectrophotometric detection (Gardner et al., 1998). The DPPH radical that has an unpaired electron can generate different paramagnetic properties or EPR spectra under a varying magnetic field. The peak intensity proportional to the concentration of DPPH in the EPR spectrum decreases with time in the presence of antioxidants as a result of DPPH radical scavenging by the antioxidants. The rate of decrease in DPPH signal intensity at an end point may be used as the indicator of scavenging capacity of the antioxidant against the DPPH radical (Zhong & Shahidi, 2011).

The FRAP assay is a typical SET-based method that measures the reduction of a ferroin analog, the Fe^{3+} complex of tripyridyltriazine $Fe(TPTZ)^{3+}$ to the intensely blue coloured Fe^{2+} complex $Fe(TPTZ)^{2+}$ by antioxidants in an acidic medium (pH 3.6). Antioxidant activity is obtained as

absorbance increases at 593 nm, and can be expressed as micromolar Fe^{2+} equivalents or relative to an antioxidant standard (Antolovich et al., 2002). Trolox (Pellegrini et al., 2003) or ascorbic acid (Gil et al., 2002) can be used as the standard. The reason for acidic pH conditions is to maintain iron solubility, and more importantly facilitate electron transfer. Low pH condition decreases the ionization potential that increases the redox potential, causing a shift in the dominant reaction mechanism (Simic & Jovanovic, 1994; Hagerman et al., 1998). Besides tripyridyltriazine (TPTZ), ferrozine (Molina-Diaz et al., 1998) and potassium ferricyanide are also used as the iron-binding ligand (Berker et al., 2010). The FRAP assay is totally electron transfer based rather than mixed hydrogen atom transfer and single electron transfer, so combined with other methods such as TEAC and, among others, can be very useful in distinguishing dominant mechanisms with different antioxidants. The FRAP assay is simple, fast and both manual and automated procedures were first described by Benzie and Strain (1996) to measure reducing power in plasma, but the assay was subsequently adapted and used for the assay of antioxidants in other biological fluids, foods, and plant extracts (Ou et al., 2002; Pellegrini et al., 2003). FRAP results can vary tremendously depending on the time scale of analysis, ranging from several minutes to several hour. Fast-reacting phenols that bind the iron or break down to the compounds with lower or different reactivity can be better analyzed with short analysis times, while slow-reacting polyphenols require longer reaction times for detection. Pulido et al. (2000) examined the FRAP assay of dietary polyphenols in water and methanol that the absorption of polyphenols such as caffeic acid and quercetin increased even after several hours of reaction time. Thus, a single-point absorption endpoint may not represent a completed reaction.

Other SET-based methods such as TEAC assay are similar to FRAP assay in that they rely on the hypothesis that the redox reactions proceed very rapidly. Compared with FRAP assay, TEAC assay is carried out at neutral pH and gives comparable relative values, but its values are usually higher for a given series of antioxidant compounds (Pulido et al., 2000; Cao & Prior, 2001; Erel, 2004).

2.5.2 Antioxidant evaluation in a model system

Antioxidants are important as additives for food preservation and health products, for their protective roles in the body against oxidative stress and the associated diseases and health disorders. Thus, model systems are used for antioxidant activity evaluation. The antioxidant evaluation in a model system is evaluated by monitoring the related changes by sensory, physical, chemical or instrumental methods. Model systems can be classified into two types, food model system and biological model system.

The evaluation of antioxidants in a food model system can be done in oils, emulsions, and muscle foods, among others. The antioxidant evaluation in food model systems is usually carried out under accelerated oxidation conditions by increasing temperature and oxygen supply, adding metal catalysts, or exposing the reactants to light. The process of lipid oxidation can be monitored by measuring the changes in oxygen consumption, oxidation substrate, oxidation products, and system change. Methods used to determine the extent of lipid oxidation include sensory evaluation, peroxide value (PV), conjugated dienes (CD), TBARS (thiobarbituric acid reactive substances), total carbonyl compounds, and volatile aldehydes, among others (summarized in Table 2-2; Shahidi & Zhong, 2015). In the oil systems, bulk oil, triacylglycerols and free fatty acids or their alkyl ester are used for antioxidant evaluation. The evaluation of antioxidant activity depends on the speed and rate of lipid oxidation and the oxidative state of the

model system in the absence or presence of antioxidants. Besides oil systems, the oil/water emulsion systems can be used for a more comprehensive assessment of antioxidant activity. One of the commonly practiced antioxidant evaluation in oil-in-water emulsion system is the β-carotene bleaching assay in which the emulsion composed of β-carotene, linoleic acid and water is used. Other emulsion systems include vegetable oil-in-water emulsion (e.g. sunflower oil, or soybean oil-in-water emulsions), fish oil-in-water emulsion, or fatty acids/water emulsions (Shahidi & Zhong, 2015). The methods to measure the products produced in emulsion systems by oxidation are similar with that of oil systems such as TBARS, and CD, among others.It is also reasonable to use raw or cooked muscle foods, especially cooked ground meat, to assess effectiveness of antioxidants in a food system. Evaluation of the antioxidant efficiency in cooked ground meat can help by predicting if antioxidant works in thermal processing of whole or modified tissue foods, to prevent rapid oxidation of its lipid and meat flavour deterioration (Rubin & Shahidi, 1988). Lipid oxidation in muscle foods is complex as lipid might interact with other components such as protein, haem, metal and salt (Ladikos & Lougovois, 1990). Proteins in meat can also be oxidized during thermal treatment, and produce carbonyl compounds leading to quality deterioration. Muscle food model systems used for antioxidant assessment include ground pork, beef, poultry meat or fish, ground or fillet, in fresh, refrigerated, or cooked forms. During processing, antioxidants may be added to muscle foods at different steps and their efficiency evaluated by measuring the oxidation products with or without antioxidants.

1 Table 2-2. Major methods used for measuring oxidation products.

Method	Oxidation marker	Reagent	Detection	Results expression
peroxide value (PV)	Hydroperoxide	Potassium iodide	Titration	% inhibition
		Thiocyanate or xylenol orange	Spectrophotometry	% inhibition
		Triphenylphosphine	FTIR	% inhibition
Conjugated dienes	Conjugated dienes		Spectrophotometry	% inhibition
conjugable oxidation products (COPs)	conjugated trienes and tetraenes		Spectrophotometry	% inhibition
TBARS	malonaldehyde or malondialdehyde (MDA) equivalents	Thiobarbituric acid (TBA)	Spectrophotometry	% inhibition
p-anisidine value	Aldehydic oxidation products (principally 2-alkenals and 2,4-alkadienals)	*p*-methoxyaniline (anisidine)	Spectrophotometry	% inhibition
Total carbonyls	Carbonyl compounds	2,4-Dinitrophenylhydrazine (DNPH)	Spectrophotometry	% inhibition
Headspace volatile	Volatile compounds, usually volatile aldehydes		Headspace gas chromatography	% inhibition
Oil stability index (OSI)	Volatile organic acids		The Rancimat or the Oxidative Stability Instrument	Induction period, protection factor

2

To assess the efficiency of antioxidant in protecting the body from oxidative stress and the associated diseases and health disorders, biological systems are used, including *in vitro, ex vivo* and *in vivo* biological model systems. The methods include LDL-cholesterol oxidation inhibition assay, inhibition of DNA oxidation, and cellular assays (red blood cells and HepG2 cells) for evaluation of antioxidant activity.

Oxidized LDL is a risk factor for atherosclerosis (Steinberg & Witztum, 2010). LDL can undergo peroxidation in which transition metal ions such as cupric ion play an important role (Aust & Svingen, 1982). Oxidized LDL include both lipid oxidation products and oxidized apoprotein particles that promote the atherogenic effects (Liangli et al., 2012). Oxidation of protein leads to extensive alteration in the protein composition and structure. The lipid/protein oxidation products generated during the oxidation of LDL include fatty acid oxidation products (free and esterified fatty acid peroxides and hydroxides, prostaglandin-like products, aldehydes, core aldehydes that contain esterified lipid backbone, pentane and other hydrocarbons), lipid derived products (lysophosphatidylcholine, cholesterol oxidation products, internally modified phosphatidyl ethanolamine/serine products), and protein oxidation products (protein carbonyls, non-enzymatic proteolyzed fragments, modified cysteine, cystine, histidine, methionine, lysine, arginine, tryptophan, and tyrosine, lipid–protein adducts which could be classified as ceroids (lipofuscins) (Parthasarathy et al., 2010). The formation of LDL oxidation products might depend on the type of oxidant, the fatty acid profile, the extent of oxidation, and the presence or absence of other agents such as redox metals. Generally, PUFAs in LDL are prone to the oxidation while MUFAs are less oxidizable (Reaven et al., 1993; Lada & Rudel, 2003). LDL oxidation might be due to lipoxygenase reaction, copper and ceruloplasmin-mediated oxidation, iron-mediated oxidation, peroxidase-mediated oxidation including myeloperoxidase and haem,

peroxynitrite-mediated oxidation, thiol-dependent oxidation, xanthine oxidase, NADPH oxidase, and other superoxide generators, AAPH or other means of radical generation including cytochromes (Parthasarathy et al., 2010). The LDL oxidation by different mechanisms might lead to different results. Peroxidase-mediated oxidation requires co-oxidants such as hydrogen peroxides or lipid peroxides, and generate very little aldehyde products as compared to metal-catalyzed oxidations (Heinecke, 1997). The treatment of LDL with AAPH, the radical generator, resulted in more protein oxidation than lipid peroxidation (Noguch et al., 1994; Dinis et al., 2002). Antioxidants such as some polyphenols have inhibition effect against LDL oxidation by scavenging free radicals and other ROS, chelating prooxidant metals, and binding with the apo-lipoprotein B, which promotes the access of antioxidant to the lipids and prevents interaction with prooxidants (Shahidi & Zhong, 2015). The formation of conjugated dienes during lipid peroxidation was usually uesd as the marker for measuring the oxidizability of LDL. In the assay, LDL-cholesterol, initiator metal ion (cupric ion, Cu^{2+}) or peroxyl radical and antioxidants are incubated at 37 °C for 20 h, and the formation of conjugated dienes is periodically monitored at 234 nm. Antioxidant activity is reported as % inhibition of conjugated dienes formation as compared with a control without antioxidants.

Oxidative stress and DNA damage caused by free radical attacks are related to various diseases and pathological conditions such as carcinogenesis, atherosclerosis, and ageing (Klaunig & Kamendulis, 2004; Ishii, 2007; Laviano et al., 2007; Bonomini et al., 2008). The DNA strand scission assay is used to evaluate the antioxidant activity of phenolic compounds and extracts in DNA model systems for their potential as antimutagenic agents (Chandrasekara & Shahidi, 2011). The DNA strand scission assay is performed to assess the protective effect of antioxidants on hydroxyl and peroxyl radical-induced DNA scission of plasmid pBR322. DNA stand scission

results in the reduction of supercoiled circular DNA into increased levels of an open circular form (Hiramoto et al., 1996). Hydroxyl and peroxyl radicals are used in this assay due to being responsible for oxidative damage to DNA, especially the mitochondrial DNA (Perron et al., 2008). Hydroxyl radicals can be generated by the reaction between $O_2^{\cdot -}$ and H_2O_2, in the presence of metal ions, while the peroxyl radical is usually generated by AAPH. After incubation with radicals and antioxidants at 37 °C, the DNA fractions are separated by gel electrophoresis and bands are visualized under trans-illumination of UV light. DNA stand scission results in the reduction of supercoiled circular DNA into increased levels of an open circular form (Hiramoto et al., 1996). Thus, both supercoiled circular DNA and open circular form may be observed as results of DNA oxidation. The concentration of the supercoiled and nicked DNA fractions is obtained from densitometry as indicated by the intensity or density of the corresponding bands. Antioxidants inhibit DNA scission possibly through a combination of radical scavenging and ferrous ion chelation mechanisms, and their inhibition efficiency can be calculated as DNA retention (% DNA retained un-oxidized and supercoiled). In the presence of antioxidants, the concentration of DNA with the open circular form decreases and concentration of DNA with supercoiled form increases, when compared with that devoid of any antioxidant.

2.6 Bioactivities

It is well known that phenolic compounds have antioxidant, antiviral, anti-inflammatory, anticancer, antidiabetic, anti-allergic, and antimicrobial activities, among others. The mechanisms of these biological activities of phenolics and their related health effects have been reviewed (Scalbert et al. 2005; Aron & Kennedy 2008). Phenolics can act as antioxidants and inhibit oxidation of biomolecules (e.g. membrane lipids, LDL, proteins and DNA) and thus prevent or inhibit pathologies such as inflammation, atherosclerosis and carcinogenesis

(Biesalski 2007). Olives have multiple benefits and health-promoting bioactives due to their nutrients and functional ingredients such as tyrosol, hydroxytyrosol and their derivatives. The polyphenolic compounds contained in olives have been reported to exert various bioactivities, including antioxidant, anti-inflammatory, and antimicrobial activities against bacteria, fungi, and mycoplasma.

2.6.1 Antiviral

In early times, the ‘virus’ was used to describe microbial pathogens that could not be removed by filtration (Knight, 1974). Now, it is defined as infective agents which depend on living host cells for their replication (Lycke & Norrby, 2014). Viruses can be either enveloped or non-enveloped, with DNA or RNA genomes (nucleic acid core). Compared to non-enveloped viruses, enveloped viruses have lipid bilayer membranes acquired through budding from the hosts' cell membrane. In all viruses, a capsid consisting of a protein shell surrounds the viral nucleic acid. Viral infection (viral replication) involves the incorporation of viral DNA or RNA into a host cell, replication of that material, and the release of the new viruses. The viruses will attach to receptors on the host cell surface and enter through the host cell membrane and then get uncoated. During replication, early regulatory proteins, new viral RNA or DNA, and late structural proteins are synthesized. Finally, they are assembled and release from the cell. Viral infection can cause mild, moderate, and severe diseases, including influenza, liver infection (hepatitis), encephalitis, and acquired immune deficiency syndrome (AIDS), among others. Millions of people have been infected by virus, and even died of these disease. For instance, 370 million chronic infections were caused by hepatitis B virus (HBV), 130 million people were infected by HCV, 40 million people by HIV as reported by Alter (2006). Therefore, antiviral therapy is necessary. Several targets for antiviral therapy have been found, including viral

attachment to cell and fusion, protein translation in infected cells, protein processing, DNA synthetic enzymes, DNA integrase, and immune system, among others (Pawlotsky, Chevaliez, & McHutchison,2007; Pommier, Johnson, & Marchand, 2005; Giri, Ugen, & Weiner,2004). Thus, antiviral agents can act as fusion inhibitors, interferon, specific protease inhibitors, reverse transcriptase inhibitors, DNA polymerase inhibitors, DNA integrase inhibitors, and effective vaccines, in order to restore immune surveillance, among others.

HCV is a positive strand RNA enveloped virus with six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) which aid in either viral assembly and/or viral replication (Lindenbach & Rice, 2005; Lindenbach et al., 2005). HCV is the major cause of several severe liver diseases including chronic hepatitis, liver fibrosis, cirrhosis, and hepatocellular carcinoma. Unfortunately, there is no effective vaccine, and the current treatments are expensive and associated with severe side effects (McHutchison et al., 1998; Patel & McHutchison, 2004). For instance, the combinations of pegylated interferon-α (Peg-IFNα) and ribavirin have been reported to be only successful for approximately 50% of individuals infected with HCV (McHutchison et al., 1998). Peg-IFN-α is a general antiviral agent supporting the immunological response while the mechanism of action of ribavirin has not yet been completely understood (Feld & Hoofnagle, 2005; Parker, 2005). The side effects caused by this treatment include fatigue, flu-like symptoms, mild anxiety, skin rash, nausea, diarrhea, autoimmune diseases, haemolytic anemia, depression, and other neuropsychiatric side effects (Fried et al., 2002; Hauser, 2004). Recently, specifically directed antivirals such as direct-acting antiviral compounds targeting the NS3/4A protease are being updated and tested (Jacobson et al., 2011; Poordad et al., 2011; Götte & Feld, 2016). The early NS3/4A inhibitors, including simeprevir, vaniprevir, asunaprevir and faldaprevir show overlapping resistance profiles and a limited

genotype coverage (Sarrazin et al., 2012). The new protease inhibitors MK-5172 and neceprevir showed higher antiviral activity and a broader genotype coverage with favourable resistance profiles (Clark, Peter, & Nelson, 2013). New drugs targeting other viral proteins are also under development (Poordad & Dieterich, 2012; Wartelle‐Bladou et al., 2012). Phenolic compounds are reported to exhibit the anti-HCV activity. Polyak et al. (2007) showed that flavonolignans (silibinins) present in silymarin are responsible for the anti-HCV activities, possibly due to the inhibitory action of silibinin on the NS5B RNA-dependent RNA polymerase (Ahmed–Belkacem et al., 2010). Another flavonoid, (−)-epigallocatechin-3-gallate (EGCG), showed a dose-dependent inhibition against HCV infection (Ciesek et al., 2011; Calland et al., 2012; Zhong, Ma, & Shahidi, 2012). Bachmetov et al. (2012) found that quercetin inhibited the activity of NS3 protease. Duan et al. (2004) reported three polyphenol components from the ethyl acetate fraction of the traditional Chinese medicine Galla which could inhibit NS3 protease *in vitro*. Gallic acid (GA), a natural phenolic compound, inhibited and decreased HCV expression through its antioxidant capacity (Govea Salas et al., 2016). Hydroxyanthraquinones showed inhibition activity against NS3 helicase, depending on the number and position of the phenolic hydroxyl group (Furuta et al., 2015).

The HIV is a Ientivirus of the Retroviridae family and there are two main subtypes, HIV-1 and HIV-2. Both viruses lead to AIDS but the pathogenic course of HIV-2 appears to be longer (Kong et al., 1988; Evans et al., 1988). It has been reported by Joint United Nations Programme on HIV/AIDS (UNAIDS) (2016) that there were almost 36.7 million people worldwide infected by HIV at the end of 2015 and 1.8 million people were children. HIV-1 reverse transcriptase, protease, and integrase play an important role in the viral life cycle. The first antiretroviral drug were azidothymidine (AZT) that blocked reverse transcription by binding the reverse

transcriptase (Mitsuya et al., 1985). Later, the antiretroviral drugs were classified into nucleoside analog RT inhibitors (NRTIs) and non-nucleoside RT inhibitors (NNRTls). NRTIs are nucleoside derivatives that block reverse transcription by competitively binding to the active site of RT, including AZT, ddI, ddC, d4T, and abacavir. NNRTls such as nevirapine, delavirdine, and efavirenz, binding to regions other than the active site and sterically block the incorporation of incoming nucleosides (De Clercq, 1992). However, the HIV reverse transcriptase lacks proof-reading activity, making it highly error-prone and capable of mutating its genome, leading to the generation of mutant viruses that can replicate even in the presence of multiple drugs (Wain-Hobson, 1993). Later, HIV protease inhibitors were developed that can prevent cleavage of gag and gag-pol precursors, and thus arrest maturation and block infectivity of nascent virions (Karacostas et al., 1989; Roberts et al., 1990), including amprenavir, indinavir, nelfinavir, saquinavir, and ritonavir (Flexner, 1998; Miller, 1999). They can reduce viral load rapidly and profoundly within a few days after the start of treatment (Ho et al., 1995; Benson, 1995). It is known that olive leaf extracts exhibit antiviral activities against HIV-1. Lee-Huang et al. (2003) found that olive leaf extract (OLE) could inhibit acute infection and cell-to-cell transmission of HIV-1. Bao et al. (2007) also reported the inhibition effect of olive leaf extract against HIV-1 and hydroxytyrosol was identified as the main molecule responsible for binding to HIV-1 envelop protein gp41. Many phenolic compounds such as EGCG have been reported to have anti-HIV activity (Singh, Bharate & Bhutani, 2005).

Phenolic compounds are also known to inhibit many other viruses. EGCG can inhibit the maturation, replication, infectivity and function of adenovirus, coronavirus, influenza virus, rotavirus, herpes simplex virus (HSV), and hepatitis A virus (HAV), among others (Zhong, Ma, & Shahidi, 2012). Polyhydroxycarboxylates derived from phenolic compounds, caffeic acid and

alkyl-esters of gallic acid have been reported to have inhibition effect against herpes simplex virus (Meerbach et al., 2001; Chiang et al., 2002; Savi et al., 2005). Hydroxytyrosol can inhibit influenza virus by morphological change of the virus, and it was revealed that hydroxytyrosol was effective against the enveloped viruses, but not against the non-enveloped viruses (Yamada et al., 2009).

2.6.2 Anti-inflammatory

Inflammation is a normal biological response of body tissues to harmful stimuli such as pathogens, damaged cells, or irritants, and is a self-protection response involving immune systems help eliminating the initial cause of cell injury, clearing out damaged cells and tissues, and initiating tissue repair. The classical signs of inflammation are redness, swelling, warmth, and sometimes pain and some immobility. However, inflammation can lead to progressive tissue damage caused by unbalanced or prolonged inflammation, and it plays a role in some chronic diseases such as cancer and diabetes. There are two types of inflammation, acute and chronic. Acute inflammation is the initial response of the body to harmful stimuli, and is usually beneficial for the host while chronic inflammation leads to a progressive shift in the type of cells present at the site of inflammation, and is associated with various chronic illnesses, including cancer (Bartsch & Nair, 2006; Lin & Karin, 2007). Inflammation is often characterized by recruitment of mast cells and leukocytes and an increased release and accumulation of soluble mediators (e.g. arachidonic acid, cytokines and chemokines, etc.) and reactive oxygen species (ROS) at the site of damage (Coussens & Werb, 2002). Under an oxidative environmental stress, ROS can be produced over a long time by and promote endothelial dysfunction by oxidation of crucial cellular signaling proteins such as tyrosine phosphatases, and thus the ROS can be both a signaling molecule and a mediator of inflammation (Mittal et al., 2014). In addition, reactive

nitrogen species (RNS), inducing nitrosative stress and adding to the pro-inflammatory burden of ROS, can be formed by combing ROS with NO at a diffusion limited rate (k=5 to 10×10^9 $M^{-1}s^{-1}$) (Beckman, 1996; Mittal et al., 2014). Therefore, production of ROS/RNS plays an important role in the activation of a variety of kinases and transcription factors mediating immediate cellular stress responses and the progression of many inflammatory diseases, depending on the redox changes. These transcription factors include nuclear factor kappa B (NF-κB), signal transducer and activator of transcription 3 (STAT3), hypoxia-inducible factor-1α (HIF1-α), activator protein-1 (AP-1), nuclear factor of activated T cells (NFAT) and NF-E2 related factor-2 (Nrf2), among others (Reuter et al., 2010). It has been reported that induction of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), aberrant expression of inflammatory cytokines [tumour necrosis factor (TNF), interleukin-1 (IL-1), IL-6 and chemokines (IL-8; CXC chemokine receptor 4, CXCR4) play a role in oxidative stress-induced inflammation (Reuter et al., 2010). As an example, NF -KB and AP-1 are redox-sensitive and become activated under oxidative/nitrosative stress leading to the up-regulation of numerous inflammatory genes, such as those coding for iNOS and COX-2, among others (Kamata & Hirata, 1999). In research, some inflammatory mediators can be used to simulate inflammation, including cytokines (e.g., TNF-α), the stress of hyperoxia, ischemia-reperfusion injury, bacterial toxins (LPS), and mediators that ligate cell surface receptors (PAF, thrombin, histamine, VEGF, and bradykinins) (Mittal et al., 2014).

