

Chapter 1

Introduction

Lipid peroxidation is an important deteriorated reaction in food during storage and processing. It not only brings about chemical spoilage in foods but also produces free radicals or active oxygens such as peroxy and hydroxyl radicals, which are purportedly associated with carcinogenesis, mutagenesis, aging, and atherosclerosis (Ohara *et al.*, 1993; Yen and Wu, 1999; Yen *et al.*, 1996). Food products undergo a chain of change in the natural matrix due to ripening, harvesting, primary processing and storage. These changes are caused by several factors including browning reactions, microbial spoilage and autoxidation of lipids. Of various factors, the oxidative deterioration of fats and oils in foods is responsible for rancid odors and flavors, with a consequent decrease in nutritional quality and safety caused by the formation of secondary potentially toxic compounds (Moure *et al.*, 2001). To reduce and delay this reaction and therefore preserve food products, synthetic antioxidants such as sulfites or butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are extensively used. However, because of their possible potential as promoters of carcinogenesis (Imida *et al.*, 1983), as well as general consumer rejection of synthetic food additives, active investigation concerning the effectiveness of naturally occurring antioxidants are being carried out. Vegetable materials contain many compounds with antioxidant activity. Several plants have been studied as sources of potentially safe natural antioxidants for the food industry. Among these, phenolic compounds, especially flavonoids have been repeatedly implicated as active antioxidants (Chimi *et al.*, 1991). Additionally, several studies have shown that phenolic compounds reduce *in vitro* oxidation of low density lipoprotein, particularly those phenolics with multiple hydroxyl groups which are generally the most efficient for preventing lipid and low density lipoprotein (LDL) oxidation (Moure *et al.*, 2001). Polyphenolic flavonoids can scavenge reactive oxygen radicals, such as the hydroxyl radical and superoxide anion radical, by

donating a hydrogen atom or electron (Terao, 1999). Green tea leaves contain up to 36% polyphenols, on a dry weight basis. Crude extracts of green tea and individual catechins also showed antioxidative potency in lard and unsaturated marine oils (Hara, 1994; Wanasundara and Shahidi, 1998).

So far, various antioxidants occurred naturally in plants has been studied (Ramarathnam *et al.*, 1988). Those antioxidants have been used as nutraceutical purposes and food application. Mulberry (*Morus alba* L.) leaves containing many nutritional components are the best feed for silkworms. Traditionally, mulberry leaves are used as a medicinal herb in Chinese culture (Yen *et al.*, 1996), but a little information regarding antioxidant activity of mulberry green tea cultivated in Thailand has been reported so far. It has been reported that the composition of green tea varies with climate, season, variety and state of maturity (Wanasundara and Shahidi, 1998). Therefore, the purpose of this research was to determine the antioxidant activity of mulberry green tea as well as some factors affecting its activity and to study the application of mulberry green tea extract to retard the oxidation of some oils and human low density lipoprotein.

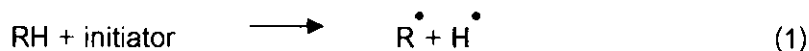
Literature review

1. Mechanism of lipid peroxidation

Atmospheric oxygen can react spontaneously with a number of organic compounds, leading to structural degradation, which is ultimately responsible for the loss of quality of numerous chemical products of economic or industrial importance. In food systems, the spontaneous oxidative reactions result in the deterioration of lipids. This direct reaction of a lipid molecule with a molecule of oxygen, termed autoxidation, is a free-radical chain reaction. The mechanism of autoxidation can be distinguished in three distinct steps: initiation, propagation and termination (Jadhav *et al.*, 1996).

A. Initiation

The autoxidation of a fat is thought to be initiated with the formation of free radicals. The formation of lipid radical R^\bullet is usually mediated by trace metals, irradiation, light or heat (Eqs. 1). Initiation reactions take place either by the abstraction of hydrogen radical from an allylic methylene group of an unsaturated fatty acid or by the addition of a radical to a double bond. The rearrangement of the double bonds results in the formation of conjugated diene ($-\text{CH}=\text{CH}-\text{CH}=\text{CH}-$), showing a characteristic UV absorption at 232-234 nm (Nakayama *et al.*, 1994)



Also, lipid hydroperoxide, which exists in trace quantities prior to the oxidation, breaks down to yield radicals as shown by Eqs. (2) and (3). Lipid hydroperoxides are formed by various pathways including the reaction of singlet oxygen with unsaturated lipids or the lipoxygenase-catalyzed oxidation of polyunsaturated fatty acids (Jadhav *et al.*, 1996).

B. Propagation

In propagation reaction, free radicals are converted into other radicals. Thus, a general feature of the reactions of free radicals is that they tend to proceed as chain reactions. In fact, propagation of free-radical oxidation processes occurs in the case of lipids by chain reactions that consume oxygen and yield new free-radical species (peroxy radicals, ROO^\bullet) or by the formation of peroxides (ROOH) as in Eqs. (4) and (5) (Jadhav *et al.*, 1996).



The product R^\bullet and ROO^\bullet can further propagate free-radical reactions.

Lipid peroxy radicals (ROO^\bullet) initiate a chain reaction with other molecules, resulting in the formation of lipid hydroperoxides and lipid free radicals. This reaction, when repeated many times, produces an accumulation of hydroperoxides. The propagation reaction becomes a continuous process as long as unsaturated lipid or fatty acid molecules are available.

The chain-propagating system here involves a bimolecular reaction of a radical with a molecule. Since lipid radicals are also highly reactive, they can easily undergo propagation reactions by two mechanisms: by reaction with an oxygen molecule in the triplet ground state (Eq. 4) or by removal of a hydrogen atom (Eq. 5). In essence, this reaction leads to the formation of a peroxy radical (ROO^\bullet) whose concentration becomes greater than that of R^\bullet in most food systems containing oxygen. The formation of hydroperoxide in reaction (Eq. 5) is conducive to the radical initiation steps shown earlier (Jadhav *et al.*, 1996).

C. Termination

A free radical has been defined as a molecular entity possessing an unpaired electron. Free radicals are electrically neutral, and salvation effects are generally very small. They are considered to be bonding-deficient and hence structurally unstable. Radicals therefore tend to react whenever possible to restore normal bonding. That is why a free radical is highly reactive. When there is a reduction in the amount of unsaturated lipids (or fatty acids) present, radicals bond to one another, forming a stable nonradical compound (Eq. 6, 7, 8). Thus the termination reactions lead to interruption of the repeating sequence of propagating steps of the chain reaction (Jadhav *et al.*, 1996).



2. Factors affecting lipid peroxidation

The rate of lipid peroxidation in foods are dependent on many factors as follows:

2.1 Fatty acid compositions

Hydrogen abstraction occurs much easier in unsaturated fatty acids than in their saturated counterparts. The number, position and geometry of double bonds affect the rate of oxidation. Relative rates of oxidation for arachidonic, linolenic, linoleic acid and oleic acid are approximately 40:20:10:1, respectively. Cis acids are oxidized more readily than their trans isomers and conjugated double bounds are more reactive than non conjugated (Nawar, 1996).

2.2 Photosensitization

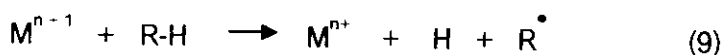
The well-known photosensitizers are chlorophyll, hematoporphyrins or flavins (including riboflavin), which are capable of converting triplet oxygen to singlet oxygen and the singlet oxygen can react with a lipid molecule to yield a hydroperoxide (Jadhav *et al.*, 1996).