The most common anti-inflammatory drugs can be classified as corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDS). Corticosteroids reduce inflammation by reducing the production of chemicals, such as prostaglandins (PGs) and leukotrienes (LT5), involved in inflammation (Vane & Botting, 1987). However, their pharmacologic actions on ocular tissue are

still unclear (Jaanus & Lesher, 1995). The more recent NSAIDS, such as aspirin and naproxen (Aleve), provide effective therapy for inflammatory by blocking the enzyme cyclooxygenase, thus inhibiting the prostaglandin synthesis pathways (Hunskaar & Hole, 1987). However, these drugs are expensive and have side effects. For instance, NSAIDS can develop serious adverse gastrointestinal events (Gabriel, Jaakkimainen & Bombardier, 1991).

Therefore, natural products and anti-inflammatory food related products are known to be lower in cost with limited side effects and intolerance compared to drugs for treating inflammation. Phenolic compounds found abundantly in plant foods have been studied for their anti-inflammatory activities in controlling the synthesis or gene expression and enzyme activity of many pro-inflammatory mediators (Shahidi & Zhong, 2009). Phenolic compounds act as anti-inflammatory agents by modulation of pro-inflammatory gene expression such as cyclooxygenase, lipoxygenase, nitric oxide synthases and several pivotal cytokines, mainly by acting through NF-κB and mitogen-activated protein kinase signalling (Santangelo et al., 2007). Curcumin, a low molecular weight polyphenol, has an anti-inflammatory effect. Lal et al., (2000) reported that curcumin could be used as a safe therapy in the treatment of idiopathic inflammatory orbital pseudotumours. Quercitrin and rutin, the most common flavonoids, exhibited beneficial effects in experimental inflammation in the rat induced by trinitrobenzene sulphonic acid (de Medina et al., 1996). EGCG has been shown to possess anti-inflammatory activity by scavenging NO and the peroxynitrite anion (Paquay et al. 2000). The phenolic compounds, Baicalein, oroxylin A, and wogonin, isolated from S. baicalensis showed strong anti-inflammatory activities by inhibiting the production of NO (Huang, Lee, & Yang, 2006). The herb S. japonica, containing high amount of phenolics and flavonoids, displayed anti-

inflammatory effect by in vitro inhibition of the production of NO and TNF-α (Zhang et al., 2011).

Tyrosol and hydroxytyrosol, the olive phenolics, have been found to have anti-inflammatory activity. Tyrosol have been demonstrated to inhibit, *in vitro* and *in vivo*, pro-inflammatory gene expression by scavenging reactive oxygen species (de la Puerta et al., 2001). Giovannini et al. (2001) reported that tyrosol and caffeic acid could inhibit inflammatory reactions by inhibiting LPS-induced TNF-alpha release. Tyrosol could also prevent inflammation by inhibiting iNOS and COX-2 gene expression (De Stefano et al., 2007). Tyrosol derived from extra virgin olive oil could decrease inflammatory mediator production by human whole blood cultures (Miles, Zoubouli, & Calder, 2005). Hydroxytyrosol has been found to show strong anti-inflammatory effect by inhibiting TNF-α,interleukin 1 beta (IL-1β), iNOS, and COX-2 expression (Cicerale, Lucas, & Keast, 2012). Oleuropein, the derivative of hydroxytyosol can prevent inflammatory by inhibiting lypoxygenase activity and the production of leukotriene B4 (Omar, 2010). Oleuropein could reduce inflammatory responses by inhibiting TLR and MAPK signaling (Ryu et al., 2015).

2.6.3 Anticancer

Cancer is one of the major causes of global mortality in humans. In 2012, the worldwide burden of cancer increased to an estimated 14 million new cases per year and cancer deaths were predicted to rise from an estimated 8.2 million annually to 13 million per year (Stewart & Wild, 2016). Furthermore, increasing evidence suggests that inflammatory response plays a pivotal role in a multitude of chronic ailments, including cancer. Thus, increased production of pro-inflammatory mediators and pro-inflammatory transcription factors could drive some 90% of all cancers (Sethi et al., 2012). Thus, the incidence of cancer decreased in patients taking nonsteroidal anti-inflammatory drugs (Trinchieri, 2012). Besides, the promising anti-cancer

drugs can be classified into chemotherapeutic (alkylating agents, antimetabolites, antimitotics, antibiotics, and topoisomerase inhibitors, among others), hormonal therapeutic (the steroid drugs) and immunotherapeutic agents (interferons, interleukins and vaccine). Many successful anti-cancer drugs are natural products or their analogues as important sources of anti-cancer molecules (Cragg & Newman, 2013). Among these natural products, phenolic compounds (flavonoids, hydroxycinnamates, hydroxybenzoates, coumarins, xanthones, chalcones, stilbenes, lignins and lignans) have proven to possess anticancer activities as reviewed by Carocho and Ferreira (2013). Curcumin, resveratrol, and their related derivatives, as well as gallic acid, chlorogenic acid, caffeic acid, carnosol, capsaicin, 6-shogaol, 6-gingerol, and their corresponding derivatives are also suggested to be effective in prevention of cancer metastasis (Weng & Yen, 2012). EGCG has been known to possess promising anticancer potential, which is thought to be attributed to its antioxidant activity, induction of phase II enzymes, inhibition against TNF-α expression and release, inhibition against cell proliferation, and induction of apoptosis, among others (Pham-Huy et al. 2008). Rosemary extract has also been shown to possess anticancer effect by inhibiting 7,12-dimethylbenz(a)anthracene (DMBA)-induced mouse skin papilloma formation and rat mammary carcinogenesis, due to its high content of polyphenols such as carnosol, carnosic acid, rosmanol, rosmarinic acid, and ursolic acid (Huang et al., 1994; Singletary, MacDonald, & Wallig, 1996; Ngo, Williams, & Head, 2011).

Tyrosol and hydroxytyrosol have been shown to have promising anticancer potential. Epidemiologic data show that the Mediterranean diet has significant protective effects against cancer and coronary heart disease, mainly due to the phenolic fractions (simple phenols: tyrosol, hydroxytyrosol) of olive oil, which confers its health-promoting properties by the route of antioxidant activity (Filik & Ozyilkan, 2003). Tyrosol and hydroxytyrosol have been reported to

have breast cancer prevention benefits, by their ability to protect against DNA damage in breast cancer cell lines (Alegre et al., 2013). Fabiani et al. (2002) suggested that hydroxytyrosol might exert a protective activity against cancer by arresting the cell cycle and inducing apoptosis in tumour cells. Hydroxtyrosol and its derivative oleuropein inhibited human breast cancer by inhibiting the rate of cell proliferation, inducing cell apoptosis, and blocking of G1 to S phase transition manifested (Han et al., 2009).

2.6.4 Other activities

There have been many reports that reveal phenolic compounds possess many other properties such as antiatherogenic, antidiabetic, anti-allergic, and antimicrobial activities, among others. Among plant phenolics, tyrosol and hydroxytyrosol have been reported to reduce LDL-cholesterol activity, decreasing the risk of cardiovascular disease, preventing several chronic diseases (for example, atherosclerosis), strokes and antimicrobial activity.

Resveratrol can play a role in the prevention of human cardiovascular diseases by its cholesterol-lowering effect and inhibition against LDL-cholesterol oxidation (Frémont, 2000). Myricetin, a natural bioflavonoid, can act as a potent anticarcinogen and antimutation as well as cardioprotective agent and antidiabetic agent (Ong & Khoo, 1997). Tyrosol and hydroxytyrosol have been shown to possess antimicrobial properties against several strains of bacteria responsible for intestinal and respiratory infections *in vitro* (Cicerale, Lucas, & Keast, 2012). Cranberry proanthocyanidins can competitively inhibit cellular adherence of uropathogenic strains of P-type E coli to mucosal cells in the urinary tract and thereby show antimicrobial effect (Howell, 2002). Alves et al. (2013) found that phenolic compounds such as 2,4-dihydroxybenzoic, vanillic, syringic and *p*-coumaric acids from mushroom species could be used as antimicrobial agents. Polyphenols of millets exhibited antimicrobial activity (Viswanath,

Urooj, & Malleshi, 2009). Phenolic acids such as caffeic, *p*-coumaric, ferulic and protocatechuic acids have been reported to exhibit antifungal effects (Dragland et al., 2003). Olive phenolics such as tyrosol play an important role in dynamics of growth and morphogenesis in the human fungal pathogen Candida (Chen et al., 2004). Luteolin and its glycosides found in millets exhibit antiarrhythmic activities (Han, Shen, & Lou, 2007). Tricin can act as antitumour and anti-leukemic agents (Lee et al., 1981). Eighteen phenolics such as (-)-epicatechin, ferulic acid, chlorogenic acid, (+)-catechin and *p*-hydroxybenzoic were identified in the extracts of the Cotoneaster species and are found to possess protective effect against Alzheimer's disease (AD) and diabetes mellitus (DM), as well as antimicrobial and anti-mutagenic effect (Uysal et al., 2016). Kumar and Pandey (2013) reviewed the biological activities of flavonoids and revealed that flavonoids exhibited coronary heart disease prevention and hepatoprotective effects.

In addition, the ability of tyrosol to bind LDL had been reported, and thus it could prevent lipid peroxidation and atherosclerotic processes (Covas et al., 2002). Hydroxytyrosol can act a therapeutic tool in the prevention of neurodegenerative diseases by crossing the blood-brain barrier (Rodríguez-Morató et al, 2015). It is demonstrated that hydroxytyrosol, oleuropein, and oleuropein aglycone have the ability to prevent tau (the proteins expressed in neurons of the central nervous system) fibrillization *in vitro* (Daccache et al., 2011). Hydroxytyrosol was found to be a new multi-targeted anti-angiogenic compound due to its inhibitory effects on endothelial cell proliferation, migration and “tubule-like” structure formation on Matrigel (Fortes et al., 2012). Carluccio et al. (2003) demonstrated that hydroxytyrosol and oleuropein inhibited early stages in atherogenesis, by reducing lipopolysaccharide (LPS)-stimulated expression of vascular adhesion molecule-1 (VCAM-1) in human vascular endothelial cells. Hydroxytyrosol could

reduce the expression of ageing-related proteins as well as the infarct size and cardiomyocyte apoptosis (Mukherjee et al., 2009).

CHAPTER 3

MATERIAL AND METHODS

3.1 Materials

The lipases from *Candida antarctica* (Novozyme-435) and lipase from *Candida rugosa* (AY30, type VII) were bought from Sigma-Aldrich (St. Louis, MO, USA). Amano lipase PS from *Burkholderia cepacia* (*Pseudomonas cepacia*) was purchased from Amano Enzyme Inc. (Nagoya, Japan). Tyrosol (4-hydroxyphenethyl alcohol) and hydroxytyrosol (4-(2-Hydroxyethyl)-1, 2-benzenediol) were purchased from Sigma-Aldrich. Docosahexaenoic acid (DHA) single cell oil (DHASCO) containing about 40 % DHA was obtained from DSM (Columbia, MD, USA). The EPADEL capsules were obtained from Mochida Pharmaceutical Industries LTD (Tokyo, Japan) and kindly provided by Professor Kazuo Miyashita of Hokaido University (Hakodate, Japan). Free fatty acids (butyric, caproic, caprylic, capric, lauric, myristic, palmitic, stearic, oleic, and linolenic acids) were purchased from Nu-Chek (Elysian, MN, USA).

Trolox (6-hydroxy-2,5,7,8-tetratnethylchroman-2-carboxylic acid) was purchased from Sigma-Aldrich. The solvents and reagents such as ethanol, acetone, hexane, methanol, sulphuric acid, isooctane, chloroform, acetic acid, diethyl ether, carbon and sodium carbonate as well as mono- and dibasic sodium and potassium phosphates and methyl tertiary-butyl ether were purchased from Fisher Scientific Co. (Nepean, ON, Canada). 2,2'-Azobis (2-tnethylpropionamidine) dihydrochloride (AAPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Hydrogen peroxide, sodium hydroxide, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), 5,5-dimethyl-1 - pyrroline-N-oxide (DMPO), ferrous sulphate,

ferric chloride, ethylenediaminetetraacetic acid (EDTA), agarose, trizma acetate(Tris acetate salt) and human LDL cholesterol were also purchased from Sigma-Aldrich Canada Ltd.

Deoxyribonucleic acid (DNA) of pBR 322 (E.coli strain RRI) was purchased from Thermo fisher Scientific (Waltham, MA, USA). Silica gel thin layer chromatographic plates (TLC; 60-A, F-254; 2.5*7.5 cm; 200 Micron) were purchased from Select Scientific (Atlanta, GA, USA).

The RAW 264.7 cells, derived from murine macrophages, were obtained from the American Type Culture Collection (Rockville, MD, USA). Cell culture medium was acquired from GIBCO (Grand Island, NY, USA). The SensoLyte 520 HCV fluorimetric Protease Assay Kit (lot #1028) and HCV NS3/4A protease (lot# 103-075), purchased from Anaspec. Company (San Jose, CA, USA). α-Glucosidase (from Bacillus stearothermophilus) were purchased from Sigma-Aldrich Canada Ltd. Fetal bovine serum (FBS) was purchased from Biological Industries (Cromwell, CT, USA), and tert-butylhydroperoxide (t-BuOOH) and dichlorofluorscein-2'7'-diacetate (DCFH-DA) were purchased from Sigma-Aldrich. HepG2 cells (lot #07112007) were purchased from Health Science Research Resources Bank (Osaka, Japan). BSA (bovine serum albumin, lyophilized powder purified by heat shock fractionation) and glucose were purchased from Sigma–Aldrich.

3.2 Methods

3.2.1 Synthesis of tyrosol and hydroxytyrosol fatty acid esters

3.2.1.1 Extraction and purification of DHA

DHASCO (60 g, treated with 200 ppm butylated hydroxytoluene; BHT) was saponified by refluxing for 1 h at the boiling temperature of the mixture (62 °C) under a blanket of nitrogen using a mixture of KOH (13.8 g), water (26.4 mL) and 95% (v/v) ethanol (158.4 mL). To the

saponified mixture, distilled water (120 mL) was added and the unsaponified matter was extracted into hexane (2×200 mL) and discarded. The aqueous phase containing saponifiable matter was acidified (pH=1.0) with 3M HCl. The mixture was transferred to a separatory funnel and the liberated fatty acids were extracted into 50 ml of hexane (4 times). The hexane layer, containing free fatty acids, was then dried over anhydrous sodium sulphate and the solvent removed at 40 °C to recover DHA which was then stored at -60 °C until use. For urea complexation, the free fatty acids (60 g) were mixed with 900 ml urea (20%, w/v) in 95% ethanol, and heated at 65 °C with stirring until the whole mixture turned into a clear homogeneous solution. The mixture was left to stand for 24 h at 4 °C for urea-fatty acid adduct crystallization. The mixture was then filtered by using a Buchner funnel lined with a thin layer of glass wool. The filtrate was diluted with an equal volume of water and acidified to pH 4-5 with 6M HCl; an equal volume of hexane was subsequently added and the mixture was stirred thoroughly for 1 h, then transferred to a separatory funnel. The hexane layer, containing liberated fatty acids, was separated from the aqueous layer containing urea. The hexane layer was washed with distilled water (the separation procedure repeated twice) to remove the remaining urea and subsequently dried over anhydrous sodium sulphate and the solvent was then removed at 40 °C using a rotary evaporator.

3.2.1.2 Preliminary screening

The preliminary selection of enzymes were carried out in 20 mL vials in which the chosen enzyme (*Candida antarctica* lipase SP 435, *Candida cylindracae* lipase AY30, Amano lipase PS from *Burkholderia cepacia*, 20 mg) was added to a solution of tyrosol (20.7 mg, 0.15 mmol) in t-butyl methyl ether (5 mL), containing the selected fatty acid, such as DHA (1.5 mmol). Control reactions without enzyme were carried out under the same conditions. The mixture was shaken

under a nitrogen blanket at 40 °C for 24 h. After the reactions were quenched by filtering off the enzyme, the filtrates were subsequently taken to dryness using a rotary evaporator at 40 °C

3.2.1.3 General procedure for enzymatic esterification

The general enzymatic synthesis procedure was modified form that reported by Grasso et al. (2007). *Candida Antarctica* lipase (100 mg) and the acyl donor (DHA, EPA, 18 mmol; butyric acid, caproic acid, caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid, and linolenic acid, 6 mmol) were added to a solution of the substrate (TY or HTY, 3 mmol) in t-butyl methyl ether (100 mL) and the mixture was shaken (400 rpm) in an orbital shaker at 40 °C for 24 h. The reactions were quenched by filtering off the enzyme and concentrated to yield a crude residue, which was further purified.

3.2.1.4 Purification and identification of tyrosol and hydroxytyrosol esters

Purification of the TY and HTY esters from the crude product mixture was achieved by using a simplified base extraction method in order to remove the unreacted free fatty acids. The extra free fatty acids (1 g) was removed by refluxing for 20 min (after that there will be only the target compounds and solvent left) at the boiling temperature of the mixture (45 °C) with stirring under a blanket of nitrogen using a mixture of sodium carbonate (equal weight to the synthesis mixture, 1 g), water (5 mL) and 95% (v/v) ethanol (15 mL). After the reaction, extra undissolved sodium carbonate was removed by filtering through a Whatman No.1 filter paper. The target compound was extracted with n-hexane (3 × 20 mL), and the organic phase was dried by filtering through a layer of anhydrous sodium sulphate. Finally, the filtrates were taken to dryness using a rotary evaporator at 40 °C.

The mixture is then analyzed by using thin layer chromatographic (TLC) analysis. The reaction mixture (0.1 ml) from synthesis procedure was transferred to a 4 mL vial, and the solvent was removed under a stream of nitrogen. The sample was redissolved in 2 mL of methanol/water (95:5, v/v) for TLC analysis and HPLC analysis. After loading the samples, the TLC plates were developed in a mixture of hexane/ethyl acetate/formic acid (3:3:0.12, v/v/v) by adding 3 mL of hexane, 3 mL of ethyl acetate, and 0.12 mL of formic acid to the 100 mL chromatography tank. After that, the plate was loaded into the tank and allowed to develope. Normally, three to five samples could be analyzed in one plate.

The composition of the reaction mixture was determined by using reversed phase high-performance liquid chromatography-mass spectrometry (HPLC-MS), using an Agilent 1100 HPLC unit (Agilent Technologies, Palo Alto, CA, USA) with a UV diode array detector (UV-DAD). Separation was achieved on a SUPELCOSIL™ C-18 column (4.6 mm×250 mm, 5 μm) (Sigma-Aldrich) coupled with a SUPELCOSIL™ LC-18 Supelguard™ Cartridge (4.0 mm×20 mm, 5 μm) (Sigma-Aldrich) by gradient elution with a methanol/water mobile phase (80:20-95:5, v/v, from 0 to 20 min, and 95:5, v/v, for 10 min) at a flow rate of 1 mL/min, and fractions were detected at 216-232 nm by UV detector. HPLC flow was further analyzed online by the MS detector system (LC-MSD-Trap-SL, Agilent) with atmospheric pressure chemical ionization (APCI) at positive mode for identification of each fraction. The MS conditions were as follows: drying gas flow rate, 5 L/min; nebulizer pressure, 60 psi; drying gas temperature, 350 °C; APCI temperature, 400 °C; and capillary voltage, 110 V.

3.2.2 Antioxidant evaluation

3.2.2.1 DPPH radical scavenging activity

DPPH radical scavenging activity of TY, HTY and their esters was determined according to the method described by Wang and Shahidi (2013) with slight modification. TY, HTY and their esters in ethanol (250 μL) were mixed with 1 mL of ethanolic solution of DPPH (0.18 mM). Different samples were used at different concentrations from 100 μM to 25 mM. Contents of each test solution were thoroughly mixed and allowed to stand in the dark for 10 min. The mixture was subsequently injected into the sample cavity of a Bruker E-scan electron paramagnetic resonance (EPR) spectrometer (Bruker Biospin Co., Billercia, MA, USA). Ethanol was used as the control instead of the test compounds. The operating parameters of the Bruker E-scan were set as follows: 5.02 × 102 receiver gain, 1.86 G modulation amplitude, 2.621 s sweep time, 8 scans, 100.00 G sweep width, 3495.53 G center field, 5.12 ms time constant, 9.795 GHz microwave frequency, and 86.00 kHz modulation frequency. Meanwhile, trolox solutions (12.5−400 μM) were evaluated for their DPPH radical scavenging activity as described above.

The DPPH scavenging activity of the test compounds was calculated using the equation below.

DPPH scavenging activity (%) = 100 * (1 − signal intensitysample/signal intensitycontrol)

where the control contained no trolox or test compounds.