2.3 Lipoxygenase

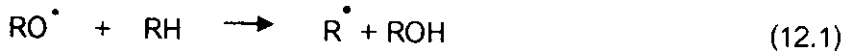
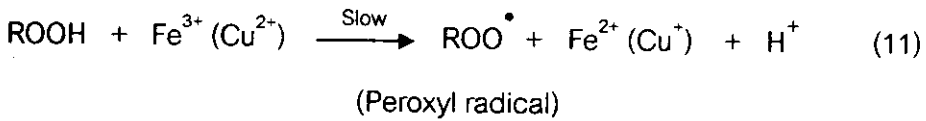
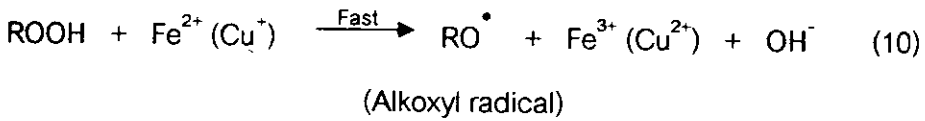
Lipoxygenase is present in spices, wheat flour and vegetables and catalyzes the oxidation of unsaturated fats in dried product and generates peroxides and volatile breakdown products. Hydroperoxide can be formed in which the reaction of polyunsaturated with oxygen is catalyzed by lipoxygenase. Free-radical intermediates occur during lipoxygenase catalysis and these can lead to cooxidation of easily oxidized compounds such as carotenoids and polyphenols (Rajalakshmi and Narasimhan, 1996).

2.4 Metals: Iron and Copper

Transition metal ions are remarkably good promoters of free-radical reaction because of singlet electron transfer during their change in oxidation states. Transition metal ions having variable oxidation number (iron Fe^{2+} or Fe^{3+} ; copper as Cu^+ or Cu^{2+} ; Mn, Ni, Co, etc.) were implicated in enhancing the rate of oxidation. A direct reaction between a metal catalyst and a lipid molecule is envisaged in the chain initiation step (Eq. 9).



In the presence of transition metal ions, the catalytic decomposition of hydroperoxides by the metal appears to be the major source of free radicals. Transition metal ions such as iron and copper can accelerate peroxidation by decomposing lipid hydroperoxide in both their lower (Eq. 10) and higher (Eq. 11) oxidation states. The alkoxy and peroxy radicals that are produced during these reactions can abstract hydrogen and perpetuate the chain reaction (Eq. 12) of lipid peroxidation (Jadhav *et al.*, 1996).



2.5 Other factors

A. Heat treatment

The rate of oxidation increases with temperature. Oxidation generally proceeds via the initial formation of hydroperoxides. The high temperature can cause many isomerization and scission reaction to take place, producing a myriad of secondary or breakdown products such as epoxides, dihydroperoxides, cyclized fatty acids, dimers and with scission reactions, aldehydes and ketones are found (Jadhav *et al.*, 1996).

B. Surface area

The rate of oxidation increases in direct proportion to the surface area of the lipid exposed to air. Furthermore, as surface-volume ratio is increased, a given reduction in oxygen partial pressure becomes less effective in decreasing the rate of oxidation (Nawar, 1996).

C. Moisture

The rate of oxidation depends strongly on water activity. Foods with very low moisture content (a_w value of less than about 0.1) undergo oxidation very rapidly. Increasing the a_w to about 0.3 retards lipid oxidation and often produces a minimum rate. This protective effect of small amount of water is believed to occur by reducing the catalytic activity of metal catalysis, by quenching free radicals, and/or by impeding

access of oxygen to the lipid. At somewhat higher water activities ($a_w = 0.55-0.85$), the rate of oxidation increases again, presumably as a result of increased mobilization of catalysts and oxygen (Nawar, 1996).

D. Radiation

Radiation generates radicals, including hydroxyl radical. The measurement of one or more of the products resulting from the radiation-generated hydroxyl radical on DNA can serve as an index of oxidative damage to DNA in the whole organism or in isolated cells and also that of the prooxidant state. The free-radical-induced modified base in DNA is detectable using a combination of gas chromatography, mass spectrometry and selected ion monitoring (Jadhav *et al.*, 1996).

3. Antioxidants

Antioxidants are a group of chemicals effective in extending the shelf-life of a wide variety of food products. The use of antioxidants was extended to a wide variety of food products including high-fat foods, cereal and even products containing very low levels of lipids. In general, antioxidants function by reducing the rate of initiation reaction in the free-radical chain reactions and are functional at very low concentration, 0.01% or less (Rajalakshmi and Narasimhan, 1996).

Lipid peroxidation is a free-radical chain reaction that causes a total change in the sensory properties and nutritive value of food products. Changes in color, texture, odor and flavor; loss of vitamins and damage of proteins are some of the effects of lipid peroxidation. The onset of lipid peroxidation can be delayed by the addition of antioxidants (Rajalakshmi and Narasimhan, 1996).

The use of antioxidants in food products is governed by regulatory laws of the country or by international standards. Even though many natural and synthetic compounds have antioxidant properties, only a few of them have been accepted as "generally recognized as safe" (GRAS) substances for use in food products by international bodies such as the Joint FAO/WHO Expert Committee for Food Additives

(JECFA) and the European Community's Scientific Committee for Food (SCF). Table 1 presents the antioxidants permitted for use in food products.

Table 1 Antioxidants permitted in foods

Ascorbic acid, sodium, calcium salts	Glycine
Ascorbyl palmitate and stearate	Gum gyaiaic
Anoxomer	Lecithin
Butylated hydroxyanisole	Ionox – 100
Butylated hydroxytoluene	Polyphosphates
<i>tert</i> – Butyl hydroquinone ^a	Propyl, Octyl and dodecyl gallates
Citric acid, stearyl and isopropyl esters	Tartaric acid
Erythroic acid and sodium salt	Thiodipropionic acid, dilauryl and Distearyl esters
Ethoxyquin	Tocopherols
Ethylenediaminetetraacetic acid and Calcium disodium salt	Trihydroxy butyrophenone

^aNot permitted for food use in European Economic Community countries

Source: Rajalakshmi and Narasimhan (1996)

3.1 Classification of food antioxidants

3.1.1 Primary antioxidants

Primary antioxidants terminate the free-radical chain reaction by donating hydrogen or electron to free radicals and converting them to more stable products. They may also function by addition in reactions with the lipid radicals, forming lipid-antioxidant complexes. Many of the naturally occurring phenolic compounds like flavonoids, eugenol, vanilin and rosemary antioxidant also have chain-breaking properties. Primary antioxidants are effective at very low concentrations but at higher levels they may become prooxidants (Rajalakshmi and Narasimhan, 1996).

3.1.2 Synergistic antioxidant

Synergistic antioxidants can be broadly classified as oxygen scavengers and chelators. They may act as hydrogen donors to the phenoxy radical, thereby regenerating the primary antioxidant. Hence phenolic antioxidants can be used at lower levels if a synergist is added simultaneously to the food product. Oxygen scavengers such as ascorbic acid, ascorbyl palmitate, sulfites and erythorbates react with free oxygen and remove it in a closed system (Rajalakshmi and Narasimhan, 1996). Chelators like ethylene diaminetetraacetic acid, citric acid and phosphates are not antioxidants but they are highly effective as synergists with both primary antioxidants and oxygen scavengers (Rajalakshmi and Narasimhan, 1996).

3.1.3 Secondary antioxidants

Secondary or preventive antioxidants such as thiopropionic acid and dialkyl thiodipropionate function by decomposing the lipid peroxides into stable end products (Rajalakshmi and Narasimhan, 1996).

3.1.4 Miscellaneous antioxidants

Compounds listed under miscellaneous antioxidants such as flavonoids and related compounds and amino acids function as both primary antioxidants and synergists. Nitrites and nitrates, which are used mainly in meat curing, probably function as antioxidants by converting heme proteins to inactive nitric oxide forms and by chelating the metal ions, especially nonheme iron, copper and cobalt that are present in meat. β -carotene and related carotenoids are effective quenchers of singlet oxygen and also prevent the formation of hydroperoxides. Zinc strongly inhibits lipid peroxidation at the membrane level, possibly by altering or preventing iron binding. Selenium is necessary for the synthesis and activity of glutathione peroxidase, a primary cellular antioxidant enzyme. Glucose oxidase and catalase function by removing dissolved or headspace oxygen and preventing the accumulation of hydrogen peroxide, respectively (Rajalakshmi and Narasimhan, 1996).

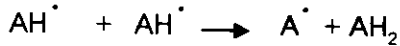
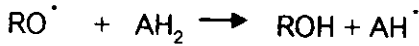
3.2 Mode of action of antioxidants in food

Antioxidants have different modes of action. Several antioxidants function as discussed below.

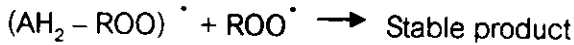
3.2.1 Radical scavenger

Two different mechanisms as radical scavengers have been proposed for antioxidant: either as hydrogen donors or as electron donor that form charge-transfer complexes (Namiki, 1990; Osawa, 1994).

Hydrogen donor



Electron donor



Antioxidants may either delay or inhibit the initiation step by reacting with a fat free radical or inhibit the propagation step by reacting with the peroxy (ROO^\cdot) or alkoxy (RO^\cdot) radicals. Reaction of an antioxidant with free radical results in the formation of a free phenoxy (A^\cdot) radical (Rajalakshmi and Narasimhan, 1996). The phenoxy radicals are stabilized by delocalization of the unpaired electron in the aromatic ring and are further stabilized by bulky group at the ortho position (Figure 1) (Shahidi and Wanasundara, 1992).

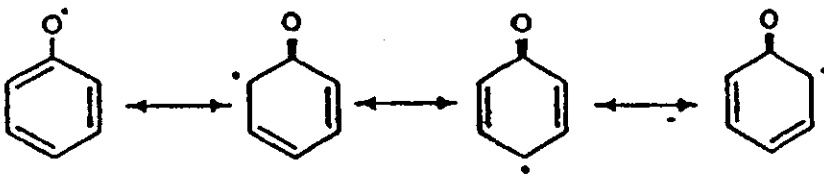


Figure 1 Delocalization of the unpaired electron in the aromatic ring of phenoxy radicals

Source: Shahidi and Wanasundara (1992)

3.2.2 Peroxide decomposer

Some phenols, amine, dithiopropionic acid and thiopropionic acid function by decomposing the lipid peroxide into stable end products such as alcohol, ketone and aldehyde (Dziezak, 1986; Namiki, 1990).

3.2.3 Singlet oxygen quenchers

Singlet oxygen is generated from the triplet state oxygen. The mechanism of conversion the triplet oxygen to singlet oxygen is initiated by the transfer of the photosensitizer to its electronically excited state due to the absorption of light in the visible or near-UV region. Subsequently, the photosensitizer is able to transfer its excess energy to an oxygen molecule, giving rise to singlet oxygen (Shahidi and Wanasundara, 1992). Thus, the singlet oxygen can react with a lipid molecule to yield a hydroperoxide. Singlet oxygen reacts about 1,000-10,000 times as fast as normal oxygen with methyl linoleates (Jadhav *et al.*, 1996). Lipid oxidation initiated by xanthine oxidase can be inhibited by β -carotene because of its ability to quench singlet oxygen (Rajalakshmi and Narasimhan, 1996; Namiki, 1990). β -carotene and related carotenoids are effective quencher of singlet oxygen and can act as antioxidants by preventing the formation of hydroperoxides (Jadhav *et al.*, 1996).

3.2.4 Enzyme inhibitor

Lipoxygenase (LOX; linoleate oxygen oxidoreductase, EC 1.13.11.12) is a non-heme iron-containing enzyme that catalyzes the oxygenation of the 1,4-pentadiene sequence of polyunsaturated fatty acid to produce their corresponding hydroperoxide (Salas *et al.*, 1999). Free-radical intermediates occur during lipoxygenase catalysis, and these can lead to cooxidation of easily oxidized compounds, e.g. carotenoids and polyphenols (Rajalakshmi and Narasimhan, 1996).

3.2.5 Synergists

A. Chelating agents

Chelating agents are not antioxidants, however, they play a valuable role in stabilizing foods. Chelating agents that improve the shelf life of lipid-containing food are EDTA, citric acid and phosphoric acid derivatives. Chelating agents form stable complexes with pro-oxidant metals such as iron and copper. Chelating agents bind metal ions and forms sigma bonds with a metal. It is considered as an effective secondary antioxidant because of the stabilized oxidation form of the metal ion. An unshared pair electrons in their molecule structure promotes the chelating action (Dziezak, 1986; Rajalakshmi and Narasimhan, 1996; Jadhav *et al.*, 1996)

B. Reducing agents or oxygen scavengers

Reducing agents or oxygen scavengers function by various mechanisms. They may act as hydrogen donors to the phenoxy radical, thereby regenerating the primary antioxidant or react with free oxygen and remove it in a closed system (Giese, 1996; Rajalakshmi and Narasimhan, 1996). Ascorbic acid is a strong reducing agent, readily losing H^+ to become dehydroascorbic acid, which also has vitamin C activity. However, vitamin C activity is lost, when the lactone ring of dehydroascorbic acid is hydrolyzed to yield diketogluconic acid. Ascorbic acid acts as an antioxidant by inhibiting radical formation at double bonds of mono or polyunsaturated fatty acids, quenching free radicals, scavenging oxygen and serving as a reductant (Cort, 1982; Cabelli and Bielski, 1983). Ascorbic acid is a good antioxidant for peroxidations initiated in the aqueous phase, but does not trap peroxy radicals in the lipid phase (Doba *et al.*, 1985).

3.3 Ideal food grade antioxidant

Food grade antioxidants should possess a high antioxidant activity and provide other advantages without retarding or destroying the food quality. Coppen (1983) suggested the characteristics of the ideal food grade antioxidants as follows:

- A. Safe
- B. Not impart color, odor, or flavor to the fat even on long storage
- C. Effective at low concentration
- D. Easy to incorporate
- E. Effective for at least 1 year at a temperature of 25-30 °C
- F. Stable to heat processing and protect the finished product (carry-through effect)
- G. Low cost

4. Sources of food antioxidants

4.1 Synthetic antioxidants

Synthetic antioxidants are mainly phenolic such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butyl hydroquinone (TBHQ), and propyl, octyl, and dodecyl gallates. The differences in their antioxidant activity are related to their physical properties such as volatility, solubility and thermal stability. In general, the use of primary antioxidants (BHA, BHT, or TBHQ) is limited to 100-200 ppm, and gallates are used at levels up to 200-500 ppm for the stabilization of fat and oils (Rajalakshmi and Narasimhan, 1996).

4.2 Natural antioxidants

In recent years, consumers and food manufacturers have been interested in products with "all natural" labels. The volume of such products increases 175% from 1989 to 1990, and the number of products claimed to be without additives or preservatives rose by 99% during the same period (Rajalakshmi and Narasimhan, 1996). Consequently, a lot of emphasis has been given to the identification and incorporation of novel natural antioxidants in food products. The area of natural antioxidants has been developed enormously in the past decade, mainly because of the increasing limitation on the use of synthetic antioxidants and enhanced public awareness of health issues. Natural antioxidants are generally preferred by consumers since they are considered safe. Rajalakshmi and Narasimhan (1996) listed some

advantages and disadvantages of natural antioxidants compared to synthetic antioxidants (Table 2).

Table 2 Advantages and disadvantages of natural antioxidants in comparison with synthetic antioxidants

Advantages	Disadvantages
<ul style="list-style-type: none"> ● Readily accepted by the consumer, as considered to be safe and not a "chemical". ● No safety tests required by legislation if a component of a food that is "generally recognized as safe" (GRAS). 	<ul style="list-style-type: none"> ● Usually more expensive if purified and less efficient if not purified ● Properties of different preparations vary if not purified. ● Safety often not known. ● Many impart color, aftertaste, or off-flavor to the product.