The trolox solutions (12.5−400 μM) used in this study yield a linear DPPH scavenging activity (%) versus trolox concentration regression line. The DPPH radical scavenging capacities of the test compounds were expressed as micromolar (μM) trolox equivalents per millimolar sample (mM), which was calculated according to the trolox standard curve.

3.2.2.2 Cupric ion-induced human low-density lipoprotein oxidation

The inhibitory effect of TY, HTY and their esters on cupric ion-induced human LDL peroxidation was measured according to the method described by Ambigaipalan and Shahidi

(2015) with slight modification. LDL (5 mg/mL) was dialyzed in 10 mM phosphate buffer (pH 7.4, 0.15 M NaCl) using a dialysis tube with a molecular weight cut off of 12−14 kDa (Fisher Scientific) with stirring at 4 °C under a nitrogen blanket in the dark for 12 h. Diluted LDL cholesterol (0.03 mg LDL/mL, 0.8 mL) was mixed with TY, HTY and their esters (10 μM, 100 μL). The samples were pre-incubated at 37 °C for 15 min. The reaction was initiated by adding a solution of cupric sulphate (50 μM, 100 μL), and the samples were then incubated at 37 °C for 15 h. Appropriate blanks were run for each sample by replacing LDL and cupric sulphate with PBS for background correction. A control sample (with LDL and cupric ion but without testing compound) and a blank sample (with LDL but without cupric ion and testing compound) were prepared. The formation of conjugated dienes was recorded at 234 nm using a diode array spectrophotometer (Agilent). The oxidative status of the reactive mixture at the testing interval (3, 9 and 15 h post incubation) was followed by monitoring its absorbance at 234 nm with respect to the zero point (without incubation at 37 °C).

3.2.2.3 DNA strand scission assay

The protective effect of TY, HTY and their esters on peroxyl and hydroxyl radical-induced supercoiled DNA damage was according to a procedure described by Ambigaipalan and Shahidi (2015) with slight modification. The plasmid pBR322 DNA was dissolved in 0.5 mM phosphate buffer (PBS, pH 7.4) to 50 μg/mL. Sample solution in ethanol (1 mM) was diluted in 0.5 mM PBS (pH 7.4) to 10 μM. In a 0.5 mL Eppendorf tube, PBS (2 μL), pBR 322 DNA (2 μL), sample (2 μL), H_2O_2 (1.0 mM, 2 μL) and $FeSO_4$ (0.5 mM, 2 μL) were added in the order stated to test the inhibition of hydroxyl radical-induced DNA scission. In another Eppendorf tube, PBS (2 μL), pBR 322 DNA (2 μL), sample (2 μL), and AAPH (11.25 mM, 4 μL) were added in the order stated to determine the inhibition against peroxyl radical-induced DNA scission. A control with

DNA alone and a blank devoid of sample were prepared with each set. The mixture was incubated at 37 °C for 1 h in the dark.

After incubation, 1 μL of the loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, and 50% glycerol) were added to the reaction mixture. The mixture (10 μL) was then loaded onto 0.7% agarose gel prepared in Tris−acetic acid−EDTA (TAE) buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.5). SYBR safe was added into agarose gel solution at a concentration of 100 μL/L of TAE buffer as a DNA gel stain. Electrophoresis was run at 80 V for 1 h using a model B1A horizontal mini gel electrophoresis system (Owl Separation Systems Inc., Portsmonth, NH, USA) and a model 300 V power supply (VWR International Inc., West Chester, PA, USA) at room temperature in TAE buffer. The bands were visualized under trans-illumination of UV light using the Alpha-Imager gel documentation system (Cell Biosciences, Santa Clara, CA, USA). The intensity (area %) of bands was analyzed using Chemilmager 4400 software (Cell Biosciences) to quantify DNA scission.

The retention of supercoiled DNA strand (%) was calculated using the following equation.

DNA retention (%) = 100 (area of supercoiled DNA with oxidative radical and sample/area of supercoiled DNA in control)

3.2.3 Antiviral Activities

3.2.3.1 HCV protease inhibitory activity

Inhibitory activity of TY, HTY and their selected derivatives (compounds containing C4:0; C8:0; C18:0; C18:1; EPA; DHA) against HCV protease was evaluated as an indicator for their antiviral activity. The assay was slightly modified from the method described by Zhong, Ma, and Shahidi (2012). The SensoLyte 520 HCV fluorimetric Protease Assay Kit (lot #1028) and HCV NS3/4A

protease (lot# 103-075) from Anaspec. Company (San Jose, CA, USA) were used for the HCV protease inhibition assay. HCV protease inhibition assay was carried out in a 384-well black plate (BD Falcon) as follows: 2 μL of sample solutions (in dimethyl sulphoxide (DMSO)) and 10 μL of a freshly prepared 1 in 50 dilution (made in assay buffers) of the enzyme substrate were put in each well. This was followed by the addition of 8 μL of freshly prepared 0.5 μg/mL of the enzyme into each well and subsequent incubation at 37 °C for 30 min. The fluorescence was then measured at excitation/emission wavelengths of 490 nm/520 nm, respectively, by a Tecan Infinite F200 PRO microplate reader (Männedorf, Switzerland**)**. Percentage inhibition was calculated as follows:

$$\% \text{ Inhibition} = 100 \times (F_{control} - F_{sample}) / F_{control}$$

where, $F_{control}$ and F_{sample} represent the fluorescence value of the control without test compounds and of those with added test compounds. Samples were assayed at different concentrations to plot a concentration versus inhibition percentage curve, and IC50 values, i.e., concentration resulting in 50% inhibition, were determined. A known HCV protease inhibitor, embelin,, was used as a reference.

3.2.3.2 Determination of inhibitory activity against alpha-glucosidase

The inhibitory activities of TY, HTY and their selected derivatives (compounds containing C4:0; C8:0; C18:0; C18:1; EPA; DHA) on alpha-glucosidase were determined using the method reported by Zhong, Ma, and Shahidi (2012) on 96-well plates. Ten microlitres of sample solution (in DMSO) and 80 μL of substrate solution (2 mM of 4-nitrophenyl α-D-glucopyranoside in 100 mM potassium phosphate buffer, pH 7.0) were added to each well. After 10 μL (0.50 U/mL) of enzyme from Bacillus Stearothermophilus (Sigma, Lot# SLBP7209V) in buffer were added per

well, the plates were incubated at 37 ºC for 20 min. In the control wells, sample solution was replaced with DMSO. The absorbance at 405 nm was measured on a plate reader before and after incubation. The increase in absorbance (ΔA) was used to calculate the inhibition.

$$\text{Inhibition\%} = (\Delta A_{control} - \Delta A_{sample})/\Delta A_{control}$$

where, $\Delta A_{control}$ and ΔA_{sample} represent the absorbance change after incubation of control with DMSO only and of those with added test compounds.

IC50 values (the concentration at which the compound inhibits 50% of enzyme activity) were calculated from the inhibition%-versus concentration curves. Acarbose was used as a reference inhibitor (positive control) for alpha-glucosidase.

3.2.4 Cell Culture and Cytotoxic Assay

The cell viabilities and cytotoxic properties of selected fatty acid esters were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay modified from Wang et al. (2016). Human hepatoma carcinoma cells (HepG2) were grown in DMEM medium containing 10% FBS and 100 U penicillin and streptomycin, the cells were maintained in humidified atmosphere of 5% CO_2 at 37℃. HepG2 cells were seeds in 96-well plate (about 5000 cells per well) and incubated for 16 hours, after then added various compounds keep the concentration at 10 μg/ml, incubated for 24 or 48 h, then added 20 μL MTT (5 mg/mL) and continue incubating for 3 h. Removed the medium and added 100 μL DMSO to dissolve the crystal before measuring the absorption at 570 nm. The inhibition rate was calculated by using the following formula.

$$\text{Inhibition rate (\%)} = 100*(OD_{control} - OD_{sample})/OD_{control}$$

3.2.5 Determination of ROS generation in HepG2 Cells

Determination of ROS generation in HepG2 cell was carried out using the dichlorofluorescin (DCFH) assay of Wang et al. (2016). Before experiment, HepG2 cells were seeded in 96-well black plate (about 30000 cells per well) and incubated for 14 h, then various concentrations of compounds were added and incubated for 4 h. Removed the culture medium, washed with PBS and added 10 μM DCFH-DA, continue incubating for 30 min. Then the medium was removed and washed with PBS again. Added 400 uM t-BuOOH and incubated for another 90 min, and washed with PBS before measuring the fluorescence by using a Tecan Infinite F200 PRO microplate reader (excitation and emission wavelengths were 485 and 535 nm, respectively). The control groups were added FBS-free medium instead for t-BuOOH.

3.2.6 Nitrite assay

The LPS-induced NO production by the macrophages was determined by a modified method reported by Zhong et al. (2012). The RAW 264.7 cells, derived from murine macrophages, were cultured in DMEM supplemented with 10% endotoxin-free, heat-inactivated fetal calf serum (GIBCO, Grand Island, NY, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin. RAW 264.7 cells were plated at a density of 1×106 cells/mL into 24 well plates, the culture medium was changed to serum-free DMEM without phenol red and incubated overnight where they were activated by medium containing LPS (Escherichia coli O127:E8, molecular weight, 60 kDa, Sigma Chemical Co.). The RAW 264.7 cells were treated with various compounds and LPS or LPS only for 24 h. The supernatants are harvested and the amount of nitrite, an indicator of NO synthesis, is measured by use of the Griess reaction. Briefly, supernatants (100 μL) are mixed with the same volume of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) in duplicate on 96-well plates. Finally, the

absorbance at 570 nm was measured with an ELISA reader. A serial dilution of sodium nitrite (NaNO2) was used as the standard.

3.2.7 Bovine Serum Albumin (BSA)-Glucose Assay

The assay used to test the antiglycation activities *in vitro* was modified from Wang et al. (2016). BSA (2 mg/mL) was co-incubated with 33 mM D-glucose in 0.1 M, pH 7 PBS at 37 °C for 7 days. Aminoguanidine (AG, 1 mM) was used as a positive control and tyrosol and hydroxytyrosol derivatives were added to the glycation model at 100 μM. After incubation, 100 μL sample solution were pippeted to each well of the 96-well plate and fluorescent AGEs (advanced glycation end-products) were indicated by fluorescence intensity with excitation wavelength of 355/40 nm and emission wavelength of 405/10 nm (Victor X4 Multilabel Plate Reader, PerkinElmer, Santa Clara, CA, USA).

3.3 Statistical method

All the tests were conducted with three replicates (three separate sample preparations). Data were presented as mean ± standard deviation (SD). The statistical analysis was performed by using SPSS 16.0 software (SPSS Inc. Chicago, IL, USA). Differences between means were evaluated by one-way analysis of variance (Student-Newman-Keuls post-hoc test). Comparisons that yielded P values < 0.05 were considered significant.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Synthesis of tyrosol and hydroxytyrosol fatty acid esters

4.1.1 Enzyme screening

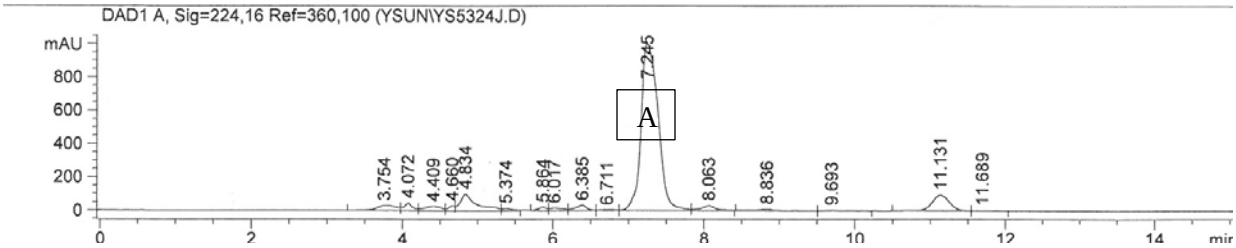
Three lipases from *Candida antarctica* (Novozyme-435), *Candida rugosa* (AY30, type VII), and *Pseudomonas cepacia* (*Burkholderia cepacia*) were screened for their ability to combine DHA with tyrosol (Table 4-1), and the compound was identified by HPLC-MS (Figures 4-1 and 4-2). These lipases catalysed synthesis of the free fatty acid with tyrosol and hydroxytyrosol afforded varying yields. The yield of tyrosol DHA ester with various lipases was in the order of *Candida antarctica* > *Pseudomonas cepacia* > *Candida rugosa*. The synthesis of tyrosol DHA ester can be catalysed by all three lipases used. However, there was no significant difference ($p>0.05$) in this synthesis when lipases from *Pseudomonas cepacia* and *Candida rugosa* were tested and their efficiency was very low. *Candida antarctica* catalysed this synthesis very effectively, and showed the highest yield for production of tyrosol DHA ester (86.28 %, after 24 h). The results reported here agree with the findings of Grasso et al. (2007) who synthesized hydroxytyrosol lipophilic analogues with 12 different lipases, and reported that lipase from *Candida antarctica* gave the highest yield. Buisman et al. (1998), using hydroxytyrosol, octanoic acid and immobilized lipases from *Candida antarctica*, found the yield was 85% in diethyl ether within 15 hours (35 ºC). Lipase from *Candida antarctica* is known to have regioselectivity which ensures that the HTY and TY are esterified to the primary hydroxyl group (alcoholic hydroxyl groups) (Bouallagui et al., 2011; Crauste et al., 2016; Mateos et al., 2008). Lipase from *Candida antarctica* was selected for carrying out the subsequent experiments.

Table 4-1. Effect of enzyme type on the synthesis of tyrosol DHA ester (yield %).

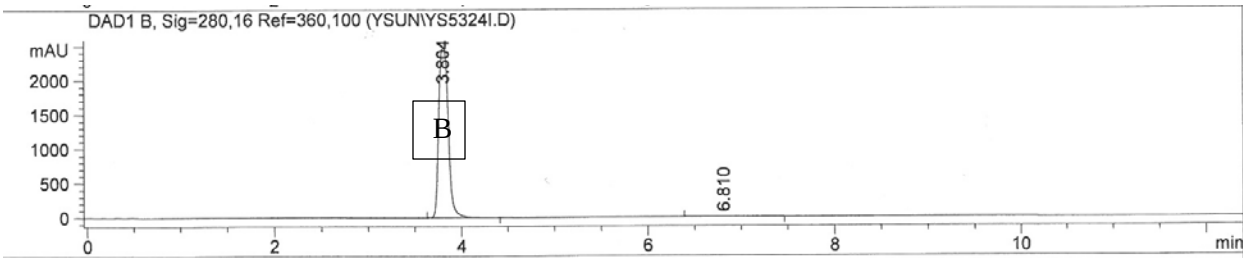
Source of enzyme	Yield (%)
Candida antarctica	86.28 ± 2.37
Candida rugosa	0.46 ± 0.24
Burkholderia cepacia	1.48 ± 0.11

Values are mean values of triplicate determinations±standard deviation.

(a)DHA



(b)Tyrosol



(c) TY DHA ester

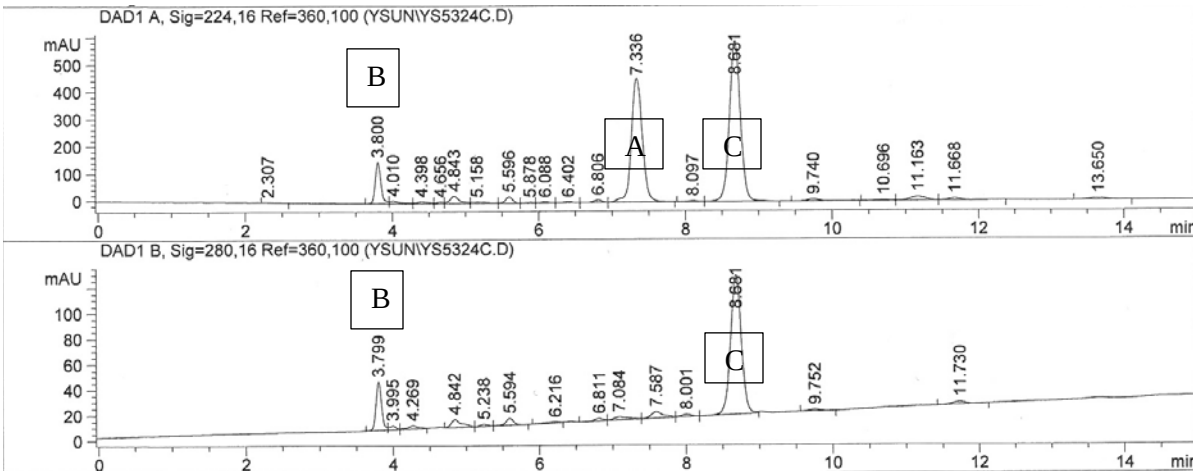


Figure 4-1. High-performance liquid chromatography (HPLC) chromatograms of starting materials (a, b) and resultants (c) of synthesis of tyrosol DHA ester at 280 nm. A: DHA; B: tyrosol; and C: tyrosol DHA ester.

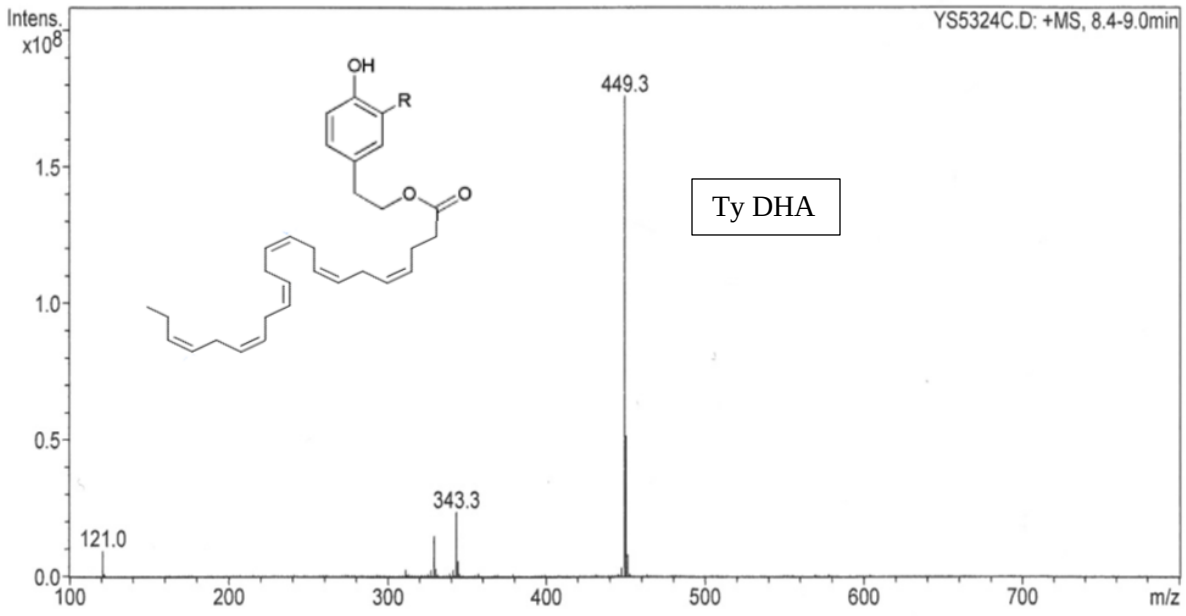


Figure 4-2. Chemical structures and mass spectrometric data of tyrosol DHA ester.

4.1.2 Mole ratio effect

In order to obtain good yields of the esterification with lower cost, different tyrosol to DHA mole ratios were used. The effect of mole ratio of substrates on the synthesis of tyrosol DHA is shown in Table 4-2. As the number of moles of DHA increased from 1 to 10, the yield of synthesis of the ester increased. In this experiment, TLC was used first to see if there was a spot for the tyrosol DHA ester, which means the ester was succesfully synthesized. The spots of synthesized ester (tyrosol /DHA) were not found in the TLC plates at 1:1 and 1:2 mole ratio, while that at 1:6 and 1:10 mole ratio (tyrosol /DHA) was clearly seen which was further confirmed by HPLC-MS. Good yield of tyrosol DHA ester can be obtained at a tyrosol to DHA mole ratio of 1:6 (84.87%) and 1:10 (86.28%) because excess DHA helped the reaction of tyrosol with DHA by enzyme. In the experiment by Grasso et al. (2007), the mole ratio of the acyl donor and hydroxytyrosol or homovanillic alcohol was 20:1, affording a yield of 90.9 - 98.1%. De Pinedo et al. (2005) synthesized phenols with ethyl fatty acid ester at a mole ratio of 1:30 at 37 °**C** for 16 h and the yields are 29 - 97%. Thus, it is reasonable to use more acylating agent. However, considering the economic factors, the mole ratio of tyrosol to DHA was selected to be 1:6 as there was no significant difference with that at a ratio of 1:10. In addition, the yield (84.87%) of tyrosol DHA ester at a mole ratio of 1:6 was quite good and acceptable as it is known that yields above 70% are *good* (Furniss, 1989).

Table 4-2. Effect of mole ratio of substrates on synthesis of tyrosol DHA ester.*

Mole ratio tyrosol /DHA**	Yield (%)
1:1	Not Detected
1:2	Not Detected
1:6	84.87 ± 0.54*
1:10	86.28 ± 2.37*

*Values are mean values of triplicate determinations ± standard deviation. **Mole ratios of tyrosol to DHA were 1:2, 1:6 and 1:10.