Source: Rajalakshmi and Narasimhan (1996)

A. Herbs and spices

Antioxidants from spices and herbs have the potential for large-scale applications. Spices have been used not only for their flavoring properties but also for their food-preserving ability. Over the past few years, a number of medicinal plants such as *Echinacea purpurea*, *Ginkgo biloba* (ginkgo) and pine bark extract (Oligopin) have been investigated for their quenching activity of specific reactive oxygen species (ROS), such as the hydroxyl radical, the superoxide anion, singlet oxygen, and lipid peroxides (Masaki *et al.*, 1995). The seeds of *Cassia tora* L. have been conventionally used in traditional Chinese medicine for several centuries. Yen and Chuang (2000) reported that methanolic extracts from *C. tora* L. exhibited a strong antioxidant activity on lipid peroxidation. This herb has been reported to contain many active components, including chrysophanol, emodin, aloe-emodin, rhein, physcion, obtusin, aurantio-obtusin, rubrofusarin, torachryson, and toralactone. Among these compounds, emodin

exhibited the strongest scavenging effect on hydroxyl radicals (Yen *et al.*, 2000). Emodin has been known to be pharmacologically potent and this characteristic was reported to be associated with its scavenging of hydroxyl radical (Huang *et al.*, 1991). Spices are known for both its antioxidative and microbial properties. Cheah and Gan (2000) reported that the aqueous extracts of galangal (*Alpinia galanga*) inhibited lipid oxidation and microbial growth in minced beef. Aruoma *et al.* (1996) reported that an extract from a mixture of aromatic spices (rosemary, sage, thyme and oregano), called Spice Cocktail Provençal effectively stabilized animal fats and frozen fish products. Rosemary and sage had very high antioxidative activity in lard, while clove was the most effective spice in oil-in-water emulsion. In French dressing and mayonnaise (oil-in-water emulsion), oregano was found to be the most potent spice in retarding oxidative deterioration (Chipault *et al.*, 1995). Lagouri and Boskou (1996) demonstrated the presence of tocopherols, mainly γ -tocopherol, in the non-polar fraction obtained from oregano by hexane extraction. Tocopherols are best known as efficient naturally occurring liposoluble antioxidants and its defence against reactive oxidative species (Diplock, 1992).

B. Cereals and oil seed

Active compounds were detected in hulls from peanut, mung bean and buckwheat. Mung bean (*Phaseolus aureus*) is a leguminous seed, and its hulls have been demonstrated to possess antioxidant activity, which has a reducing power and scavenges DPPH radicals (Duh *et al.*, 1997). Navy bean (*Phaseolus vulgaris*) hull extract was a stronger antioxidant than BHA and BHT, at similar concentrations, in soybean and sun flower oils (Onyeneho and Hettiarachchy, 1991). Hydrophilic phenolic extract of pea bean (*Phaseolus vulgaris*) showed strong antioxidant activity while its hydrophobic fraction possessed a weak activity (Tsuda *et al.*, 1993). The bran extract has been reported to have more antioxidant activity than the extracts from durum wheat or from coat of tamarind seeds, with strong oxidation-inhibiting activity, whereas no activity was detected in the germ (Moure *et al.*, 2001). Rice bran has been reported

to inhibit pancreatic lipase *in vitro*. This action indicates that administration of rice bran to animals may cause a reduction in plasma triglycerides and suppression of fat accumulation (Tsutsumi, 2000). Corn kernel was found to contain the antioxidant compound (Kurilich and Juvik, 1999). Malt and barley have shown antioxidant properties mainly due to the presence of phenolic compounds, especially flavonoids and hydroxycinnamic acids (Maillard *et al.*, 1996).

Soybean broth possessed good antioxidant properties, including scavenging abilities on DPPH and hydroxyl radicals and chelating abilities on both ferrous and cupric ions (Esaki *et al.*, 1999). As compared to soybean broth, fermented soybean broth exhibited comparable antioxidant activity and ferrous chelating capability, and better scavenging effects on DPPH[•], superoxide anion and hydroxyl free radical, but less cupric chelating capability. Soybean broth and fermented soybean broth both acted as both primary antioxidant and oxygen scavengers (Yang *et al.*, 2000). In addition, Esaki *et al.* (1999) reported that fermented soybean products have been found to be more stable against lipid peroxidation than unfermented soybeans. Free isoflavones such as daidzein (7,4'-dihydroxyisoflavone) and genistein (5,7,4'-trihydroxyisoflavone) that are produced during fermentation by microorganisms are generally known to be antioxidative components in these products.

During the extraction of oil from oilseeds, the antioxidant compounds present in the hulls could be incorporated in the oil, as reported for peanut oil extracted from the coated seeds, which contained higher oxidative stability than the oil from dehulled seeds (Shahidi *et al.*, 1997).

C. Fruits and vegetables

Fruits and vegetables contain many different antioxidant components. The consumption of fruits and vegetables has been associated with low incidences and mortality rates of cancer and heart disease (Wang and Lin, 2000). The phytochemicals in plant tissues responsible for the antioxidant capacity can largely be attributed to the phenolics, anthocyanins, and other flavonoid compounds (Cao *et al.*, 1997). Blackberries, raspberries, and strawberries are good sources of natural antioxidants.

Extracts of berry fruits from several cultivars showed a remarkably high scavenging activity toward chemically generated superoxide radicals. In addition, berry fruits were shown to be effective in inhibiting oxidation of human low-density lipoprotein, which may have potential health effects (Heinonen *et al.*, 1998). Grapes, wines, and grape byproducts contain large amounts of phenolic compounds, mostly flavonoids, at high concentrations of 1000-1800 mg/mL. Some of these compounds may act at very low concentrations to inhibit LDL oxidation *in vitro* (Frankel *et al.*, 1993). Fuhrman *et al.* (1995) found that chronic red wine consumption (400 mL/day) reduced the susceptibility of LDL to undergo lipid peroxidation catalyzed by copper. One of the major polyphenols in wine is known to be catechins. Red wine contained substantial quantities of catechins, whereas low to negligible amounts were found in white wine and commercially available fruit juices (Arts *et al.*, 2000).

Many aromatic plants, especially citrus fruits, and their essential oils are used as flavoring agent in a wide range of food. They have been known to support various biological activities such as antimicrobial and antioxidant properties. Choi *et al.* (2000) reported that the radical scavenging activity of citrus essential oils from lemon was highest, meanwhile Tahiti lime and Eureka lemon oil were also strong radical scavengers. The higher efficiency may have been caused by a higher content of terpenes with the exception of limonene and myrcene. Radical scavenging activity was found to be high when the terpenes included a higher content of γ -terpinene and terpinolene.

Burdock, a vegetable which is consumed and has been used in beverages in China for centuries, has been reported to have antioxidant property. Addition of burdock extracts resulted in lower malondialdehyde in both linoleic acid and liposome model system, compared to the control (Duh, 1998).

D. Leaves

Several reports have shown that olive leaf extract had the capacity to lower blood pressure in animals and increased blood flow in the coronary arteries (Zarzuolo, 1991). The bitter compound oleuropein, the major constituent of the secoiridoid family in

the olive (*Olea europaea* L.) trees, has been shown to be a potent antioxidant endowed with anti-inflammatory properties. For oleuropeosides and the other phenols present in olive leaf extract, it is mainly the *o* - dihydroxy (catechol) structure present in their moieties which confers antioxidant properties to these compounds (Benavente-García *et al.*, 2000). The extract from young green barley leaves (*Hordium vulgare* L.) possessed antioxidant activity and the active component was identified as 2''(3'')-*O*-glycosylisovitexin (Osawa *et al.*, 1992). The antioxidant activity of mulberry leaves (*Morus alba* L.) was slightly less than of BHA but was stronger than that of α -tocopherol and the major components were identified as β -carotene and α -tocopherol (Yen *et al.*, 1996). Lai *et al.* (2001) reported that hsian-tiao (*Mesona procumbens* Hemsl) leaf gum can be attributed as an electron donor that could react with free radicals, and convert them to more stable products, and terminate radical chain reactions. Moreover, it also has scavenging activity on DPPH and superoxide radical.

E. Agricultural and industrial residues

Agricultural and industrial residues are attractive sources of natural antioxidants. Koga *et al.* (1999) reported that potato peel waste, rape of olive, olive mill waste waters, grape seeds and grape pomace peels could be used as cheap sources of antioxidants. Identification of polyphenolic compounds from apple pomace, grape pomace, citrus seeds and peels, carrot pulp waste, old tea leaves, cocoa by-products, non-volatile residue from orange essential oil and soy bean molasses has also been reported (Moure *et al.*, 2001). Spent ground coffee oil from the residue from the production of instant coffee was also used as an antioxidant for food preservation and for aroma stabilization. The antioxidant activity was due to the 5-hydroxytryptamide carboxylic acids (10-75% dry wt. of the product) (Moure *et al.*, 2001).

F. Enzymatic antioxidant (Kawakami *et al.*, 2000)

The following are a few examples as enzymatic scavengers in plants:

- Superoxide dismutase (SOD) reacts with superoxide radicals ($O_2^{\cdot -}$) and converts them to O_2 and H_2O_2

- Catalase (CAT) detoxifies H_2O_2 into water
- Ascorbate peroxidase (APx) scavenges H_2O_2 by using ascorbic acid

as an electron donor

G. Tea

Tea beverage has been considered to be "functional food" in China for centuries. The tea plant is an evergreen laurel tree and is taxonomically classified as *Camellia sinensis* (L.) O. Kuntze of the family of *Theaceae* (Chu, 1997). Tea is prepared from the leaves of two-defined varieties of the tea plant *Camellia sinensis*: *assamica* and *sinensis* (Wiseman *et al.*, 1999). *C. sinensis* var. *assamica*, whose leaf is large (leaf length and width, 16 – 19 x 7 – 9 cm) and trunk is tall, are in the areas ranging from Yun-nan province of China to the northern region of Myanmar and Assam region of India. On the other hand, that of var. *sinensis*, whose leaf is small (leaf length and width, 5.5 – 6.1 x 2.2 – 2.4 cm) and the trunk is bush type, are observed in the eastern and southeastern districts of China. The cultivars of var. *sinensis* can survive in winter as cold as $-12^{\circ}C$, whereas those of var. *assamica* perish at $-4^{\circ}C$ in a few weeks. Therefore, the former cultivars have been cultivated in the temperated regions and the latter grows in the tropical and subtropical regions (Chu, 1997).

The manufacturing of tea

The processed tea leaves are classified into three types according to the degree of fermentation: unfermented, semi-fermented, and fully-fermented tea leaves, and they are called green tea, oo-long tea, and black tea, respectively (Chu, 1997).

1. Green tea

For green teas production, the harvested fresh leaves are immediately steamed at $95-100^{\circ}C$ for 30-45 sec to inactivate the enzymes, especially polyphenol oxidase. The steaming treatment protects degradation of vitamins and thus, the content of vitamins in green tea is much higher than fermented teas. The steamed leaves are then rolled to make a slender pickle form followed by drying in current air of moderate temperature. The moisture content of fresh leaves that is usually 78-80%, decrease to

about 10% during the rolling process. The rolling process disrupts the leaf tissue and mixes them uniformly in the shape as flat as possible. The drying subsequently applied increases aroma and preserves the products. The rolled and dried leaves called Aracha are finally fired (roasted) and cut to prepare the final products by tea dealers. The firing treatment is also important to remove the original coarse aroma and add a fired tea flavor (Chu, 1997).

One of the most important processes in tea manufacturing for drink is fermentation. It is known that the conversion of tannin in tea leaves is not achieved by microorganisms but by enzymes present in the leaves, and so, this phenomenon should be called "enzymation". The degree of fermentation greatly affects the quality and type of tea. The fermentability (activity of polyphenol oxidase) and content of polyphenols in young tea leaves greatly differ depending on the kind of cultivars. Those cultivars which show better fermentability and higher content of polyphenols are evaluated to be a good quality for black tea manufacture. Almost all of the cultivars belonging to the var. *sinesis* group have relatively low fermentability and less content of polyphenols (Chu, 1997). The processing of the fresh tea leaf is associated with significant changes in flavonoid composition (Figure 2).

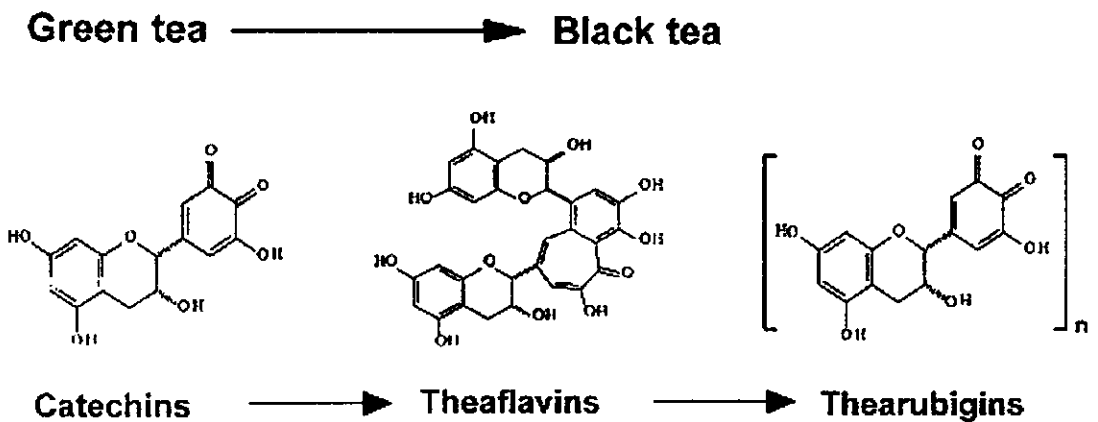


Figure 2 Chemical conversion of catechins during tea processing

Source: Wiseman *et al.* (1999)

2. Semifermented or Oolong tea

Plants of var. *sinesis* are used for this type of tea with a wide range of leaf characters.

Fresh shoots are withered (reduces the moisture content of the leaves from 75-80 % to 55-70 %) by spreading out on bamboo mats for 30-60 min in sunlight. The withering then continues on a floor in a room for 6-8 h with gentle agitation once an hour. The leaves ferment during withering and develop a red color. The fermentation is stopped by heating the leaves in a pan at 250-300 °C for 15 min. The leaves are then rolled and finally dried (Willson, 1999).

3. Fermented or Black tea

Unlike green or oolong teas, black teas undergo a full fermentation process which causes the leaves to turn black. This is what gives the leaves their characteristic flavor.

Teas are plucked only from the top two leaves and the bud. After plucking, the leaves are brought to the drying sheds about 16-17 h and allowed to withered by exposing it to the air. This reduces the moisture content by about 40% and then the leaves are rolled twice (45 min in each time) and keep at temperature 20-26 °C for 2-3 h to be fermented. This fermentation, which is completely stopped in Green Tea, turns the tea leaves darker and darker and gives them their black color (hence the name "Black" Tea). Then the leaves are quickly dried for stopping the fermentation and making the tea shelf life stable. For drying process, it has been twice drying. First: drying at 80-90 °C for 15 min and second: drying at 75 °C for 20 min. The world's finest black teas are produced in India (Assam, Darjeeling and Nilgiri), Sri Lanka (Ceylon) and China (Fukatsu, 1992).