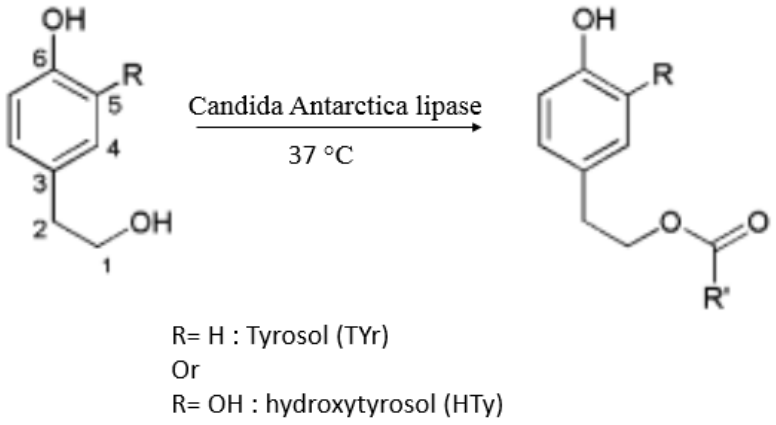
4.1.3 Comparison of the enzymatic and chemical methods

The chemical method to synthesis tyrosol DHA esters was modified from that of Zhong (2010). DHA was converted to the corresponding acyl chloride by reaction with thionyl chloride. Stearoyl chloride, a commercial product was used as such. Esterification of tyrosol was carried out with acyl chlorides (stearoyl chloride, docosahexaenoyl chloride) at a mole ratio of 1:1. Acyl chloride was added dropwise to tyrosol which was dissolved in ethyl acetate. The reaction was carried out in the presence of pyridine which removed the released HCl from the medium. The mixture was then heated in an oil bath at 50°C under a nitrogen blanket with constant stirring. The reaction mixture upon completion of the esterification was cooled to ambient temperature and filtered. The filtrate was then washed 3 times with distilled water (60°C), and the ethyl acetate layer was collected and passed through a cone of anhydrous sodium sulphate. The dry powder of crude products containing a mixture of tyrosol esters (at different degrees of substitution) may be obtained by evaporating the solvent.

Using the Chemical esterification method, TY was known to be esterified on the primary hydroxyl group (alcoholic hydroxyl groups) as confirmed by HPLC-MS (Figure 4-1 and 4-2). In

this study, with the chemical method, a good yield (88.09±1.20%) may be obtained when using a tyrosol to DHA mole ratio of 1:1, while enzymatic method needs a higher ratio of DHA (acylating agent) to tyrosol with a similiar yield.

With regard to the labor requirement, both methods appear to be comparable. From the purely experimental standpoint, the enzymatic method is more time-consuming in this study, as synthesis of esters takes more time (24 h), but it is simpler and requires less energy. Meanwhile, with esters of DHA, good yields (chemical method, 88.09%; enzymatic method, Table 4-2, 86.28%) were obtained by both methods. In addition, the enzymatic reaction workup was easier, making the chromatographic purification unnecessary as only t-butyl methyl ether and extra DHA left after synthesis. Moreover, the chemicals used such as pyridine are toxic (International Agency for Research on Cancer, 2007). Thus, removal of these toxic compounds and purification are necessary. Purification needs flash chromatography which is time consuming, and might also cause loss of target compounds (Roge et al., 2011). Therefore, in this study, enzymatic synthesis was followed.



R'	Compound
C_3H_7	Butyrate ester
C_5H_{11}	Caproate ester
C_7H_{15}	Caprylate ester
C_9H_{19}	Caprate ester
$C_{11}H_{23}$	Laurate ester
$C_{13}H_{27}$	Myristate ester
$C_{15}H_{31}$	Palmitate ester
$C_{17}H_{35}$	Stearate ester
$C_{17}H_{33}$	Oleate ester
$C_{17}H_{29}$	α-linolenate ester
$C_{19}H_{29}$	EPA ester
$C_{21}H_{31}$	DHA ester

Figure 4-3. General structures of synthesized fatty acid esters.

4.1.4 Syntheis of TY and HTY fatty acid esters

The study undertaken in a previous section was extended to other free fatty acids to investigate the influence of the length and degree of unsaturation of the fatty acid in the esterification reaction. The lipophilic TY/HTY esters have been synthesized by a simple, chemoselective procedure (Figure 4-3). Enzymes (lipase from *Candida Antarctica*) were used for the transesterification reactions, and the results obtained are shown in Table 4-3. Higher yields of ester formation were observed for shorter chain fatty acids under the same experimental conditions. For instance, the yield of TY caproate (69.3%) was higher than that of TY stearate (41.6%). Compared with saturated fatty acid ester (TY stearate), higher yields were obtained when unsaturated fatty acids (oleic acid and linolenic acid) were used as the acylating agent in the reaction, possibly due to a better solubility in the reaction medium. Stamatis et al. (1999) also found a decrease in the esterification yields with increasing chain length while Yan et al.

(1999) found just the opposite effect. The results on the effect of the chain length of the saturated carboxylic acids in the literature are contradictory and unclear (De Pinedo et al., 2005; Mateos et al., 2008). For obtaining better yields of TY DHA and TY EPA, higher amounts of acylating agents and longer reaction times were needed.

Table 4-3. Reaction yields for some phenolic fatty acid esters.

Phenol	**Acylating agent**	**Product**	**Yield (%)**
TY	Butyric acid[a]	TY butyrate	63.8
TY	caproic acid[a]	TY caproate	69.3
TY	lauric acid[a]	TY laurate	45.8
TY	stearic acid[a]	TY stearate	41.6
TY	oleic acid[a]	TY oleate	44.8
TY	linolenic acid[a]	TY linolenate	56.0
TY	EPA[b]	TY EPA	88.1
TY	DHA[b]	TY DHA	84.9
HTY	EPA[b]	HTY EPA	75.3
HTY	DHA[b]	HTY DHA	63.2

a. Reaction conditions: phenol to fatty acid ratio 1:2, *Candida Antarctica* lipase, t-butyl methyl ether, 37 °C, 24 h. b. Reaction conditions: phenol to fatty acid ratio 1:6, *Candida Antarctica* lipase, t-butyl methyl ether, 37 °C, 24 h. Values are mean values of triplicate determinations.

4.2 Antioxidant evaluation

4.2.1 DPPH radical scavenging activity

DPPH radical scavenging method is a rapid, simple and inexpensive method for evaluating the antioxidant potential. DPPH can react with the sample in both ethanol and water, whereas other methods analyzing antioxidant can just be run in a selected solvent (Kedare & Singh, 2011).

Thus, in this work, the stable and commercially-available artificial radical DPPH was used to directly measure the radical quenching ability of TY, HTY and their esters. In this study, all test compounds exhibited DPPH radical scavenging activity to different extent, as reflected in the signal intensity in the EPR spectra of the DPPH radical which decreased by all the test compounds (Figure 4-4).

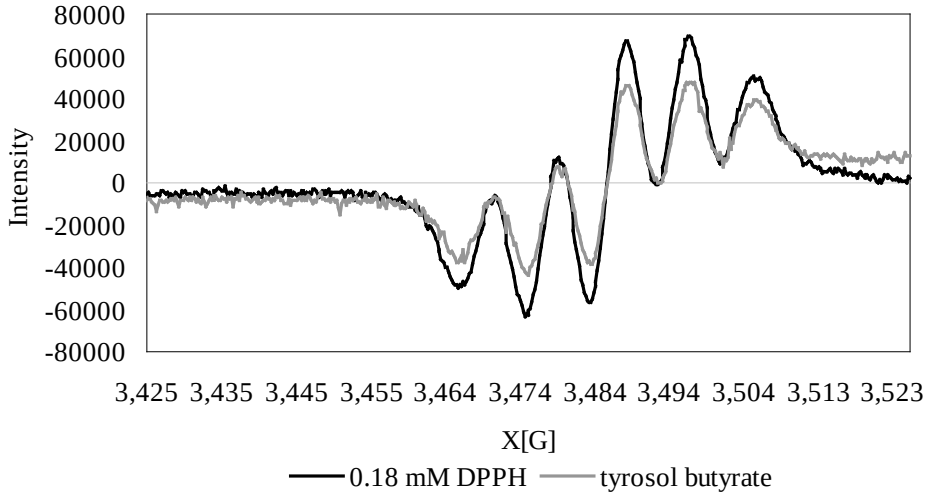


Figure 4-4. Signal intensity of DPPH (0.18 mM) and the TY butyrate with DPPH (1 mM) as observed by EPR.

The quantitative results of the DPPH radical scavenging assay of the samples are summarized in Table 4-4. Among all samples tested, HTY showed the highest capacity of 1005.14 µM trolox equivalents per mM of sample. This means that HTY had the same DPPH radical scavenging capacity as trolox. It was previously reported that HTY has the highest activity because it is the most polar antioxidant, as explained by the polar paradox theory and the interface phenomenon (Porter, 1993) that explain more polar antioxidants are more effective in their DPPH radical scavenging activity. In contrast, the DPPH radical scavenging capacity of TY observed in this

study was much lower. A previous study by Vlachogianni et al. (2015) found that low concentration (5–400 µM) of TY did not reveal any capacity to scavenge the DPPH radical.

As shown in Figure 4-5a, TY and its saturated fatty acid esters except TY butyrate all exhibited very weak DPPH radical scavenging ability. When comparing the radical scavenging capacity of TY saturated fatty acid (SFA) esters, TY butyrate showed the highest capacity as trolox equivalents, followed by TY myristate (Figure 4-5a). Meanwhile, the DPPH radical scavenging ability of TY was lower than that of TY butyrate, but higher than that of other SFA esters. As Figure 4-5b shows, all HTY SFA esters exhibited much higher DPPH radical scavenging ability compared to their TY analogues which ranged from 20.39 to 578.94 µM trolox equivalents per mM sample. Obviously, the introduction of the lipid part into HTY decreased its DPPH radical scavenging ability. The reason for high capacity of TY butyrate might because the lipophilic derivatives of TY may have greater accessibility/affinity to the lipophilic DPPH radical than the TY itself. Moreover, acylation may have an effect on the hydrogen atom donation capability of TY by altering its electron density and distribution on the aromatic ring.

As shown in Figure 4-6a, TY and its unsaturated esters all exhibited very weak DPPH radical scavenging ability which ranged from 1.25 to 5.43 µM trolox equivalents per mM sample. Overall, the DPPH radical scavenging ability of TY esters positively correlated with the number of unsaturations in the fatty acid. In addition, the DPPH radical scavenging ability of TY was lower than that of TY DHA, but higher than that of other esters. As Figure 4-6b shows, all HTY unsaturated fatty acid (UFA) esters exhibited much higher DPPH radical scavenging ability compared to their TY analogs which ranged from 174.05 to 414.03 µM trolox equivalents per mM sample. This is similar to SFA esters that the introduction of the lipid part into HTY decreased its DPPH radical scavenging ability. For HTY esters containing C18, the DPPH

radical scavenging ability increased with the number of unsaturations in the fatty acids. In contrast, the DPPH radical scavenging ability of HTY DHA and HTY EPA was similar and lower than that of HTY C18:1 and HTY C18:3.

According to Braude et al. (1954), phenols can transfer electrons from the phenolic hydroxyl groups to DPPH radicals to quench them. Therefore, the number and position of the active group (phenolic hydroxyl group) of phenols may affect DPPH radical quenching properties. In this study, HTY exhibited a higher DPPH radical quenching ability than TY. Similar results were also observed by Carrasco-Pancorbo et al. (2005). It was speculated that the lower antioxidant activity of TY compared to HTY can be attributed to the absence of the ortho-diphenolic hydroxyl grouping in its chemical structure (Mateos at al., 2003). It is known that ortho-diphenols are more effective antioxidants than simple phenols, due to the stabilisation of the phenoxyl radical through hydrogen bonding (Foti & Ruberto, 2001; Goupy et al., 2003).

According to Grasso et al. (2007), the results show that the antiradical activity of HTY is not notably influenced by the presence and length of saturated acyl chain at C-1, while de Pinedo et al. (2007) reported there was a small effect of the length of the alkyl chain in radical-scavenging activity and found an increase of the side chain length leads to an increase in the radical-scavenging capacity. However, in this study, the introduction of SFA decreased the DPPH scavenging capacity except for TY butyrate, possibly due to the reduced ability of the product to undergo conformational changes. A previous study showed that isoquercitrin esters exhibited a lower radical scavenging activity than isoquercitrin itself, and the antiradical activity decreased with increasing carbon chain length. Isoquercitrin butyrate exhibited the highest antiradical activity (Salem et al., 2010). Similarly, Takahashi et al. (2003) also showed similar results in the case of alkylaminophenols of various alkyl chain lengths. In addition, Jakovetić et al. (2013)

showed that all-synthesized cinnamic acid esters exhibited better antioxidant potential than cinnamic acid itself and their radical-scavenging effectiveness decreased with increasing of their alkyl chain length, with ethyl cinnamate being the most potent antioxidant. Therefore, introduction of an alkyl ester side chain had different results on the antioxidant activity of phenolic acid systems (Silva et al., 2000; Reis et al., 2010; Gaspar et al., 2009; Roleira et al., 2010). Gaspar et al. (2010) reported that sinapic acid had a higher activity when compared to that of its alkyl esters, and they assume the effect of the alkyl ester side chain in hydroxycinnamic systems is strongly related to the number of hydroxyl groups and the aromatic substitution pattern. Other studies also found that caffeic acid alkyl esters had lower DPPH radical scavenging activities than caffeic acid itself, dependent on the extension, or type, of the ester side chain (Roleira et al., 2010; Silva et al., 2000). Similar results have been reported by Kikuzaki et al. (2002) that introduction of alkyl part to ferulic acid decreases its activity against DPPH. Overall, the present study and previous papars revealed that the effect of alkyl esterification on the antioxidant activity may differ depending on the type of phenolic acids, possibly due to different mechanisms of action of phenolic acids, which are mainly determined by their ring substitution. Therefore, the antioxidant activity of the phenolic compounds is influenced by their molecular structure, hydrogen-donating ability and subsequent stabilization of the formed phenoxyl radical (Silva et al., 2000). The dissimilarity in the antioxidant capacity of phenols and their derivatives might be related with to steric hindrance caused by the bulkiness of the alkyl groups (Miller & Rice-Evans, 1997), and the antioxidant activity might be higher when a catechol group is present (Roleira et al., 2010).

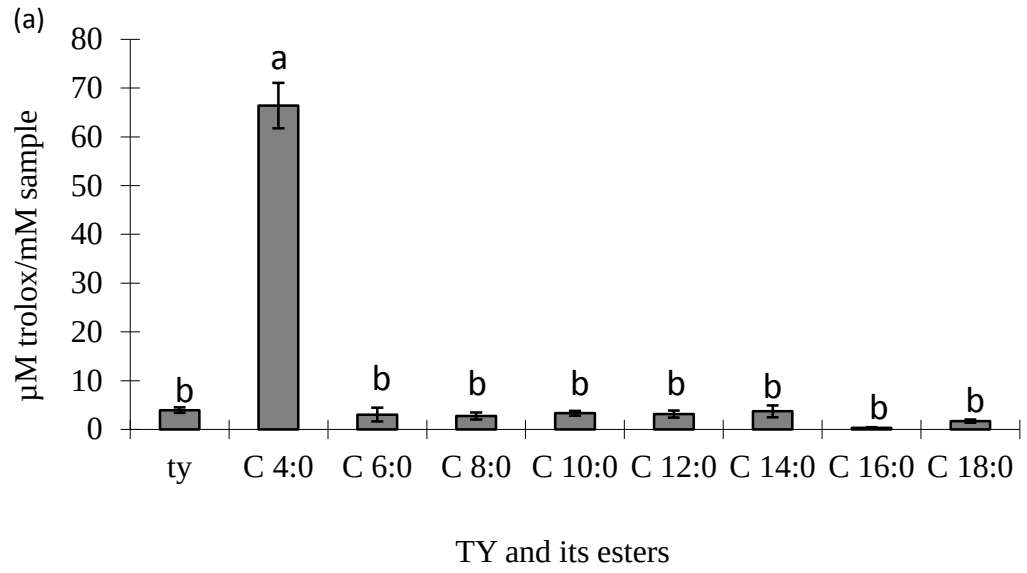
According to Crauste et al. (2016), all n-3 PUFA-phenol esters tested in the literature showed radical scavenging ability in the DPPH radical assay. However, the correlation between the

introduction of the unsaturated lipid part and the increase or decrease in the radical scavenging ability of the phenolic derivatives is still uncertain (Crauste et al., 2016). For example, Zhong and Shahidi (2011) reported that EPA and DHA tetra acyl esters of epigallocatechin gallate (EGCG) (3', 5', 3", 5"-esters) both exhibited a greater ability in scavenging DPPH radical than EGCG itself. They speculated that the enhanced lipophilicity and reduced electron density of the O-H bound at position 4' and 4" caused by the acylation contributed to this enhancement effect (Zhong & Shahidi, 2011). However, researchers have found that HTY acetate and HTY C18:1 exhibited a lower DPPH radical scavenging activity than HTY (Bouallagui et al., 2011; Gordon et al., 2001). According to Mbatia, et al. (2011), this drop in radical scavenging ability could be attributed to the increased hydrophobicity, which may result in decreased solubility in the assay medium. Meanwhile, the present study showed that the DPPH radical scavenging ability of all TY esters and HTY esters containing C18 increased with the number of unsaturations in the FA. Actually, similar unsaturation-dependent antioxidant effect has previously been reported for PUFA-quercetin esters (Mainini et al., 2013).

Table 4-4. DPPH radical-scavenging activities of tyrosl (TY) and hydroxytyrosol (HTY) esters.

Compound	uM trolox/mM TY ester	uM trolox/mM HTY ester
Parent compound	$3.95\pm0.55^{b,c}$	1005.14 ± 57.80^{a}
butyrate ester	66.41 ± 4.63^{a}	475.09 ± 12.06^{c}
caproate ester	$3.04\pm1.41^{b,c}$	578.94 ± 57.80^{b}
caprylate ester	$2.76\pm0.74^{b,c}$	113.38 ± 10.59^{f}
caprate ester	$3.33\pm0.72^{b,c}$	321.60 ± 14.93^{d}
laurate ester	$3.17\pm0.73^{b,c}$	20.39 ± 8.19^{g}
myristate ester	$3.72\pm1.21^{b,c}$	448.04 ± 58.28^{c}
palmitate ester	0.31 ± 0.15^{c}	550.37 ± 27.48^{b}
stearate ester	$1.72\pm0.35^{b,c}$	$174.05\pm27.31^{e,f}$
oleate ester	$1.25\pm0.27^{b,c}$	328.20 ± 58.86^{d}
α-linolenate ester	$1.69\pm1.34^{b,c}$	414.03 ± 48.82^{c}
EPA ester	$2.47\pm0.53^{b,c}$	220.26 ± 4.40^{e}
DHA ester	5.43 ± 0.83^{b}	$182.89\pm10.46^{e,f}$

Values are mean values of triplicate determinations±standard deviation. Values with different superscripts are different ($p<0.05$) from one another.



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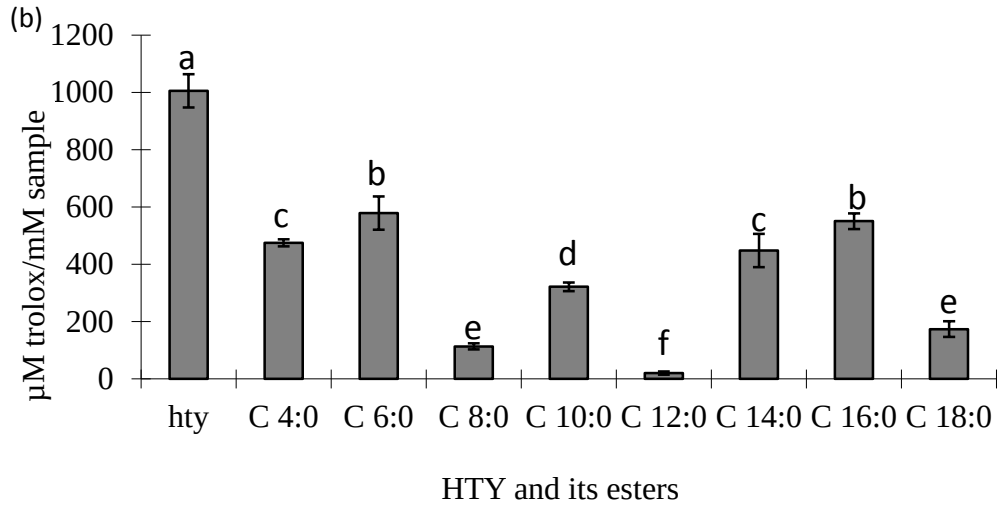

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3 Figure 4-5. DPPH scavenging capacity of saturated fatty acid esters in µmol trolox
4 equivalents/mmol. (a) DPPH scavenging capacity for tyrosol and its saturated fatty acid esters;
5 (b) DPPH scavenging capacity for hydroxytyrosol and its saturated fatty acid esters. Bars with
6 different letters are significantly different at $P < 0.05$ (triplicate determinations).

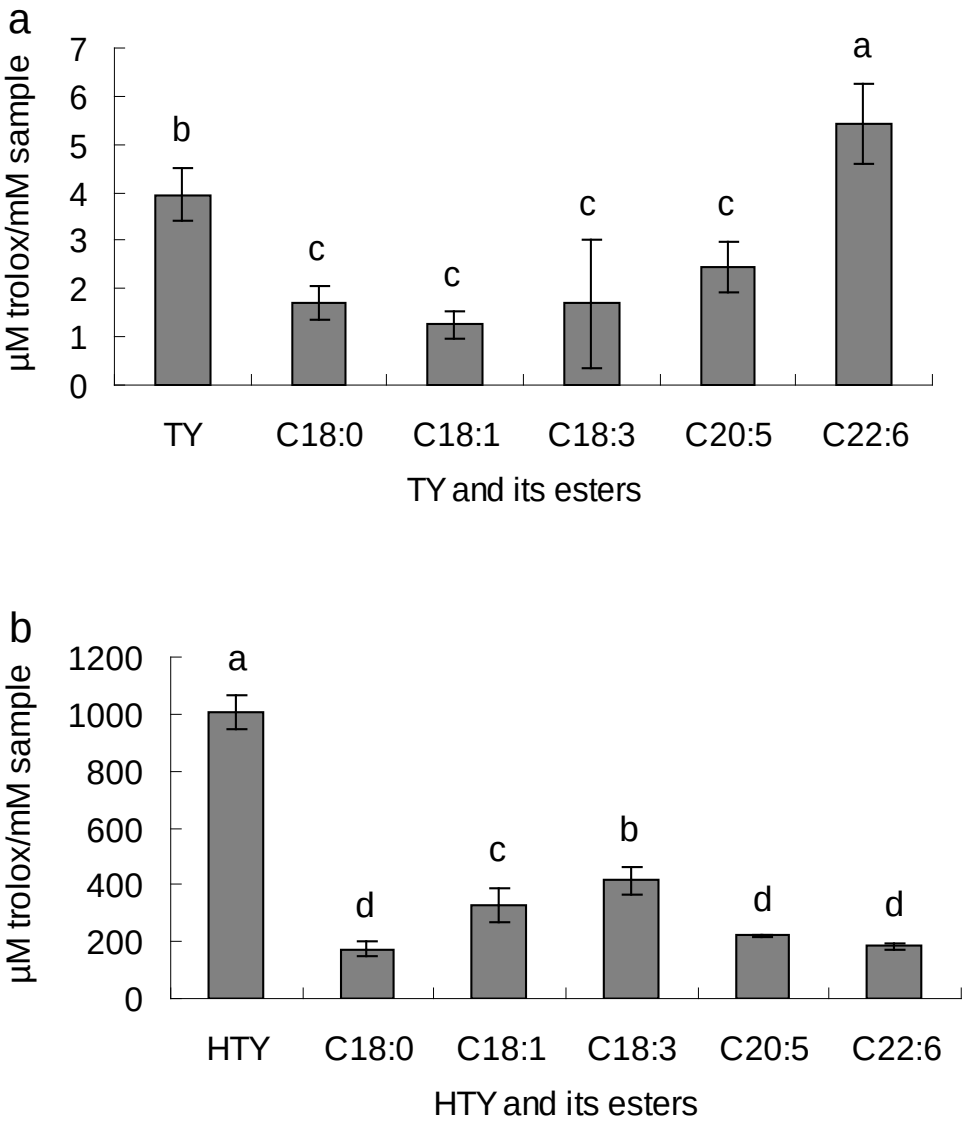
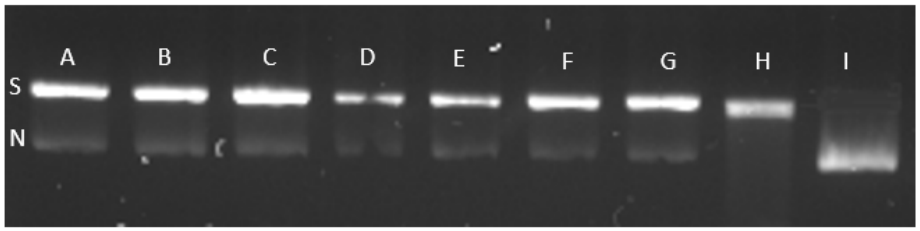


Figure 4-6. DPPH scavenging capacity of unsaturated fatty acid esters in µmol trolox equivalents/mmol. (a) DPPH scavenging capacity for tyrosol and its stearate and unsaturated fatty acid esters; (b) DPPH scavenging capacity for hydroxytyrosol and its stearate and unsaturated fatty acid esters. Bars with different letters are significantly different at $P < 0.05$ (triplicate determinations).

4.2.2 DNA strand scission assay

DNA strand scission assay was performed to assess the protective effect of tyrosol (TY), hydroxytyrosol (HTY) and their different fatty acid esters on hydroxyl and peroxyl radical-induced DNA scission of plasmid pBR322. More than 90% of undamaged pBR322 DNA is generally in the supercoiled form. The damage of the pBR322 DNA results in the reduction of the supercoiled form into increased levels of an open circular form (Hiramoto et al., 1996). Figure 4-7 shows the electrophoretic pattern of DNA strand scission induced by peroxyl and hydroxyl radicals with and without the presence of antioxidative agents. In agarose, the undamaged supercoiled circular form of DNA (I lane) has a relatively high electrophoretic mobility whereas the open circular DNA (H lane) has a reduced electrophoretic mobility as reported previously (Lin et al., 2008). However, for TY esters, the undamaged supercoiled circular DNA and the open circular DNA were both present in the agarose gel.

a) hydroxyl radical-induced DNA scission



b) peroxyl radical-induced DNA scission

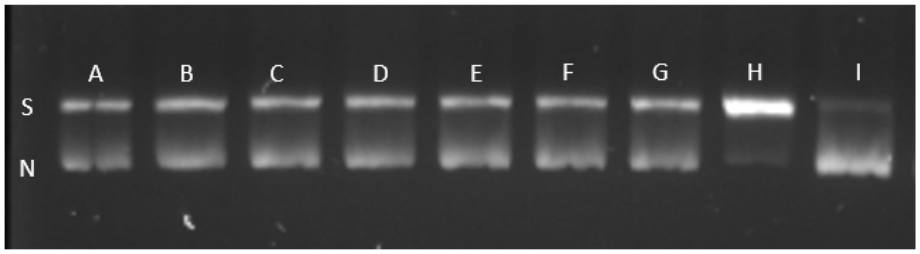


Figure 4-7. (a) Agarose gel electrophoresis of inhibition of hydroxyl radical-induced DNA scission by tyrosol and tyrosol esters (10 uM); (b) peroxyl radical-induced DNA scission by tyrosol and tyrosol esters (10 uM). Lane designations are: A, DNA + tyrosol; B, DNA + tyrosol butyrate; C, DNA + tyrosol caproate; D, DNA + tyrosol caprylate; E, DNA + tyrosol caprate; F, DNA + tyrosol laurate; G, DNA + tyrosol myristate; H, DNA + radical; I, DNA; S, supercoiled DNA strands; and N, nicked DNA strands.

Table 4-5 presents the percentage inhibitory effects of TY, HTY and their esters on DNA strand scission induced by peroxyl and hydroxyl radicals. TY and its esters exhibited a protective effect ranging from 10.48 to 28.85% against hydroxyl radical-induced DNA scission at a concentration of 10 μM, while HTY and its esters exhibited a higher inhibitory effect that ranged from 18.58 to 53.49% at the same concentration. For peroxyl radical-induced DNA scission, TY and its esters showed inhibition that ranged from 47.36 to 63.19% at a concentration of 10 μM, whereas inhibition of HTY and its ester varied from 56.13 to 77.60% at the same concentration.

As shown in Figure 4-8a, TY, HTY and their saturated FA esters all exhibited protective effects against hydroxyl radical-induced DNA scission to some extent at a concentration of 10 μM. TY SFA esters achieved the DNA retention rates which ranged from 11.15 to 26.94%, while HTY esters achieved the DNA retention rates which ranged from 18.58 to 50.35%. Among SFA esters, HTY butyrate ester showed the highest inhibitory effect. Furthermore, HTY exhibited a higher protective effect than TY. Meanwhile, for a pair of esters with the same saturated lipid part, the HTY ester also exhibited a higher protective effect than the TY saturated analogues except for caprate (decanoate**)** ester. For TY, the introduction of the lipid part increased the protective effect on hydroxyl radical-induced DNA damage as TY showed the lowest DNA retention (10.48%). However, for HTY, the introduction of saturated fatty acids (SFA) decreased the protective effect.

From Figure 4-8b, it can found that TY, HTY and their SFA esters exhibited very good protective effects against peroxyl radical-induced DNA scission at a concentration of 10 μM. TY and its SFA esters showed a similar DNA retention which ranged from 47.36 to 63.19 %, whereas that for the corresponding values of HTY and its esters varied from 56.13 to 65.72%. Meanwhile, HTY showed a similar protective effect against peroxyl radical-induced DNA scission to most of its esters. Among the TY esters with SFA, tyrosol caprylate ester and tyrosol laurate ester showed the highest inhibitory effect, while the HTY butyrate ester showed the highest inhibitory effect among HTY esters with SFA.

As shown in Figure 4-9a, TY, HTY and their UFA esters all exhibited protective effects against hydroxyl radical-induced DNA scission to some extent at a concentration of 10 μM. TY and its UFA esters achieved the DNA retention which ranged from 10.48 to 28.85%, while HTY and its UFA esters achieved the DNA retention rates which ranged from 19.71 to 53.49%. In contrast,

HTY exhibited a higher protective effect than TY. Meanwhile, for a pair of esters with the same lipid part, the HTY ester also exhibited a higher protective effect than the TY analogues. Furthermore, for TY or HTY esters, their DNA retention rates were all positively correlated with the number of unsaturations in the FA. For TY, the introduction of the unsaturated lipid part increased the protective effect on hydroxyl radical-induced DNA damage due to their higher lipophilicity. However, for HTY, the introduction of the lipid part decreased the protective effect except for DHA, possibly due to a combined influence of lipophilicity and the loss of the alcoholic hydroxyl group.

As shown in Figure 4-9b, TY, HTY and their UFA esters also exhibited the protective effects against peroxyl radical-induced DNA scission at a concentration of 10 μM. TY and its UFA esters showed a similar DNA retention which ranged from 50.39 to 62.22%, whereas the corresponding values of HTY and its UFA esters varied from 56.13 to 77.60%. For the HTY UFA esters, their DNA retention increased slightly with the degree of unsaturation in the FA. Meanwhile, HTY showed a similar protective effect against peroxyl radical-induced DNA scission to most of its UFA esters except for HTY DHA.

ROS, such as hydroxyl radical, hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet -}$) are major sources of oxidative stress in cells, which can damage proteins, lipids, and DNA (Orrenius et al, 2007). Oxidative DNA damage is involved in tissue damage resulting from heart attack and stroke, cardiovascular diseases including arteriosclerosis, as well as cancer, aging, Alzheimer's and Parkinson's diseases (Perron et al., 2008). The protective effects on oxidative DNA damage of TY and HTY have previously been observed (Aruoma et al., 1998; Quiles et al., 2002; Grasso et al., 2007). In contrast, HTY exhibited a higher protective effect than TY (Quiles et al., 2002; Grasso et al., 2007). Perron et al. (2008) suggested that polyphenols can prevent hydrogen

peroxide-induced DNA damage by binding to iron. Liao e al. (2004) suggested that the ortho-phenolic hydroxyl groups of polyphenol are responsible for chelating metal ions. Therefore, the difference in protective effects of HTY and TY on oxidative DNA damage may be attributed to the ortho-diphenolic hydroxyl group in the chemical structure of HTY.

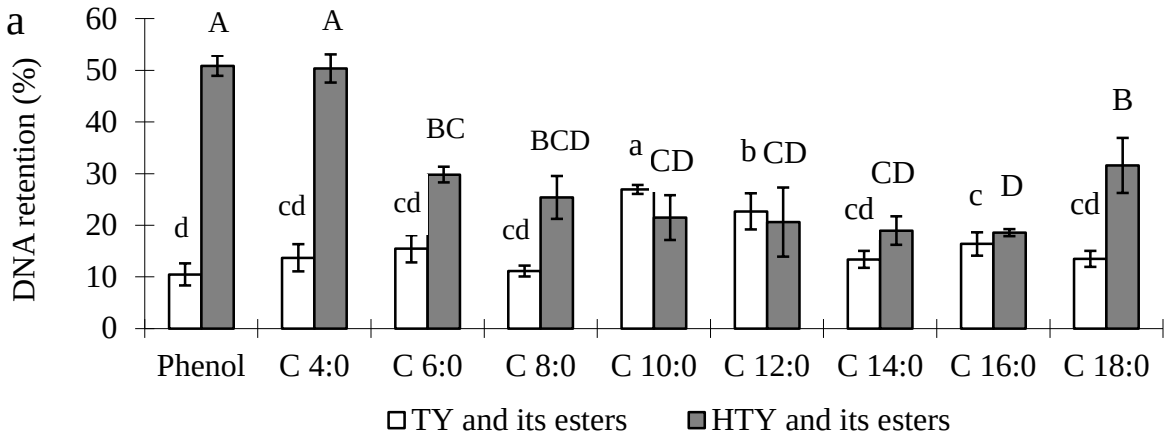
TY and HTY esters all exhibited the protective effects against hydroxyl and peroxyl radical-induced DNA scission at a concentration of 10 μM, possibly due a combination of radical scavenging and ferrous ion chelation. The results of this work indicate that the introduction of fatty acids in general (except for DHA) to HTY decreases the protective effect on hydroxyl radical-induced DNA damage, whereas introduction of fatty acids to TY increases the protective effect on hydroxyl radical-induced DNA damage. Grasso et al. (2007) pointed out within the group of lipophilic analogues of HTY that protective effects of these compounds against DNA damage were adversely proportional to their chain length. In this study, we also found introduction of longer chain saturated fatty acids to HTY decreased the protective effect on hydroxyl radical-induced DNA damage except for stearate HTY, possibly due to the polarity and nature of fatty acid side chain. In a previous study, Grasso et al. (2007) have also found that HTY esters were less effective than HTY in their protective effect on hydrogen peroxide-induced DNA damage. Zhong and Shahidi (2012) reported that the ester derivatives of EGCG were more effective than EGCG in protecting against DNA scission, which was thought to be due to cumulative factors of lipophilicity, steric features (DNA binding affinity), hydroxyl radical scavenging and metal chelation capacity, all of which could play a role in the overall antioxidant efficacy of the test compounds. In our study, the greater antioxidant efficacy of TY esters was possibly due to its higher lipophilicity. In a previous study, it was found that the radical-scavenging activity of phenolic antioxidants increased with the introduction of electron-donating

groups (methyl, methoxy, and hydroxy) in the ortho- or para-position of 4-OH, while the activity decreased in the presence of electron-withdrawing groups (trifluoromethyl and nitro) (Shang et al., 2009). We further found that the protective effects on oxidative DNA damage of TY and HTY were all positively correlated with the number of unsaturations in the FA. Actually, Mainini et al. (2013) reported that the antioxidant activity of PUFA-quercetin increased with the number of unsaturations in the FA. According to Richard et al. (2008), LC-PUFA may act as a kind of antioxidant, which could scavenge superoxide in an unsaturation-dependent manner (Grasso et al., 2007).

Table 4-5. Inhibition of supercoiled DNA scission induced by peroxyl and hydroxyl radicals by tyrosol, hydroxytyrosol and their fatty acid esters.

Compound	Hydroxyl radical retention%		Peroxyl radical retention%	
	Tyrosol	Hydroxytyrosol	Tyrosol	Hydroxytyrosol
Parent compound	10.48 ± 2.13^{d}	50.86 ± 1.95^{a}	60.69 ± 6.62^{a}	$61.78\pm4.90^{a,b}$
butyrate ester	13.71 ± 2.63^{d}	50.35 ± 2.72^{a}	$59.48\pm1.67^{a,b}$	$65.72\pm5.56^{a,b}$
caproate ester	$15.44\pm2.63^{c,d}$	29.81 ± 1.54^{b}	$59.17\pm6.01^{a,b}$	59.83 ± 5.56^{b}
caprylate ester	11.15 ± 1.05^{d}	$25.39\pm4.14^{b,c}$	63.19 ± 4.77^{a}	61.73 ± 5.71^{b}
caprate ester	26.94 ± 0.88^{a}	$21.48\pm4.36^{b,c}$	60.10 ± 4.37^{a}	58.44 ± 3.79^{b}
laurate ester	$22.68\pm3.50^{a,b,c}$	$22.81\pm4.55^{b,c}$	63.19 ± 2.22^{a}	$65.52\pm7.81^{a,b}$
myristate ester	13.39 ± 1.65^{d}	$22.40\pm3.24^{b,c}$	$57.22\pm4.32^{a,b,c}$	$64.94\pm5.29^{a,b}$
palmitate ester	$16.38\pm2.27^{b,c,d}$	18.58 ± 0.70^{c}	47.36 ± 1.32^{c}	58.65 ± 3.03^{b}
stearate ester	13.48 ± 1.55^{d}	19.71 ± 5.68^{c}	$55.05\pm3.07^{a,b,c}$	56.13 ± 3.74^{b}
oleate ester	14.49 ± 5.79^{d}	$22.25\pm4.19^{b,c}$	$53.91\pm1.76^{a,b}$	$63.92\pm6.13^{a,b}$
α-linolenate ester	$16.79\pm2.57^{b,c,d}$	$22.98\pm2.36^{b,c}$	$50.39\pm2.15^{b,c}$	$64.72\pm3.73^{a,b}$
EPA ester	$23.32\pm2.57^{a,b,c}$	31.29 ± 7.54^{b}	$56.46\pm4.14^{a,b,c}$	$64.78\pm3.73^{a,b}$
DHA ester	28.85 ± 3.31^{a}	53.49 ± 7.02^{a}	62.22 ± 4.43^{a}	77.60 ± 8.10^{a}

*Values are mean values of triplicate determinations±standard deviation. Values with different superscripts are different ($p<0.05$) from one another.



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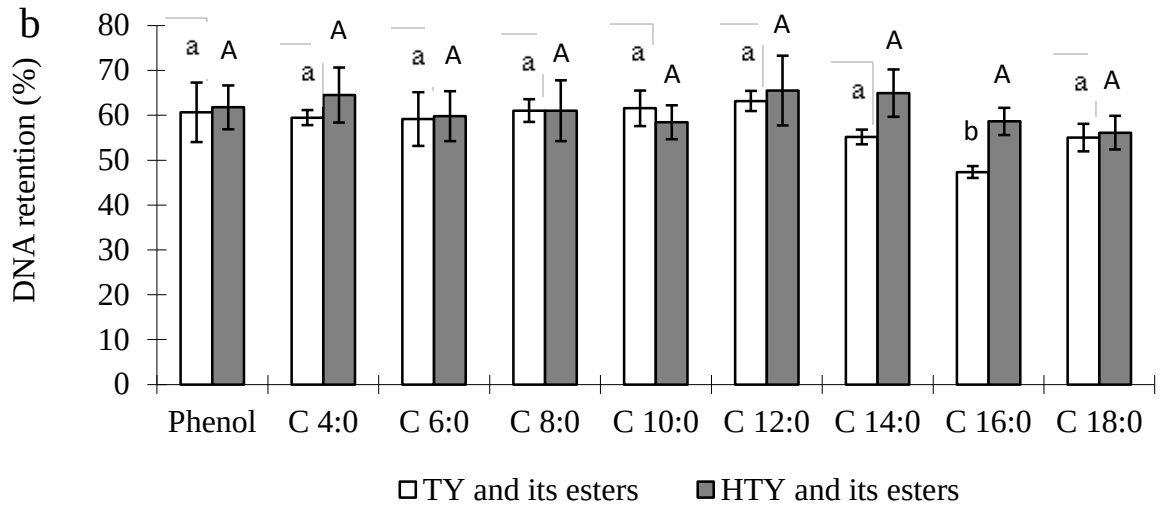

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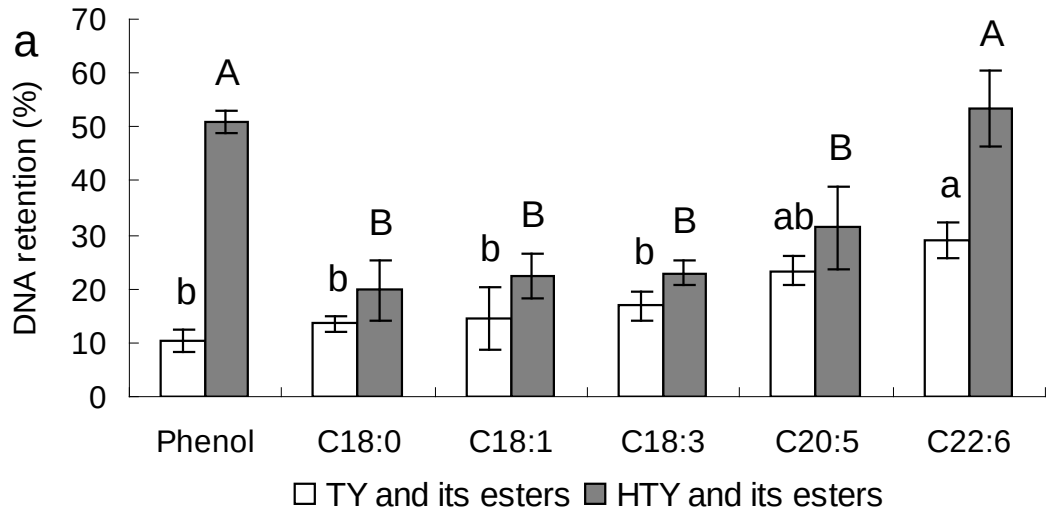
3 Figure 4-8. DNA retention (%) of saturated fatty acid esters in hydroxyl and peroxyl radical
4 induced oxidative scission. (a) DNA retention (%) in hydroxyl radical induced oxidative scission
5 for TY, HTY and their saturated fatty acid esters; (b) DNA retention (%) in peroxyl radical
6 induced oxidative scission for TY, HTY and their saturated fatty acid esters. Bars with different
7 letters are significantly different at $P < 0.05$ (triplicate determinations).



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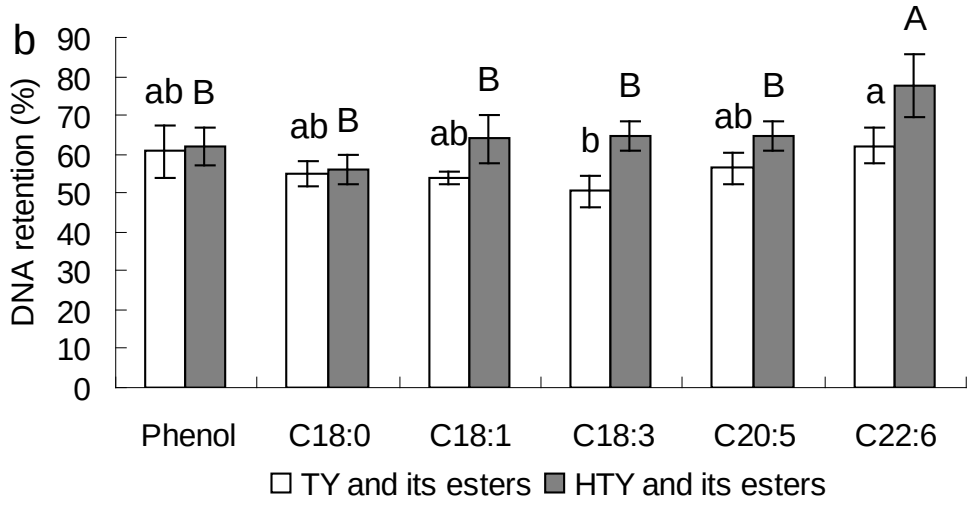

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3 Figure 4-9. DNA retention (%) of unsaturated fatty acid esters in hydroxyl and peroxyl radical
4 induced oxidative scission. (a) DNA retention (%) in hydroxyl radical induced oxidative scission
5 for TY, HTY and their stearate and unsaturated fatty acid esters; (b) DNA retention (%) in
6 peroxyl radical induced oxidative scission TY, HTY and their stearate and unsaturated fatty acid
7 esters. Bars with different letters are significantly different at $P < 0.05$ (triplicate determinations).