Composition of tea

A unique feature of tea leaves is that they contain significant quantities of polyphenolic phytochemicals termed flavonoids. Flavonoids are distributed widely in plant foods such as vegetables and fruits. They possess a unique C6-C3-C6 structure (diphenylpropane structure) with phenolic OH groups (Figure 3). Flavonoids can be classified as calcones, flavone, flavanones, flavanols, and flavonols (Terao, 1999). More than 30% of the dry weight of the tea leaf comprises flavonoid compounds with the predominant form being the flavan-3-ols or catechin (Wiseman *et al.*, 1999). Catechins are distributed widely in plants; however, they are only rich in tea leaves, where catechins may constitute up to 25% of dry leaf weight (Pietta and Simonetti, 1999). Tea catechins are flavanol-type flavonoids in which phenolic OH groups are bound to the 5 and 7 positions (Terao, 1999). Flavanols, a subclass of flavonoids found in foods in nearly as high a concentration as the anthocyanidins, are characterized as catechin, epicatechin, and similar compounds. These flavonoids are found in a limited number of foods and beverages. It is interesting to note that black tea contains about one-half the concentration of flavanols as green tea (Table 3). This may be due to the oxidative formation of flavanol dimers, oligomers, and polymers during the processing of black tea (Beecher, 1999).

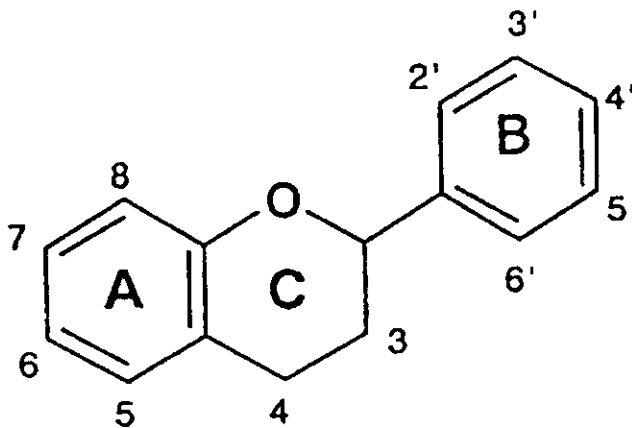


Figure 3 Basic structure of flavonoid

Source: Terao (1999)

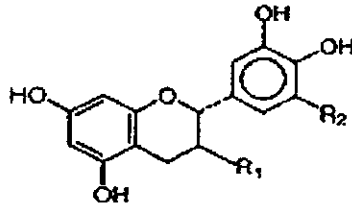
Table 3 Flavanol content of selected beverages

Beverage	Total flavanol ^a (mg/100 ml)
Tea, black	9 – 349
Tea, green	16 – 719
Wine, red	5 – 19

^a Total flavanol is the sum of the concentrations of epicatechin, epigallocatechin, epicatechin-3-gallate, and epigallocatechin-3-gallate

Source: Beecher (1999)

Wiseman *et al.* (1999) reported that the fresh tea leaf contain six major catechins, including (+)-catechin, (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (+)-gallocatechin, (-)-epigallocatechin (EGC), and (-)-epigallocatechin gallate (EGCG) (Figure 4). Young tea leaves contain the highest catechin concentrations and the leaf bud and the first leaf are generally rich in epigallocatechin gallate (Bhatia and Ullah, 1968). Catechins are colorless, water-soluble compounds that function as secondary metabolites in the tea leaf, possibly as inhibitors of bacterial and viral infestation due to their astringent properties (Wiseman *et al.*, 1999).



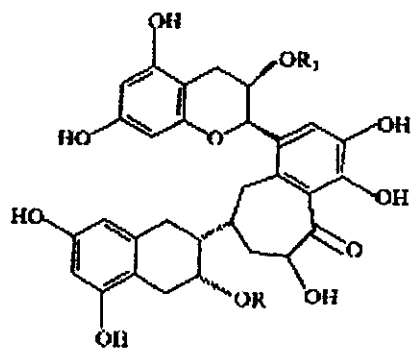
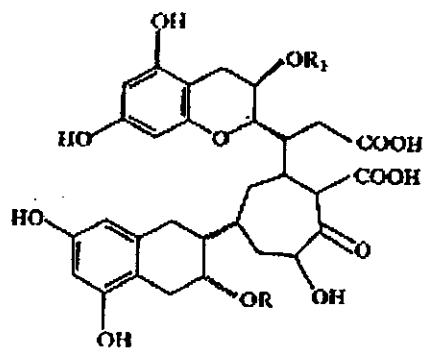
Catechins

Compound		R ₁	R ₂	M.W.
Epicatechin	EC		H	290.3
Epigallocatechin	EGC		OH	306.4
Epicatechin-gallate	ECg		H	442.4
Epigallocatechin-gallate	EGCg		OH	458.4
Catechin	C		H	290.3

Figure 4 Structure of catechins

Source: Pietta and Simonetti (1999)

Oxidation of the colorless catechin molecules results initially in the formation of catechin quinones, which can undergo complex condensation and coupling reactions to form a range of orange-yellow to reddish-brown colored flavonoids and several volatile constituents (Graham, 1992). The best known fermentation products are the thearubigin, theaflavin, and related compounds (Figure 5).



Thearubigin and related compounds

Compound	R	R ₁
Thearubigin	H	H
Thearubigin-3-gallate	galloyl	H
Thearubigin-3'-gallate	H	Galloyl
Thearubigin-3,3'-digallate	galloyl	Galloyl

Theaflavin and related compounds

Compound	R	R ₁
Theaflavin	H	H
Theaflavin-3-gallate	galloyl	H
Theaflavin-3'-gallate	H	galloyl
Theaflavin-3,3'-digallate	galloyl	galloyl

Figure 5 Structures of thearubigin, theaflavin, epitheaflavin, and related compounds

Source: Pietta and Simonetti (1999)

H. Mulberry leave

Mulberry belongs to the genus *Morus* of the family Moraceae. Koidzumi (1917) classified the genus *Morus* into 24 species and one subspecies. Mulberry is distributed in a wide area of tropical, subtropical, temperate and sub-Arctic zones. Most of mulberry varieties cultivated in Japan belong to *Morus bombysis* Koidz, *M. alba* and *M. latifolia* Poir. Varieties belonging to *M. bombysis*, have lobed and shallow bottom leaves. Branches are brown or gray, and the winter buds are oval and sharp-pointed. Varieties of *M. alba* have lobed/unlobed leaves with whitish-gray or grayish-brown branches. *M. latifolia* varieties have large, unlobed, lustrous leaves and greenish-gray or whitish-gray branches. "Ichinose" and "Kairyo-nezumigaeshi" most widely cultivated in Japan, are *M. alba* varieties, and "Kenmochi", intensively cultivated in cold areas, is a variety of *M. bombysis*.

Composition of mulberry leaves

Mulberry leaves are particularly rich in calcium, potassium, sodium, phosphorous, and magnesium. Apart from a high ascorbic acid content, mulberry leaves also contain carotene, vitamin A, B1, B2, D, E, and folic acid. The presence of glutathione, gamma-amino butyric acid, lutein, quercetin and phytosterols have been reported in these leaves, lending to explain mulberry's other beneficial ability: decrease cholesterol and blood pressure levels. Seventeen of the 20 amino acids are likewise found in mulberry leaves. Mulberry is also a very rich source of flavonoids, anthocyanins and insoluble and soluble fiber.

Mulberry (*morus alba*) leaves, a maple-looking, are from the mulberry tree (Figure 6). In nature, mulberry leaves is the silkworm's exclusive food, but in Japan and other parts of Asia, people are using it to take advantage of its blood glucose, cholesterol, and blood pressure regulating properties (Asano, 2001).