8

4.2.3 Cupric Ion-Induced Human LDL oxidation

Oxidised LDL is considered a risk factor for atherosclerosis (Steinberg & Witztum, 2010). LDL can undergo peroxidation (Morel et al., 1983) in which transition metal ions such as cupric ion play an important role (Aust & Svingen., 1982). In this study, human LDL was used to assess the inhibitory effect of TY, HTY and their esters on cupric ion-induced lipid peroxidation. In *vitro*, the oxidation of LDL may be initiated by cupric ion, resulting in the formation of conjugated dienes which can be measured as the change in the absorbance at 234 nm (Wagner & Heinecke, 1997).

As shown in Figures 4-10 and 4-11, the UV absorbance for the control, the blank and the test samples in general (except for HTY and HTY caproate) were increased during the incubation time up to 9 h, and were maintained thereafter. The time-dependent increase in the UV absorbance for the blank sample (with LDL but without cupric ion and testing compound) indicated continuous formation of conjugated dienes from LDL during 37 °C incubation even in the absence of cupric ion. From Figure 4-10a, it can be seen that the absorbances of all the TY SFA esters were always lower than that of the control samples (with LDL and cupric ion but without testing compound) during the 15 h of incubation, indicating that all the TY SFA esters can inhibit LDL oxidation within this period, which was also the same for HTY UFA esters as their absorbance was lower than that of the control sample at any point in time during the test period (Figure 4-11b). However, for HTY SFA esters and TY UFA esters, their absorbance at 234 nm was lower than that of the control sample during the initial 3 h, indicating that all these esters can inhibit LDL oxidation during this period (Figure 4-10b and 4-11a). After that, the UV absorbance at 234 nm for HTY caprylate, TY linolenate, TY EPA and TY DHA increased quickly, and these were even beyond that of the control sample after 15 h of incubation,

indicating that HTY caprylate had little protective effect against LDL oxidation and the PUFA moiety of the TY esters may be oxidized, thus resulting in the formation of conjugated dienes.

The inhibitory effects on cupric ion-induced LDL oxidation of all test samples are reported in Table 4-6. For SFA esters, the compounds containing C6:0 showed the best inhibitory ability among all the TY and HTY SFA esters during the entire incubation period of up to 15 h. For UFA esters, TY oleate showed a higher inhibitory activity than other UFA TY esters during the 15 h incubation period, while HTY linolenate showed the best inhibitory ability among all the esters during the initial 3 h of incubation.

The LDL used in this study contained 20-22% protein, 10-15% triacylglycerol, 20-28% phospholipid, 37-48% cholesteryl ester and 8-10% cholesterol. The average LDL comprises 86% linoleic acid, 12% arachidonic acid and 2% DHA, which provides a rich source of lipid peroxidation substrate (Abuja & Esterbauer, 1995). In this study, the HTY UFA esters exhibited higher inhibitory effects on LDL oxidation than that of the TY analogues. However, the results for SFA esters are opposite for UFA esters and TY esters showed a higher inhibitory activity than most of the HTY esters. The inhibitory effects on LDL oxidation of TY and HTY have previously been reported (Aruoma et al., 1998; Di Benedetto et al., 2007). According to Di Benedetto et al. (2007), HTY exhibited a higher inhibitory effect on cell-mediated oxidation of LDL than TY. We propose that the extra ortho-diphenolic hydroxyl group of HTY compared to TY contributes to the difference in this inhibitory effect. Tyrosol SFA esters showed a higher protective effect, possibly due to their ability as antioxidants to modulate human LDL.

LDL oxidation is a HAT-based antioxidant assay, which can be used to measure hydroxyl radical ($HO^{\bullet}$) and lipid peroxyl radicals ($ROO^{\bullet}$) quenching ability, reducing power (especially for donating hydrogen atom), and transition metal ion chelating ability (Craft et al., 2012; Tan &

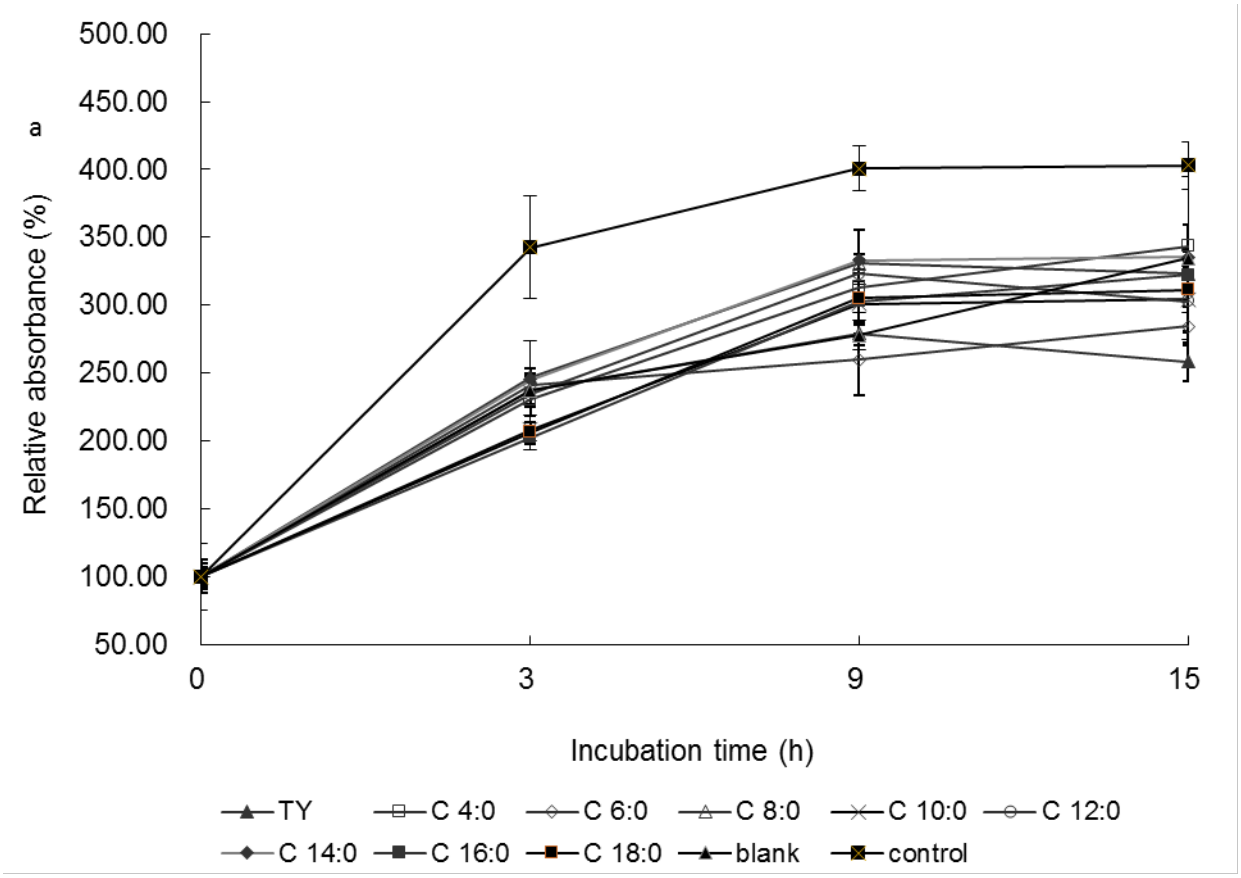
Lim, 2015). In this study, all esters of TY and HTY exhibited inhibitory effects on cupric ion-induced LDL oxidation during the first 3 h of incubation. Among SFA esters, HTY caproate showed the highest inhibition effect while for UFA esters, HTY linolenate displayed the best effect. In a previous study, Trujillo et al. (2006) reported that the HTY esters containing acetate, C14:0 C16:0, C18:1 and C18:2 all showed a protective effect on lipids against oxidation caused by peroxyl radicals and linoleate ester showed the best inhibition effect. In this work, our results showed that the HTY esters, except the caproate ester, exhibited much lower inhibitory effect on cupric ion-induced LDL oxidation than that of HTY. However, according to Trujillo et al. (2006), the protective effect of HTY on lipids against oxidation caused by peroxyl radicals was similar to that of HTY containing acetate, C14:0, C16:0 and C18:1, and was lower than that of HTY 18:2. This is in agreement with the results that HTY caproate had a similar protective effect to HTY against LDL oxidation. Furthermore, most of the esters did not show good inhibition as TY and HTY themselves. A previous study also showed this trend that hydroxytyrosol glucuronide only maintained a slight activity in protecting LDL from cupric ion-induced oxidation (Khymenets et al., 2010). The difference in experimental methods used in the two studies might be responsible for the observed results. Crauste et al. (2016) have suggested that the discrepancies between the observed activities are unavoidable for phenol esters when using two different methods.

Table 4-6. Inhibition (%) against human LDL cholesterol oxidation by tyrosol, hydroxytyrosol and its esters incubated at 37 °C for 15 h.

Compounds	Incubation time (h)			
	0.00	3.00	9.00	15.00
TY	100±9.63^{a}	237.25±10.12^{def}	279.05±8.81^{hij}	258.29±14.18^{i}
C 4:0	100±2.78^{a}	230.56±12.47^{def}	313.03±13.46^{fgh}	343.68±15.61^{defg}
C 6:0	100±7.15^{a}	240.68±3.51^{def}	259.92±26.08^{j}	284.29±14.30^{ghi}
C 8:0	100±2.52^{a}	246.99±2.88^{cdef}	330.59±25.33^{efg}	323.01±12.63^{defghi}
C 10:0	100±8.14^{a}	208.25±5.98^{f}	301.11±6.42^{fghi}	304.19±14.22^{efghi}
C 12:0	100±12.22^{a}	234.44±9.58^{def}	323.30±13.97^{fg}	302.77±22.02^{efghi}
C 14:0	100±4.80^{a}	245.31±8.50^{def}	332.97±1.03^{efg}	335.39±3.70^{defgh}
C 16:0	100±5.10^{a}	202.63±5.33^{f}	302.83±15.22^{fghi}	322.07±19.35^{defghi}
C 18:0	100±3.88^{a}	206.42±12.61^{f}	304.99±10.73^{fghi}	311.40±17.16^{efghi}
C 18:1	100±3.43^{a}	210.82±3.78^{f}	303.09±5.17^{fghi}	297.91±6.17^{fghi}
C 18:3	100±3.75^{a}	274.45±2.61^{cd}	383.87±3.11^{bc}	384.17±4.56^{bcd}
EPA	100±9.07^{a}	250.63±0.62^{cdef}	368.75±2.99^{cd}	439.68±3.41^{a}
DHA	100±11.85^{a}	288.74±1.07^{bc}	421.95±2.57^{a}	423.84±4.04^{ab}
HTY	100±8.88^{a}	94.46±14.60^{h}	86.75±4.76^{k}	79.82±6.07^{j}
C 4:0	100±9.19^{a}	290.29±16.77^{bc}	330.91±24.41^{efg}	317.74±22.04^{defghi}
C 6:0	100±7.37^{a}	97.12±12.61^{h}	90.74±2.10^{k}	85.09±5.02^{j}
C 8:0	100±4.33^{a}	337.08±3.55^{a}	406.25±8.36^{ab}	364.40±21.69^{cdef}
C 10:0	100±3.20^{a}	321.42±5.58^{ab}	365.91±22.05^{cde}	350.28±23.89^{cdefg}
C 12:0	100±9.14^{a}	230.25±8.49^{def}	321.32±23.28^{fg}	323.74±20.51^{defghi}
C 14:0	100±2.26^{a}	223.83±9.08^{def}	305.20±5.66^{fghi}	296.83±18.84^{fghi}
C 16:0	100±8.38^{a}	236.39±9.81^{def}	328.22±8.05^{efg}	340.08±11.86^{defgh}
C 18:0	100±6.20^{a}	202.23±9.71^{f}	289.04±11.95^{ghij}	270.22±35.53^{hi}
C 18:1	100±9.94^{a}	239.82±2.31^{def}	255.10±2.98^{j}	261.61±13.64^{i}
C 18:3	100±2.07^{a}	147.57±27.44^{g}	256.54±5.25^{ij}	275.69±4.03gh^{i}

EPA	100±12.18[a]	221.95±12.93[ef]	272.92±11.32[hij]	284.44±15.37[ghi]
DHA	100±5.98[a]	263.98±14.04[cde]	341.09±2.86[def]	369.55±6.99[cde]
blank	100±4.21[a]	237.18±36.78[def]	277.75±10.75[hij]	334.60±59.86[defgh]
control	100±24.79 [a]	342.62±37.83[a]	400.84±16.89[ab]	403.18±17.46[abc]

*Values are mean values of triplicate determinations±standard deviation. Values with different superscripts are different ($p<0.05$) from one another.



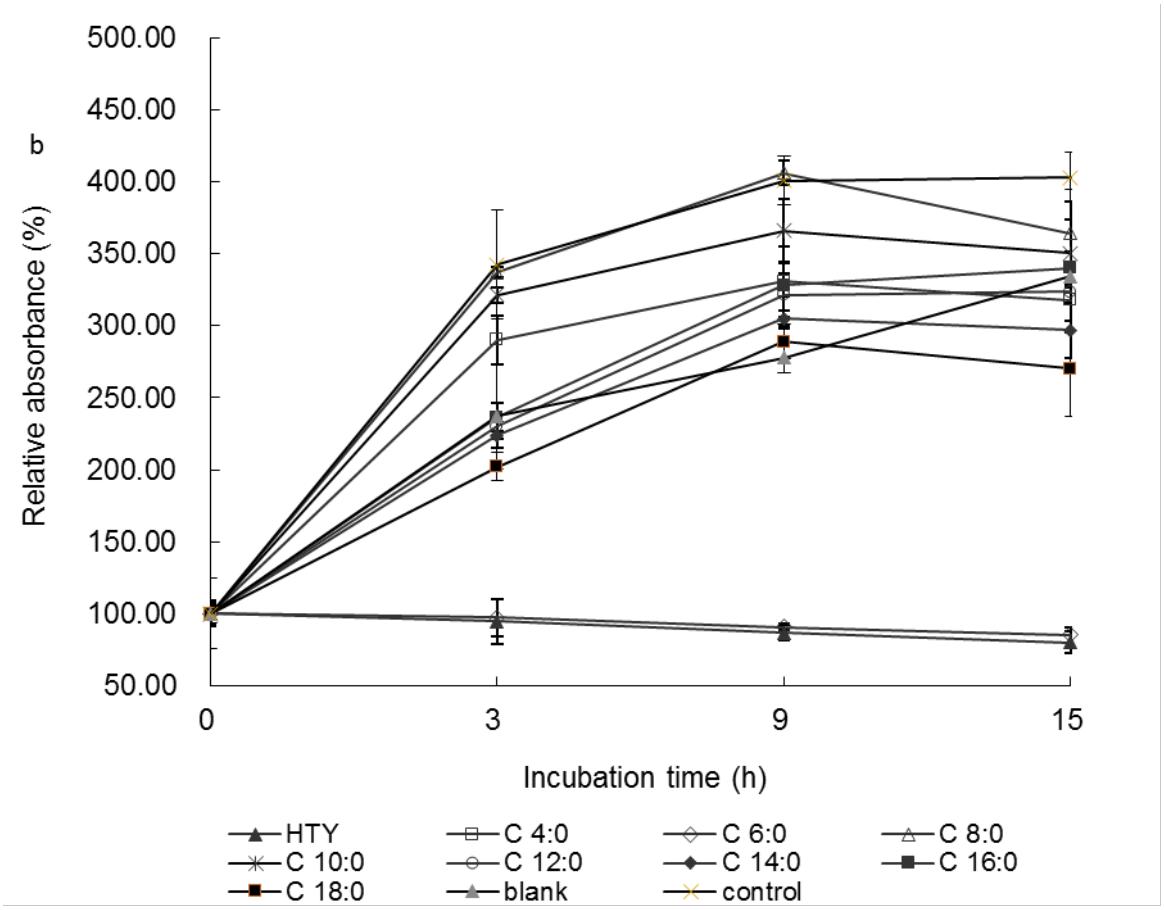
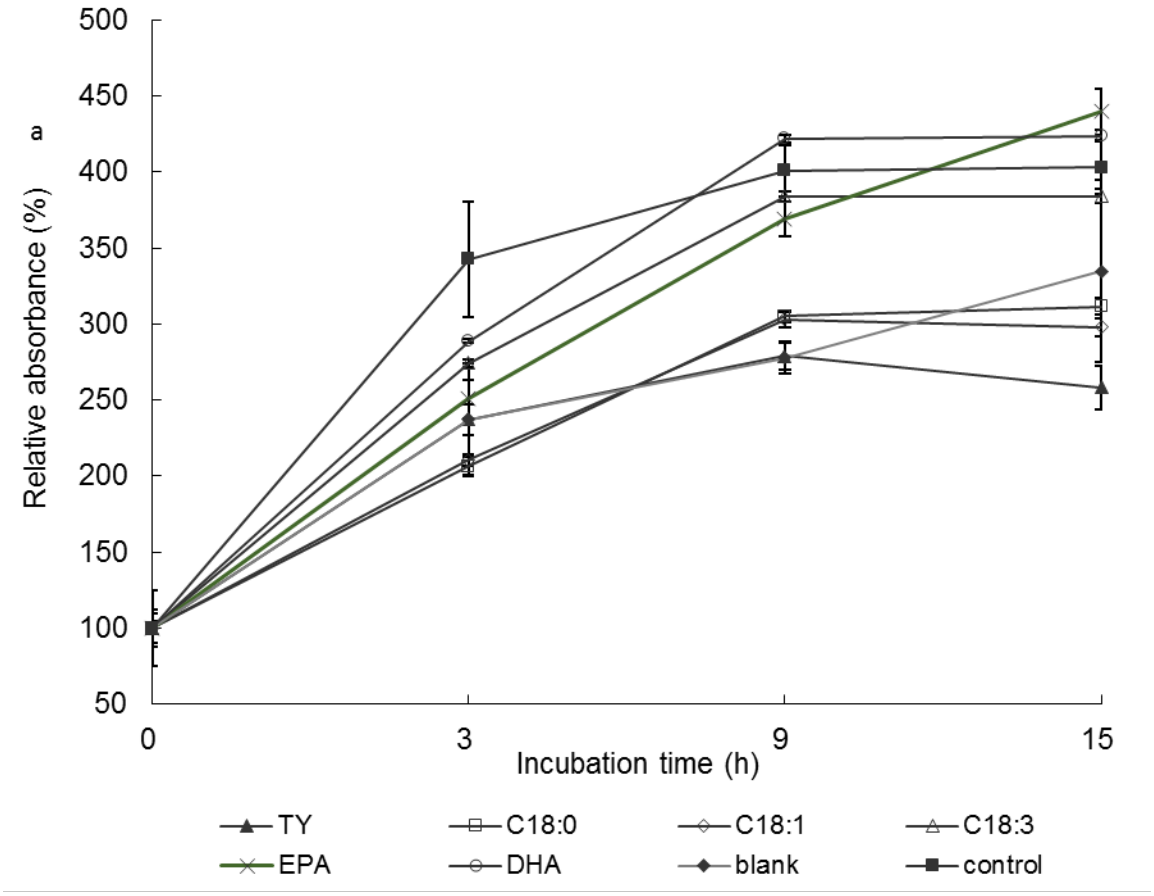


Figure 4-10. The inhibition of saturated fatty acid esters against formation of conjugated dienes at 234 nm against human LDL oxidation over a 15-hour period (triplicate determinations). (a) Relative absorbance for tyrosol and its saturated fatty acid esters; (b) relative absorbance for hydroxytyrosol and its saturated fatty acid esters.


a
Relative absorbance (%)
500
450
400
350
300
250
200
150
100
50
0
3
9
15
Incubation time (h)
TY
C18:0
C18:1
C18:3
EPA
DHA
blank
control

1

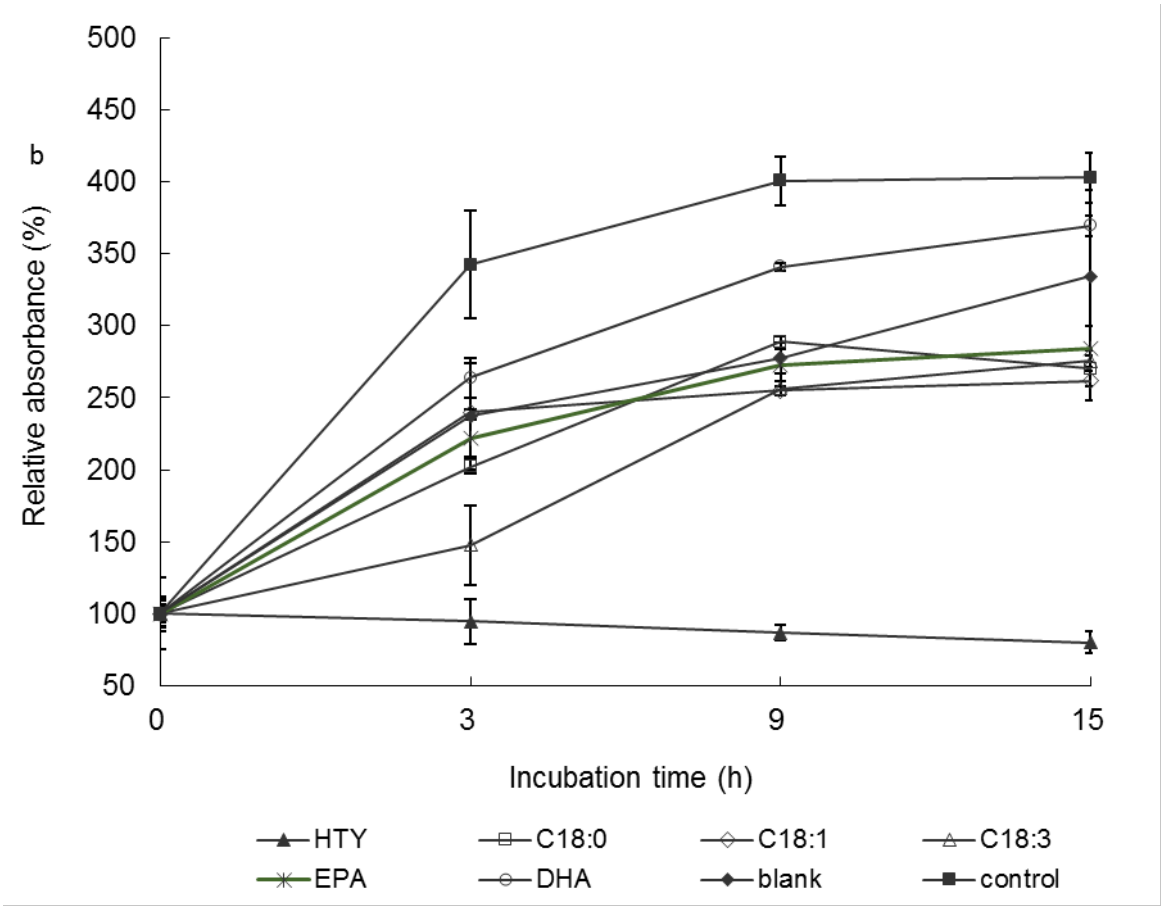


Figure 4-11. The inhibition of unsaturated fatty acid esters against formation of conjugated dienes at 234 nm against human LDL oxidation over a 15-hour period (triplicate determinations). (a) Relative absorbance for tyrosol and its stearate and unsaturated fatty acid esters; (b) relative absorbance for hydroxytyrosol and its stearate and unsaturated fatty acid esters.

4.3 Antiviral Properties

4.3.1 HCV Protease Inhibition

HCV infection is a contagious liver disease which is considered as a serious health threat globally. There are nearly 200 million people worldwide who are infected with HCV, and the chronic infection of this virus is associated with cirrhosis, hepatocellular carcinoma and liver transplantation (Stauber & Stadlbauer, 2006). Furthermore, there are limited therapies using interferon and pegylated interferon in combination with ribavirin. Also for a large population of HCV-infected patients the treatment has failed (Poordad et al., 2013). Therefore, it is urgent that we develop new drugs and agents against hepatitis C infection. It is known that HCV establishes chronic infection by using the viral Ser protease NS3/4A to cleave some cellular targets involved in innate immunity and the target can be the mitochondrial antiviral signaling protein (Li et al., 2005). The NS3/4A protease of HCV is an essential noncovalent enzyme for the maturation of the virus, and represents one of the important therapeutic targets for anti-HCV treatment (Stauber & Stadlbauer, 2006). NS3/4A protease directs posttranslational cleavage of the polyprotein expressed by the RNA virus, and also possesses RNA helicase activity and release the functional proteins that are required for HCV replication (Li et al., 2005). The inhibitors of HCV NS3/4A protease could be effective therapy options for hepatitis C patients. Inhibitors of NS3/4A may bind to the enzyme and inhibit activation of viral proteins leading to disrupting the processes relevant to the suppression of HCV (Seiwert et al., 2008).

The inhibitory effect of TY, HTY and their derivatives against HCV NS3/4A protease was measured using an *in vitro* assay and compared with embelin (positive control), a known HCV protease inhibitor; the results are shown in Table 4-7. TY and its esters did not show any significant effect in inhibiting the protease, having a high IC50 value of>100 μM compared to

that of the positive control embelin which was 32.6 μM, while HTY esters showed a protease inhibitory activity. In contrast, HTY inhibited the protease. Among the HTY SFA esters, the compounds containing C18:0 and C8: 0 displayed good protease inhibitory activity that was 4-fold stronger than that of embelin. Furthermore, the compounds with UFA were also quite acceptable, especially HTY oleate (10.0 μM).

Table 4-7. Inhibitory effect (IC50) of TY, HTY and their derivatives against HCV protease.

Compounds	**TY**	**C4:0**	**C8:0**	**C18:0**	**C18:1**	**EPA**	**DHA**	**Embelin**
IC50(μM)	>100	>100	>100	>100	>100	>100	>100	32.6±2.8
Compounds	**HTY**	**C4:0**	**C8:0**	**C18:0**	**C18:1**	**EPA**	**DHA**	**Embelin**
IC50(μM)	>100	36±6.6	8.2±3.9	8.9±2.9	10±5.8	100±2.8	34±4.1	32.6±2.8

*Values are mean values of triplicate determinations±standard deviation.

HTY esters showed better inhibition compared with HTY which might be due to the changes in steric features and hydrophobicity of the compounds leading to their superior binding affinity to the enzyme. A previous study reported that the ester derivatives of EGCG with fatty acids had better antiviral activities which revealed that the esters may be more sterically favoured than EGCG in binding to the protease (Zhong, Ma & Shahidi, 2012). Clark et al. (1998) also revealed that steric and conformational effects govern the infectivity of the virus. The difference in the inhibition of TY and HTY esters suggests that the number of phenolic hydroxyl groups plays an important role in antiviral activity. The lower inhibition of EPA and DHA esters compared with those of caprylate and stearate esters maybe due to non-specific interaction of longer acyl side chain which decreases the activity through aggregation induced by hydrophobic interaction. In our study, TY did not show any inhibition at concentrations of up to 100 μM which is in agreement with a past study (Zuo et al., 2007).

4.3.2 α-Glucosidase inhibitory activity

α-Glucosidases are hydrolytic enzymes that play a vital role in carbohydrate digestion and biosynthesis of viral envelope glycoproteins. α-Glucosidases are essential for the maturation of viral glycosylated envelope proteins and inhibitors will misfold and break down of the viral glycoproteins and subsequent reduction in virion secretion (Chang et al., 2013). In addition, α-glucosidase inhibition provides a promising strategy for the development of novel anti-HIV drugs because the glycosylation of viral envelope glycoproteins is essential for infectivity of HIV (Hattori et al., 2013). α-Glucosidases are also responsible for the final breakdown of carbohydrates, from disaccharides to absorbable monosaccharide units (He et al., 2014). Thus, inhibitors of alpha-glucosidase can serve as useful drugs for type II diabetes by decreasing carbohydrate digestion and absorption.

In this study, TY, HTY and their esters were examiined for their α-glucosidase inhibitory activity and compared with acarbose (positive control), a known α-glucosidase inhibitor used to reduce postprandial hyperglycaemia. Table 4-8 presents the IC50 values of all test compounds. As summarized in Table 4-8, acarbose was found to be the most potent inhibitor of alpha-glucosidase as it showed the lowest IC50 (0.05 µM). It was found that all esters showed lower inhibition against α-glucosidase when compared with acarbose, but the esters examined did not display inhibitory activity with IC50 value of>100 µM. Furhtermore, TY itself did not show any inhibition effect. In contrast, TY esters such as TY EPA had α-glucosidase inhibitory activity which means important hydrophobic interactions occurring between these compounds and α-glucosidase. TY oleate showed the lowest IC50 value (78 µM) among all esters which means it had the highest potency as alpha-glucosidase inhibitor among all tested derivatives. The TY esters showed inhibition effect which might be due to enhanced lipophilicity.

These results suggest that the inhibition effects of TY and their derivatives against α-glucosidase are possibly due to the binding affinity of the compounds which are influenced by hydrophobic interaction and steric features as TY oleate and TY EPA having a higher inhibitory activity than TY DHA which has a higher steric hindrance. Zhong, Ma and Shahidi (2012) reported that EGCG tetraesters with saturated fatty acids had better antiviral activities in inhibiting α-glucosidase than EGCG EPA and DHA due to the steric features and hydrophobic interaction effects. Furthermore, while most of the TY derivatives that showed potent inhibition on α-glucosidase were not active against HIV, which is in agreement with findings of Hattori et al. (2013), thus suggesting that other factors such as the physiochemical properties affect the anti-HIV activity of these compounds. Hattori et al. (2013) also reported that derivatives of chlorogenic acid show high inhibition against α-glucosidase and the inhibition of enzymes was significantly increased by lengthening the alkyl chain. Another study also found that derivatives (alkyl chains incorporated in a heterocycle ring) of catechin exhibited much stronger inhibition against α-glucosidase than (+)-catechin (Hakamata et al., 2006). Tanaka et al. (2005) found that (S)-hexahydroxydiphenoyl (HHDP) esters of dihydrochalcone glucosides (04-1.6 μg/mL) inhibited α-glucosidase at a lower concentration than EGCG (3.1 μg/mL). Cheng et al. (2014) showed that derivatives of chrysin, diosmetin, apigenin, and luteolin had higher glucosidase inhibitory activity (IC50 < 24.396 μM) compared with that of the reference drug, acarbose (IC50 = 563.601 μM), and higher than their precursors except for luteolin derivatives.

Table 4-8. Inhibitory effect (IC50) of TY, HTY and their derivatives against a-glucosidase.

Compounds	**TY**	**C4:0**	**C8:0**	**C18:0**	**C18:1**	**EPA**	**DHA**	**Acarbose**
IC50(μM)	>100	>100	82±1.6	91±4.5	78±2.4	80±5.3	>100	0.05±1.3
Compounds	**HTY**	**C4:0**	**C8:0**	**C18:0**	**C18:1**	**EPA**	**DHA**	**Acarbose**
IC50(μM)	>100	>100	>100	>100	>100	>100	>100	0.05±1.3

*Values are mean values of triplicate determinations±standard deviation.

4.4 The Effect of Tyrosol, Hydroxytyrosol and their Esters on the Viability of HepG2

In this study MTT assay, a colorimetric assay, was performed to determine the cytotoxicity of tyrosol (TY), hydroxytyrosol (HTY) and their esters. MTT, a yellow tetrazolium compound, is positively charged and readily penetrates living cells and can be reduced to purple formazan (Mosmann, 1983), possibly due reaction with NADH or similar reducing molecules that transfer electrons to MTT (Marshall, Goodwin & Holt, 1995). Dead cells cannot convert MTT into formazan, thus colour formation serves as a useful and convenient cell-viability indicator. In this study, treated cells were incubated for 24 or 48h. It is known that longer incubation time will result in increased colour and sensitivity up to a point that the incubation time is limited, due to the cytotoxic nature of the detection reagents which have a requirement of energy from the cell to generate a signal. Reducing compounds are known to interfere with tetrazolium reduction assays. Reducing compounds such as ascorbic acid can reduce tetrazolium salts non-enzymatically and cause increased absorbance in the assay (Ulukaya, Colakogullari & Wood, 2004; Chakrabarti, 2001; Barltrop, 1991).

The results of the cell viability of TY, HTY and their esters are shown in Table 4-9. In the literature, 80% of cell viability is often used as the criterion for cytotoxicity (Iwasawa, Ayaki & Niwano, 2013). TY, HTY and their esters did not manifest any significant cytotoxicity following

24 or 48h exposure to a concentration at 10 μg/mL as their cell viability was higher than 80%. When compared with 24h treatment, there was a reduction in cell viability after 48h exposure to TY, TY stearate, TY EPA, and HTY caprylate which means they have some cytotoxic effects on HepG2 cells. Whereas TY butyrate, TY caprylate, TY oleate, TY DHA, HTY butyrate, HTY caprylate, HTY stearate, HTY EPA, and HTY DHA increased the viability of cells after 48h when compared with that after 24h that means they have little cytotoxicity. Introduction of fatty acids such as oleic acid to TY or HTY improved their cytotoxic activity. HTY has a lesser cytotoxic effect than TY after 24 or 48 h of incubation as their cell viability was much higher. Meanwhile, HTY esters were also less cytotoxic than their TY analogues after 24 h of incubation except for DHA ester.

In this study, neither TY, HTY nor their esters exhibited a cytotoxic effect which is in agreement with Wen et al. (2013) that TY, 4-hydroxyphenylacetic acid (4-HA), 3-hydroxyphenylacetic acid (3-HA),2-hydroxyphenylacetic acid (2-HA) and salidroside showed a cell viability of higher than 90%. HTY esters all showed a similar cell viability except for HTY DHA. HTY or HTY acetate which showed no significant differences in cell viability after a 24 h exposure (Pereira-Caro et al., 2012). Pereira-Caro et al. (2011) also pointed out that the alkyl hydroxytyrosyl ethers showed no cytotoxic effects and there were no differences in cell viability after 24 h of incubation in HepG2 Cells and similar results were obtained in enterocyte-like Caco-2 cells (Pereira-Caro et al., 2010). In contrast, HTY and oleuropein were cytotoxic in MCF-7 cells in a dose dependent manner (Han, Talorete, Yamada & Isoda, 2009). In addition, there was little difference in cell viability between most of the esters, possibly due to the nature of MTT assay which is based on the metabolic activity of the cells. Han, Talorete, Yamada and Isoda (2009) revealed the influence of the contact surface with hydroxytyrosol or oleuropein and suggested that phenolic

compounds of olive leaf have health protective rather than healing effects. When used for protective effects against cell damage as daily consumption, less cytotoxicity and higher cell viability is better, thus butyrate esters can provide the best choice as anti-cancer activity has requirement for enhanced toxicity. For instance, the enhanced toxicity of fatty acid-modified dendrimeric prodrugs exert good anti-cancer activity (Gao et al., 2015). Our results suggest that HTY DHA may serve best for anti-cancer activity as it has the highest cytotoxic effect among all the tested compounds after incubation of 24h while TY EPA showed the highest cytotoxicity after 48 h incubation.

Table 4-9. The effect on cell viability by (a) TY and its esters treatment; (b) HTY and its esters treatment.

(a) TY and its esters

Compound (10 μg/ml)	**Cell Viability %（24 h）**	**SD**	**Cell Viability %（48 h）**	**SD**
TY	104.63	8.49	99.85	13.27
TY C4:0	118.43	4.80	138.52	14.38
TY C8:0	94.43	6.49	118.65	1.36
TY C18:0	110.86	2.18	100.06	7.75
TY C18:1	91.52	6.59	110.02	12.13
TY EPA	96.08	8.76	75.69	8.48
TY DHA	100.24	2.74	109.83	3.19

(b) HTY and its esters

Compound (10 μg/ml)	Cell Viability %（24 h）	SD	Cell Viability %（48 h）	SD
HTY	122.01	11.75	139.98	3.25
HTY C4:0	127.55	6.61	136.19	7.67
HTY C8:0	112.81	7.32	103.84	7.90
HTY C18:0	111.35	2.89	121.81	8.17
HTYC18:1	120.13	3.35	145.84	9.35
HTY EP4	113.21	12.28	122.57	16.14
HTY DHA	84.69	12.95	97.33	3.98

*Values are mean values of triplicate determinations±standard deviation.

4.5 Protection on HepG2 against t-BuOOH induced oxidative stress

Cellular oxidative stress was measured by the dichlorofluorescein assay in the human hepatocarcinoma cell line (HepG2) to determine the effect of TY, HTY and their selected fatty acid esters on the intracellular generation of the ROS (Wang & Joseph, 1999). It is known that ROS have cell-signaling functions (Nohl, Gille & Staniek, 2005) and play an important role in the pathogenesis of ischemia-reperfusion injury and lead to cell ageing and age-related degenerative diseases, such as cancer (Zulueta et al., 1997; Valko et al., 2006). Although various methods can be used for oxidative stress assessment of cells (Holley & Cheeseman, 1993), the direct evaluation of ROS can be a very good evidence for oxidative damage to living cells (Wang & Joseph, 1999). In this study, 29, 79-dichlorofluorescin diacetate (DCFH-DA) was used as a

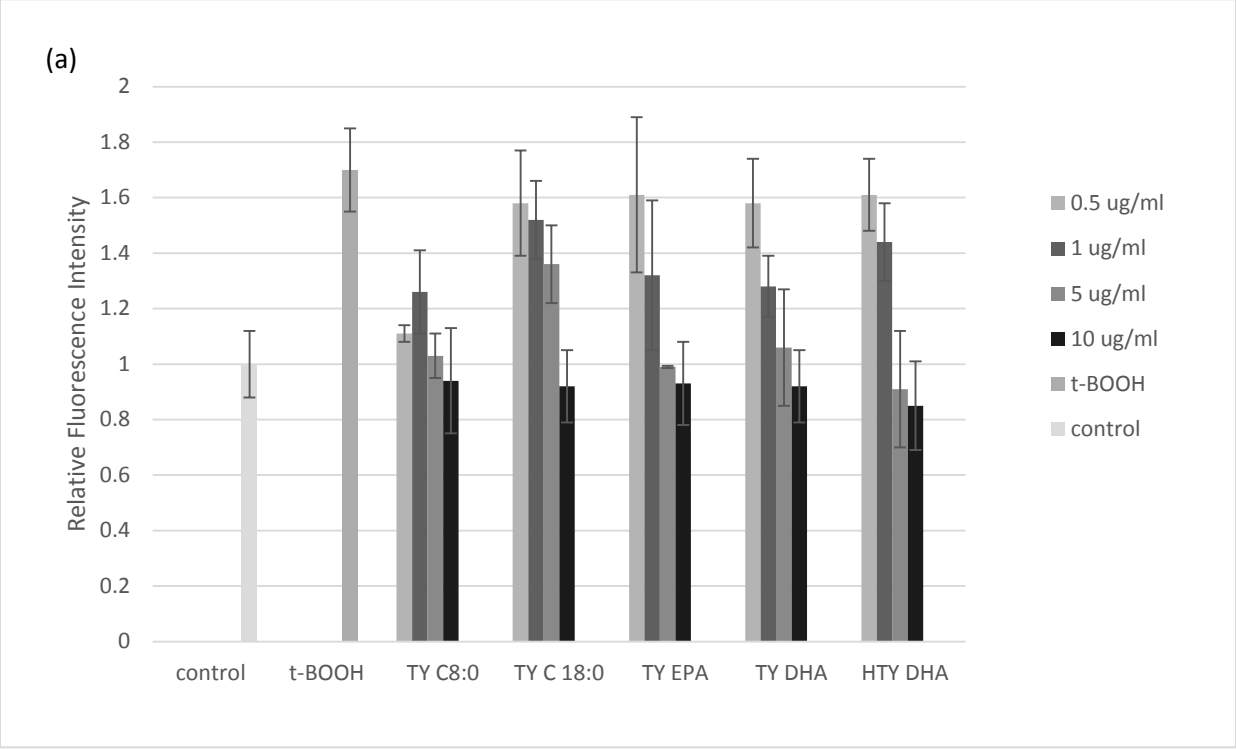
fluorometric assay for hydrogen peroxide, due to emitting fluorescence of DCFH-DA after being oxidized (LeBel, Ischiropoulos & Bondy, 1992). In cells, the nonpolar DCFH-DA is hydrolyzed enzymatically by intracellular esterases to nonfluorescent DCFH after crossing cell membranes (LeBel, Ischiropoulos & Bondy, 1992; Bass et al., 1983). The nonfluorescent DCFH is then oxidized by intracellular ROS to highly fluorescent DCF (LeBel, Ischiropoulos & Bondy, 1992) that can be used as an index to quantify the overall oxidative stress in cells (Wang & Joseph, 1999).

In this study, HepG2 cells were incubated for 14 h and then treated with different doses of TY, HTY and their esters for 4 h. A much higher ROS production was observed after 4 h in the presence of 400 μM t-BuOOH as compared to controls with FBS-free medium without t-BuOOH and the testing compounds (Figure 4-12). TY and HTY did not show any ROS inhibition even at the highest concentration (10 μg/mL). However, after being treated, ROS generation was decreased in the presence of 0.5-10 μg/mL fatty acid esters except that for 0.5 μg/mL HTY oleate ester (Figure 4-12). Treating HepG2 cultures with 1 μg/mL esters greatly decreased ROS production except for HTY oleate. It is obvious that 5 μg/mL HTY DHA, 10 μg/mL TY caprylate, TY stearate, TY EPA, TY DHA and HTY DHA reduced ROS levels to those of untreated cells (Figure 4-12a). Treating cells with 0.5 μg/mL of test samples resulted in a lower decrease in ROS generation than that observed in cells treated with higher concentrations of samples. When HepG2 cells were treated with 10 μg/mL HTY DHA, ROS production in the presence of t-BuOOH was reduced most compared to that of control untreated cells and cells treated with other samples.

Human hepatoma HepG2, used as a model in our study, is a well-differentiated transformed cell line that is often used for biochemical and nutritional studies where many antioxidants and

conditions can be assayed with minor inter-assay variations (Goya, Mateos & Bravo, 2007). In this study, t-BuOOH was used as a prooxidant that can directly oxidize DCFH to DCF while decomposing to peroxyl radicals and generating lipid peroxides and ROS, thus increasing fluorescence (Alía et al., 2006). Moreover, other prooxidants such as hydrogen peroxide cannot evoke cellular stress (Alía et al., 2005). From this study, we found that ROS generation induced by t-BuOOH in HepG2 in an oxidative stress situation could be completely inhibited by a 4 h treatment with 5 µg/mL HTY DHA, 10 µg/mL TY caprylate and TY stearate, TY EPA, TY DHA and HTY DHA while other doses of test compounds acted as full or partial inhibitors. Thus, the synthesized esters of TY and HTY containing lipophilic fatty acids strongly inhibited the generation of ROS induced by t-BuOOH in HepG2 and the effects were dose-dependent, leading to prevention or delaying conditions that cause oxidative stress in the cell. It was known that decreasing oxidative stress state could prevent the development of tumours and cancer. It has been suggested that olive oil phenolics could scavenge ROS under natural and chemically simulated oxidative stress conditions (Paiva-Martins et al., 2009). However, in this study, TY and HTY could not inhibit ROS generation. In contrast, it has been found that HTY can reduce ROS generation induced by t-BuOOH when cells were pretreated (Goya, Mateos & Bravo, 2007) and TY exerted beneficial effects in ethanol-induced oxidative stress in HepG2 cells (Stiuso et al., 2016). Stiuso et al. (2016) revealed that the generation of high intracellular ROS concentrations may be due to increased β-oxidation of fatty acids. A previous study also revealed that HTY and TY reduce H_2O_2-induced ROS level in breast epithelial MCF10A cells, whereas TY failed to reduce in human breast cancer cells and HT only reduced H_2O_2-induced ROS level slightly in breast cancer cells (Warleta et al., 2011), possibly because TY and HTY can act as direct antioxidants with a redox activity or indirect antioxidants that can provide cellular

protection against oxidative stress (Warleta et al., 2011). In our study, esters of TY and HTY showed quite high inhibition against t-BuOOH induced ROS generation in HepG2, is in agreement with a previous study that HTY acetate had antioxidative stress protective effects at physiological concentrations similar to or even slightly higher than that of HTY (Pereira-Caro et al., 2012).