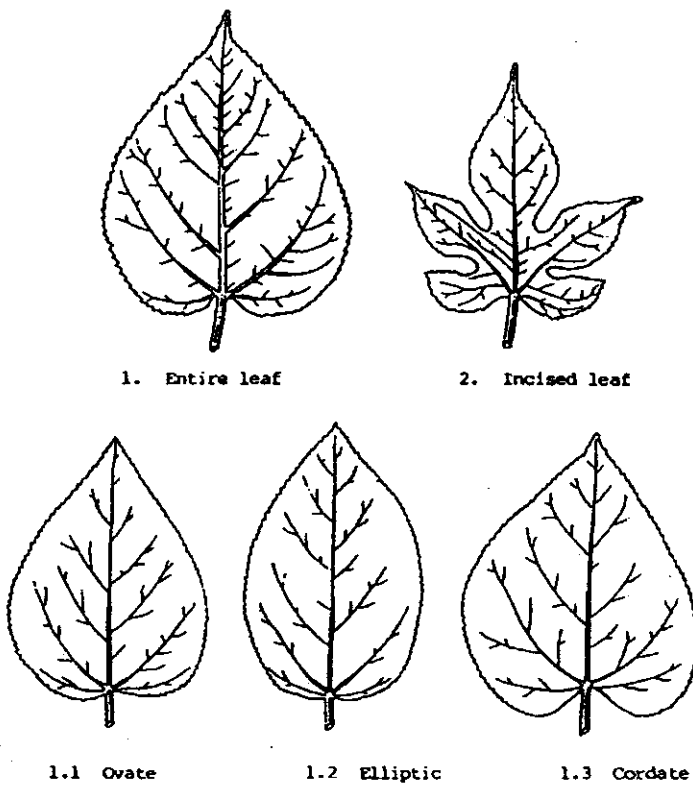


Figure 6 Shape of mulberry leaves

Source: Zheng *et al.* (1988)

Chen (1995) reported that the DNJ (1-deoxynojirimycin) was found in abundant form only in the mulberry plant and shown potent anti-hyperglycemic effect in diabetic and hyperglycemic mice. Suppressing this activity weakens digestion of sugars, thereby preventing the sudden increase in blood sugar level after meals. This is also the primary mechanism in reducing the onset and further complications of diabetes (Kojima, 2001). This mechanism also helps to explain mulberry's benefits in weight loss, as excess sugars are not absorbed into the body to cause weight gain, but eliminated from the body.

Yen *et al.* (1996) reported that the antioxidants β -carotene and α -tocopherol were identified as large components in mulberry leaves and it has been ability to prevent lipid (fats) peroxidation. In addition, Doi *et al.* (2000) reported that the presence of phytosterols and antioxidants such as lutein and quercetin help to explain mulberry leaf's ability to break down blood-vessel-clogging cholesterol and fat, and modulate blood pressure.

5. Extraction of antioxidants from natural plants

Solvent extraction is more frequently used for isolation of natural antioxidative substances. Both extraction yield and antioxidant activity of extracts are strongly dependent on the solvent, due to the different antioxidant potential of compounds with different polarity (Julkunen-Titto, 1985; Marinova and Yanishlieva, 1997). Apolar solvents are among the most employed solvents for removing polyphenols from water. Ethanol and water are the most widely employed solvents for hygienic and abundance reasons, respectively. Since the activity depends on the polyphenol compounds and the antioxidant assay, comparative studies for selecting the optimal solvent providing maximum antioxidant activity are required. Less polar solvents such as ethyl acetate, provided slightly more active extracts than mixtures with ethanol or methanol, or methanol alone for tamarind seed coats (Tsuda *et al.*, 1994b) although ethanol and methanol extracts also presented high lipid peroxidation-inhibiting activity, comparable to α -tocopherol. Tsuda *et al.* (1994a) reported that hydrophilic phenolic

extract of pea bean (*Phaseolus vulgaris*) showed strong antioxidant activity while its hydrophobic fraction possessed a weak activity.

Duh (1998) reported that the burdock roots water extracts (regardless of the temperature used) yielded the greatest amount of extract and exhibited the strongest antioxidant activity. Moure *et al.* (1999) reported that the polyphenol extraction yield was higher for the more polar solvents for extracts from *Gevuina avellana* hulls. In addition, the antioxidant activity of buckwheat extracts varied with the polarity of the solvent. Those extracted with methanol was the most active (Przybylski *et al.*, 1998). Ramarathnam *et al.* (1988) reported that antioxidative activities of the methanol extracts of rice hull showed stronger than BHA and α -tocopherol.

Cuvelier *et al.* (1996) investigated the antioxidant activities of 32 pilot-plant and commercial extracts from rosemary and sage isolated with hexane, CO₂, ethanol and found significant difference between antioxidant activities of the extracts obtained even by the same solvent. These differences could depend on synergism and antagonism between extracted phenolic acid, diterpenoids and flavones, present together in the extract. Economou *et al.* (1991) concluded that acetone was the most efficient solvent for extraction of the antioxidative materials from sage and rosemary.

6. Some properties of antioxidants from plants

6.1 Heat stability

The temperature, during drying and extraction, affects the compound stability due to chemical and enzymatic degradation, losses by volatilization or thermal decomposition (Ibáñez *et al.*, 1999). These latter have been suggested to be the main mechanism causing the reduction on polyphenol content (Larrauri *et al.*, 1997). The effect of temperature has been studied in spray-drying of carrot pulp waste (Chen and Tang, 1998). Larrauri *et al.* (1997) found a significant reduction in extractable polyphenols and condensed tannins when red grape pomace peels were dried with air at 100 °C or higher. Drying at 100 °C and 140 °C caused a reduction in the complex group of different substances (phenolic acids, anthocyanins, flavonols, flavan-3-ols, and flavanonols) by 18.6% of 32.6 %, respectively (Larrauri *et al.*, 1997).

6.2 Effect of pH

The effect of pH on antioxidant activity has also been reported. Baublis *et al.* (2000) reported increased antioxidant activity of aqueous fractions from wheat bran after treatment at acidic conditions, probably due to altered phenol composition. For aqueous extraction of antioxidant from oat fibre, the highest yield was attained at pH 6 and the highest antioxidant activity was observed at pH 10 (Lehtinen and Laakso, 1998). At alkaline pH, the fractions with high protein and fatty acid contents are solubilized and the antioxidant activity was probably carried by the protein-rich fraction. Cilliers and Singleton (1990) demonstrated ring-opening during alkaline oxidative conditions, as in non-enzymatic reactions involving polyphenolic compounds in food systems, and identified the resulting products as compounds analogous to natural lignans and neolignans. Zhu *et al.* (1997) reported that (-)-epigallocatechin gallate (EGCG) and (-)-epigallocatechin (EGC) were preferentially destroyed at pH > 6.5 (Figure 7). In contrast, (-)-epicatechin (EC) and (-)-epicatechin gallate (ECG) were more stable in both acidic and alkaline pH. EGCG and ECG have a similar backbone except for an additional hydroxyl group at position 5' in the former. By a similar observation, the structures of EGC and EC are the same except for an additional hydroxyl group at position 5' in EGC. Perhaps, the three adjacent hydroxyl groups at position 3', 4', and 5' in EGCG and EGC were more vulnerable to destruction than the two adjacent hydroxyl groups at position 3' and 4' in ECG and EC.

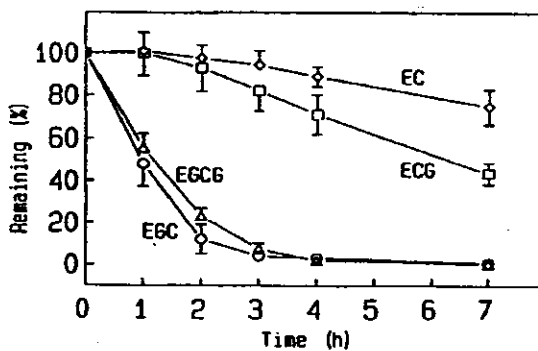


Figure 7 Stability of individual epicatechin isomers in sodium phosphate buffer (pH = 7.4). Data are expressed as mean \pm SD of $n = 5$ samples

Source: Zhu *et al.* (1997)

6.3 Effect of concentration

The antioxidant activity depends on the extract concentration. As a general trend, increased antioxidant activity is found with increasing extract concentration, but the concentration exhibiting maximum antioxidant activity is closely dependent on the extracts. For the same extract, antioxidant activity is dependent on the activity test (Yen and Wu, 1999). Dose-response curves are different for different antioxidants. Yamaguchi *et al.* (1999) compared grape seed extract with natural antioxidants, such as tocopherol and ascorbic acid and observed different effectiveness, depending on the assay. Acetone extracts from *G. avellana* hulls at concentrations lower 1000 mg/l, showed prooxidant activity, but exhibited increased antioxidant activity with increased concentration (Moure *et al.*, 1999).