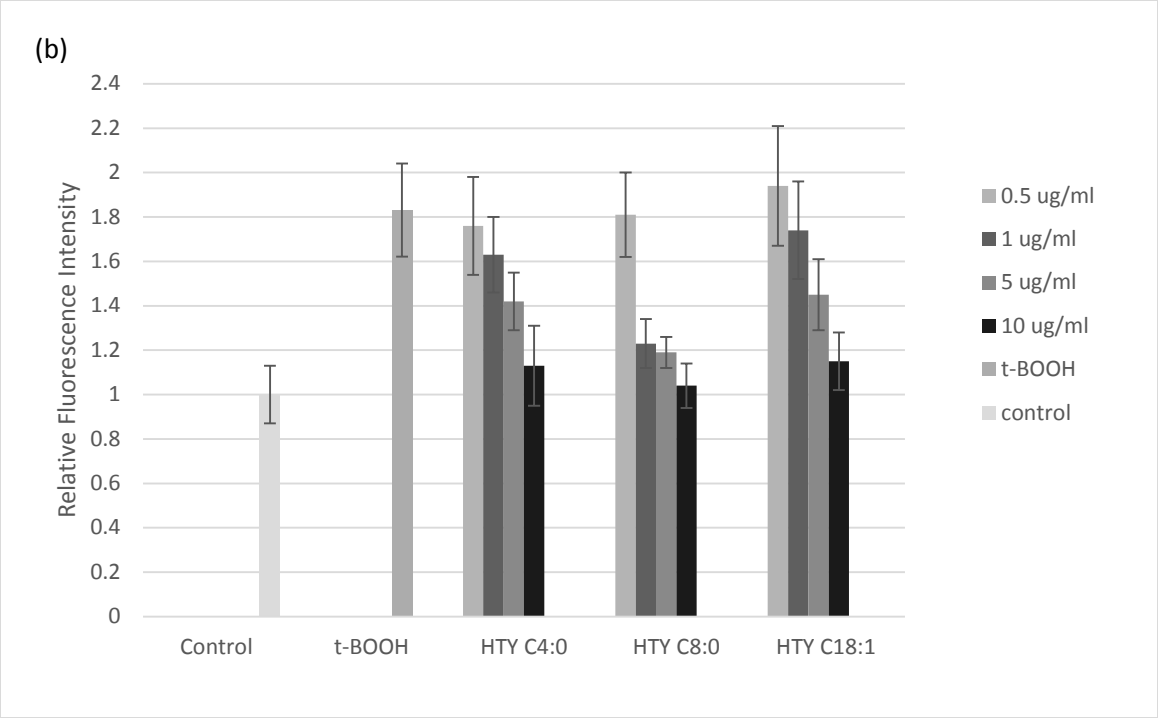


Figure 4-12. Effect of TY, HTY and their esters on intracellular reactive oxygen species (ROS) generation (triplicate determinations). HepG2 cultures were treated with the noted concentrations (0.5, 1, 5, 10 µg/mL) of TY C8:0 and TY C18:0 and HTY DHA (a), HTY C4:0, C8:0 and C18:1(b) for 4 h. In this study, TY and HTY did not show activity (NA) at the highest concentration.

4.6 Inhibition of LPS-induced nitrite production by EPA and DHA esters of tyrosol and hydroxytyrosol

Nitric oxide (NO) is an important mediator in states of inflammatory diseases (Kiemer, Müller, & Vollmar, 2002), as well as an important molecule for host defense response against various pathogens (Bogdan, Röllinghoff, & Diefenbach, 2000). NO is produced in various mammalian cells, including macrophages, neutrophils, platelets, fibroblasts, endothelium, neuronal, and smooth muscle cells, from L-arginine using NADPH and molecular oxygen by three forms of

nitric oxide synthases (NOS), namely endothelium NO synthase (eNOS), neural NO synthase (nNOS) and inducible NO synthase (iNOS) (Yang et al., 2009; Joo et al., 2014). At nanomolar concentrations, NO plays an important role in host defence and the regulation of various pathophysiological processes such as neuronal communication, vasodilatation, and neurotoxicity (Moncada, Palmer, & Higgs, 1991; Kruidenier & Verspaget, 2002). However, if NO is overproduced and uncontrolled, it will induce host cells damage associated with acute and chronic inflammations due to the cytotoxic potential of NO (Taira, Nanbu, & Ueda, 2009). LPS is a potent activator of monocytes and macrophages from the cell walls of gram-negative bacteria and involves the generation of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 (Meng & Lowell, 1997). Therefore, inhibition of NO production in LPS-stimulated RAW 264.7 cells is one of the possible ways to develop anti-inflammatory agents.

In this study, the ability of TY and HTY-EPA and -DHS esters to inhibit NO production in murine RAW 264.7 macrophages was evaluated and compared with that of their parent compounds TY and HTY. As shown in Figures 4-13 and 4-14, LPS treatment resulted in a sharp increase in the nitrite level in the macrophages as the nitrite accumulation in the cells increased. All tested compounds were effective in inhibiting the nitrite accumulation in RAW 264.7 cells in a concentration dependent manner. Treatment of cells with TY EPA and DHA esters significantly reduced nitrite accumulation at 5 and 25 µg/mL in RAW 264.7 macrophages and the inhibition effects were higher than TY itself (Figure 4-13). In contrast, HTY exhibited higher inhibition towards NO synthesis than HTY EPA and lower than HTY DHA at 5 µg/mL. Among the tested compounds, HTY showed the most potent inhibition at 25 µg/mL. This

suggests that esters could act as efficient anti-inflammatory agents by inhibiting the production of NO.

TY and HTY have been reported for their anti-inflammatory effect by NO inhibition. HTY has been demonstrated to show strong anti-inflammatory activity by inhibiting production of NO, possibly by reducing the expression of genes of iNOS and mediating via the NF-κB pathway (Richard et al., 2011). Treatment with TY increased the anti-inflammatory effects by inhibiting NO production in the anterior segment (the front third of the eye) (Mihara et al., 2016). Sato et al. (2016) revealed that TY inhibited iNOS expression and activated NF-κB translocation in LPS-stimulated RAW264.7 cells. It has been reported that HTY acetate significantly reduced nitrite levels with a significant decrease of iNOS protein expression at similar levels of HTY itself (Aparicio-Soto et al., 2015). Similar mechanisms may be involved for TY and HTY-EPA and -DHA esters with possible additional contribution from the fatty acid side chain. In addition, the phenolics (TY and HTY) found in virgin olive oil are able to directly scavenge NO (de la Puerta et al., 2001).

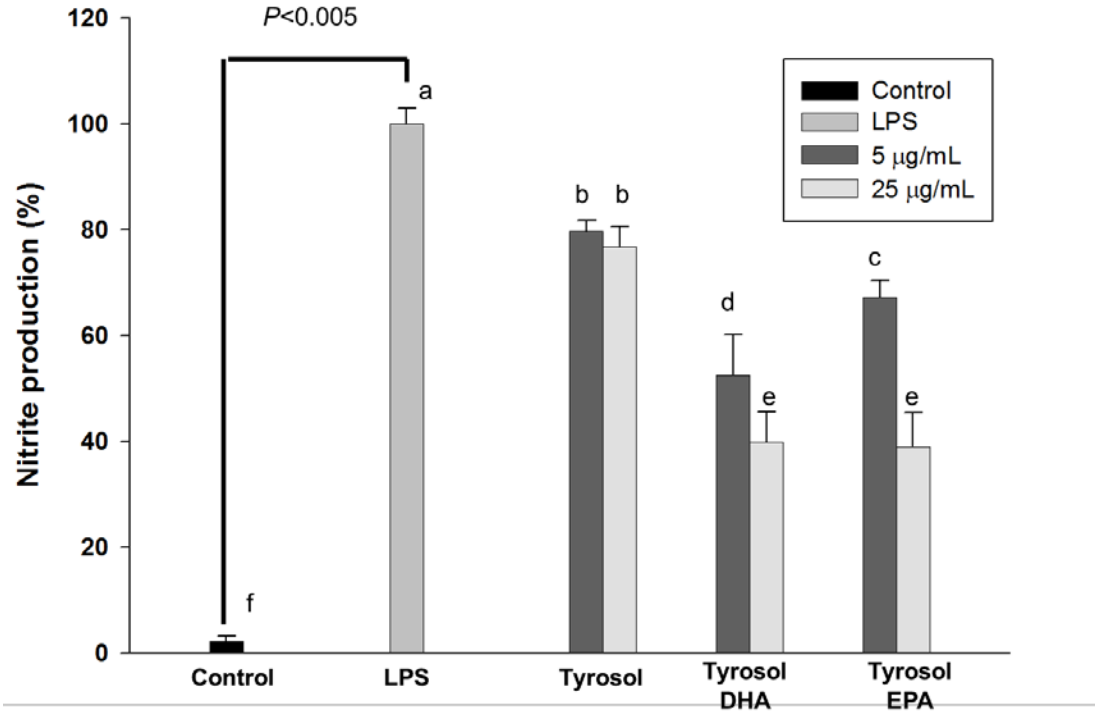


Figure 4-13. Effects of tyrosol and its derivatives compound on LPS-induced NO production in RAW264.7 macrophages. Bars with different letters are significantly different at $P < 0.05$ (triplicate determinations).

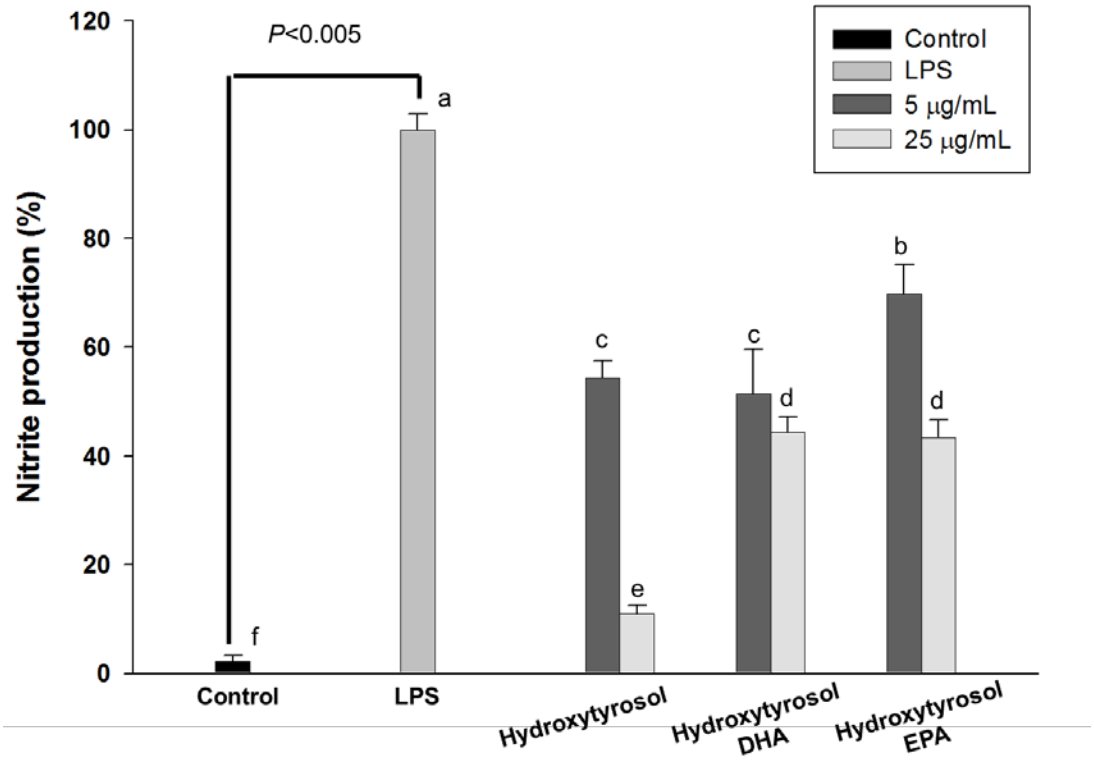


Figure 4-14. Effects of hydroxytyrosol and its derivatives compound on LPS-induced NO production in RAW264.7 macrophages. Bars with different letters are significantly different at P < 0.05 (triplicate determinations).

4.7 Antiglycation effects by BSA-glucose assay

The non-enzymatic glycation, altering the structure and functional properties of proteins, is related to the pathogenesis of some chronic diseases, especially diabetes and its associated complications (Meerwaldt et al., 2008; Vlassara & Palace, 2002), leading to the formation and accumulation of AGEs (Peng et al., 2011). AGEs are a group of complex and heterogeneous molecules such as fluorescent pentosidine and non-fluorescent carboxymethyllysine (CML) (Peng et al., 2011). Because of the harmful effects of AGEs such as diabetic complications, the

AGE formation inhibitors are recently being examined. However, many AGE inhibitors exhibit side effects and are highly toxic for diabetic patients (Thornalley, 2003). Therefore, new antiglycation drugs from natural products with lower side effects and less toxicity would be more beneficial to treat diabetic patients.

In this study, the tyrosol (TY) and hydroxytyrosol (HTY) and their selected esters were evaluated for their effects on the formation of AGEs by BSA-glucose system. D-glucose used in this experiment was allowed to react with BSA (protein resource) and served as the main glycating sugar with the highest concentration in the body (Sadowska-Bartosz, Galiniak, & Bartosz, 2014). The carbonyl scavenger aminoguanidine (AG) was used as a positive control. As shown in Figures 4-15 and 4-16, the parent TY molecule exhibited antiglycation activity whereas HTY molecule did show any inhibition activity towards AGEs formation whose relative content of fluorescent AGE was much higher than that of the control. Compared with the parent TY molecule, esters of TY with oleic acid and saturated fatty acids (SFA) with 10, 12 and 14 carbons showed slightly higher inhibitory activity against fluorescent AGEs formation. In addition,TY SFA esters containing 12 and 14 carbons showed a higher inhibition effect than AG. HTY SFA esters with 10, 12 and 14 carbons showed antiglycation effect, but at a lower level than that of AG.

TY has proven to have AGEs inhibition effect (Koko, Osman, & Galal, 2009), possibly due to the aromatic structure that are active in suppressing immune responses in both *in vitro* and *in vivo* assays (Wang et al., 1987). In this study, HTY did show any antiglycation effect. However, Navarro et al. (2015) showed that HTY exhibits antiglycative action by direct trapping of dicarbonyl compounds. Free radicals are associated with the glycation process during which superoxide radicals and dicarbonyl ketoaldehydes are generated, which may get involved in the

formation of AGEs (Sun et al., 2011). Therefore, the differences between esters are possibly influenced by their ability to scavenge free radicals or inhibit their generation which is influenced by the fatty acid side chain.

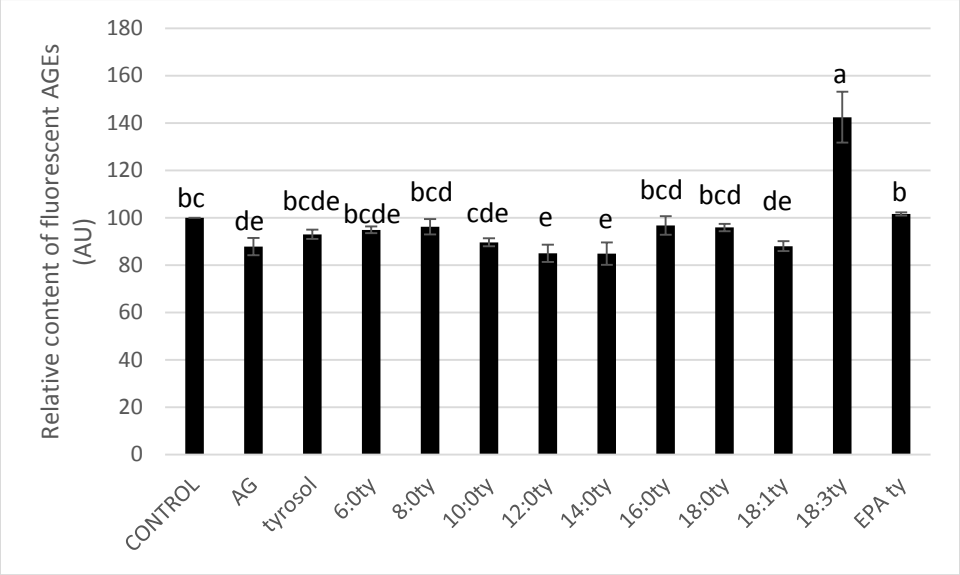


Figure 4-15. Relative content of fluorescent AGEs of aminoguanidine (AG), tyrosol (TY) and lipophilized TY derivatives. Bars with different letters are significantly different at $P < 0.05$ (triplicate determinations).

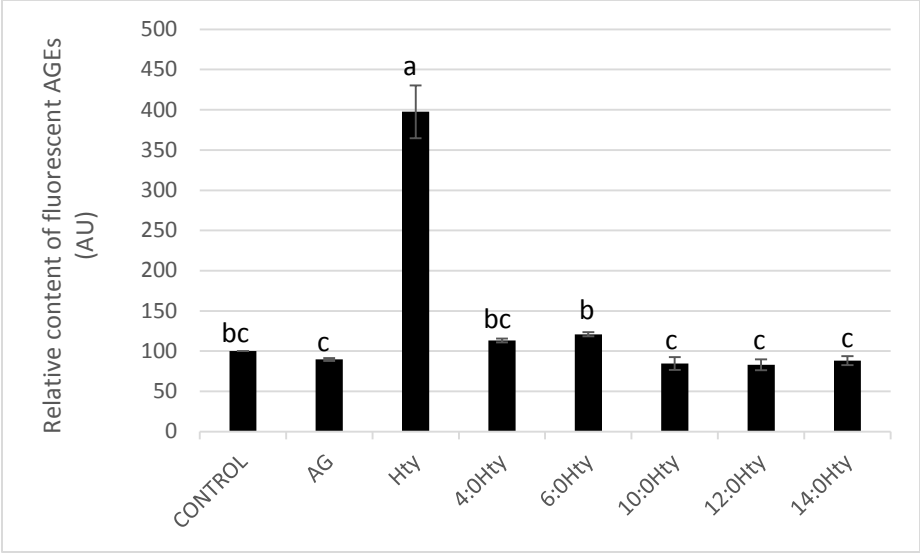


Figure 4-16. Relative content of fluorescent AGEs of aminoguanidine (AG), hydroxytyrosol (HTY) and lipophilized HTY derivatives. Bars with different letters are significantly different at $P < 0.05$ (triplicate determinations).

CHAPTER 5

SUMMARY AND RECOMMENDATIONS

The work reported in this thesis examined the lipophilization of tyrosol (TY) and hydroxytyrosol (HTY) for their expanded application and improved bioefficiency in food and natural health products. A series of TY and HTY esters of C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C18:1, C18:3, EPA and DHA were prepared enzymatically and a simplified base extraction purification procedure was employed. These ester derivatives, as expected, showed enhanced lipophilicity, and were evaluated for their bioactivities using a number of *in vitro*, *ex vivo* and *in vivo* tests. The bioactivities examined included antioxidant activity determinations using both chemical and biological model system assays,. The latter included antiviral activity, anti-inflammatory activity in LPS-stimulated murine macrophages, antiglycation activity in BSA-glucose system, ROS generation and cell viabilities in human hepatocarcinoma cell line.

The lipophilic esters of TY and HTY were all effective in scavenging DPPH radical, inhibited cupric ion-induced LDL oxidation and exhibited protective effects against hydroxyl raidical- and peroxyl radical-induced DNA scission. These results demonstrate the high influence of the ortho-diphenolic structure on the antioxidant capacity of HTY. For HTY, the introduction of the lipid part decreased its antioxidant activities. Meanwhile, an unsaturation-dependent antioxidant effect was observed for TY and HTY esters in DNA strand scission assay, and for TY esters in the DPPH assay. However, in LDL oxidation assay, the polyunsaturated fatty acid moiety of TY esters may be oxidized. In antiviral assays, HTY esters showed a HCV protease inhibitory activity while TY esters had α-glucosidase inhibitory activity. The anti-inflammatory activity of TY and HTY derivatives was evaluated in LPS-stimulated murine macrophages and were found to be effective in inhibiting LPS-induced NO. It was also found that the esterification of these

compounds could improve their antiglycation effects and inhibition against ROS generation with little cytotoxic effects. In addition, the improvement of bioavailability maybe due to their increased liposome membrane affinity and hence enhanced cellular absorption *in vivo.*

Future research on the antioxidant activities of TY and HTY may focus on their properties in food model (bulk oil and oil in water emulsion) and to examine the effect of side chain length. In this study, it was suggested that TY and HTY lipophilic esters can be used as functional food ingredients and pharmaceuticals for health promotion and disease risk reduction. Therefore, more investigation needs to be carried out on bioactivities of TY and HTY derivatives *ex vivo* and *in vivo* using cell line and animal models, followed by human clinical trials. Research should also focus on the economic feasibility of large scale production of selected esters and purification to assess their absorption and metabolism, as well as possible allergic and genotoxic potencies.

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