6.4 Synergistic effect

Synergistic actions between synthetic only, natural and synthetic, and natural antioxidants have been observed (Bandarra *et al.*, 1999). This effect is defined as the combined action which resulted in increased antioxidant potential more than that expected from a more additive effect (Moure *et al.*, 2001). Yi *et al.* (1991) observed that α -tocopherol and ascorbic acid acted highly synergistically with each other in a fish oil/lecithin/water system, requiring a minimum of 0.01-0.02 % ascorbic acid.

7. Mode of action of natural antioxidants

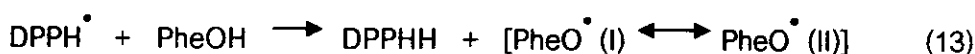
The compounds belonging to several classes of phytochemical components such as phenols and flavonoids were widely used in food products for retarding the oxidation of fats. These natural antioxidants have different modes of action, however, their main modes of action have been shown to be as free radical scavengers (Fogliano *et al.*, 1999). Two different mechanisms as radical scavengers have been proposed for natural antioxidants, either as hydrogen donor or as electron donor (Lai *et al.*, 2001).

7.1 Reducing power

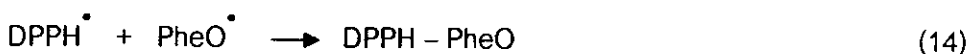
In the reducing power assay, the presence of reductants (antioxidants) in the sample would result in the reduction of the Fe^{3+} /ferricyanide complex to the ferrous form. The Fe^{2+} can therefore be monitored by measuring the formation of Per's Prussian blue at 700 nm. Increased absorbance at 700 nm indicates an increase in reducing power. Lai *et al.* (2001) reported that the reducing power of hsian-tiao (*Mesona procumbens* Hemsl) leaf gum was increased with increasing hsian-tiao leaf gum concentration. Due and Yen (1997) reported that the water extracts of three herbs, including the flower of *Chrysanthemum morifolium* Ramat at 5.0 mg, as well as the calyx of *Hibiscus sabdariffa* L. and roasted seed of *Hordeum vulgare* L. at 15.0 mg, exhibited a greater reducing power than 0.3 mg of ascorbic acid, which is a reducing agent as well as reductone (Shimada *et al.*, 1992). Yang *et al.* (2000) reported that fermented soybean broth at a concentration of 5-100 % exhibited an excellent reducing power. Yen *et al.* (2000) reported that the reducing power of active component from the seeds of *Cassia tora* L. such as anthrone and alizarin increased with an increase in concentration. However, Yen *et al.* (2000) reported that the anthraquinones, which also possessed antioxidant activity, showed almost no reducing power, indicating that reducing power of anthraquinones can not be considered as contributing to their antioxidative effect.

7.2 Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH)

The proton radical-scavenging action is known to be one of the various mechanisms for antioxidation. DPPH is one of the compounds that possess a proton free radical and shows a characteristic absorption at 517 nm (purple). When DPPH encounters proton radical scavenging, its purple color would fade rapidly (Yamaguchi *et al.*, 1998). The rate constants of the reaction of phenolic with free radicals indicate the order of reactivity (Moreno *et al.*, 1998). The main reaction is shown as follows:



Where (I), (II) are resonance structures.



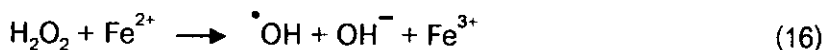
Equation (14) may occur in some cases, but it also may be forbidden depending on molecular phenolic and aromatic ring substituent volumes. Equation (15) will be complete with the PheO[•] coupling termination reaction:



Lai *et al.* (2001) reported that the scavenging effect of hsian-tsau (*Mesona procumbens* Hemsl) leaf gum on the DPPH radical was strongly concentration dependent. This result implies that the antioxidative activity of hsian-tsau leaf gum may be attributed to its proton-donating ability. Yang *et al.* (2000) reported that fermented soybean broth at a concentration of 20-100 % showed an excellent scavenging activity on DPPH radical. Thirty-four kinds of citrus essential oils shows scavenging effects on DPPH in the range of 17.7 – 64.0 % (Choi *et al.*, 2000), while soybean broth showed the scavenging activity on DPPH at concentration 100 % (Yang *et al.*, 2000).

7.3 Scavenging effect on hydroxyl radical

Flavonoids and phenols have been shown to possess an important antioxidant activity towards hydroxyl radical, which is principally based on the redox properties of their phenolic hydroxyl groups and the structural relationships between different parts of their chemical structure (Bors *et al.*, 1990). Hydroxyl radical ([•]OH) scavenging activity of flavonoids was reported in experimental systems where hydroxyl radical was generated by UV photolysis of H₂O₂. It is generally thought that hydroxyl radical is produced *in vivo* by Fenton-type reactions, in which iron ions (Fe²⁺) react with H₂O₂ (Eqs. 16) (Puppo, 1992).



Reducing agents such as ascorbic acid can accelerate $\cdot\text{OH}$ formation by re-reducing Fe^{3+} ions to Fe^{2+} (Eqs. 17):



However, some ferric chelates such as ferric-EDTA can react directly with H_2O_2 to form hydroxyl radical. The hydroxyl radical is the most toxic oxyradical that has the potential to proceed the lipid peroxidation (Puppo, 1992). Lai *et al.* (2001) reported that the addition of hsian-tsao (*Mesona procumbens* Hemsl) leaf gum to the $\text{Fe}^{2+} - \text{H}_2\text{O}_2$ system resulted in a concentration-dependent decrease in the formation of malondialdehyde, suggesting that hsion-tsao leaf gum was a scavenger of hydroxyl radical. Plant-derived antioxidants have been shown to function as singlet and triplet oxygen quenchers and free radical scavengers (Wang and Lin, 2000). Ghiselli *et al.* (1998) reported that wine and dealcoholized wine were the most efficient against hydroxyl radicals. However, the dealcoholization caused a 80% loss of the hydroxyl radical scavenging. Yen *et al.* (2000) reported that at a concentration of 0.25 mg/ml, the scavenging effects of active component from the seeds of *Cassia tora* L. such as anthrone, aloe-emodin and emodin, on hydroxyl radicals produced by the Fenton reaction were 26.2, 16.6 and 41.8 % respectively.

8. Application of natural antioxidants

The antioxidant activity of green tea catechins used in cooked ground pork, were in the order of EGCG > ECG \approx EGC \gg EC. All catechins were more effective than BHT and α -tocopherol (Shahidi and Alexander, 1998). Koketsu (1997) reported that tea polyphenols and tocopherol showed antioxidant activity in lard in a concentration-dependent manner (Figure 8).

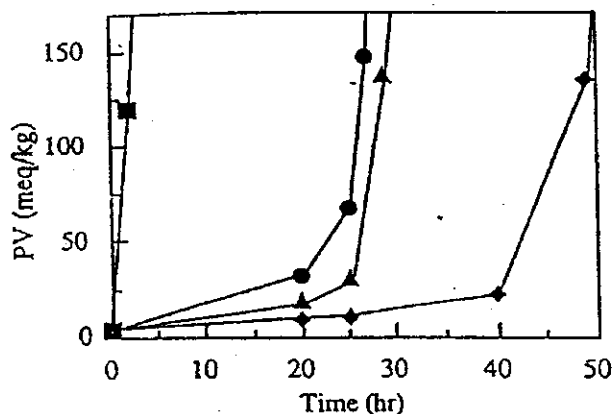


Figure 8 Antioxidative activity of tea polyphenols in lard

(-■-) control, (-●-) 400 ppm tocopherol, (-▲-) 400 ppm tea polyphenol,
 (-◆-) 600 ppm tea polyphenol

Source: Koketsu (1997)

Rajalakshmi and Narasimhan (1996) showed that retinol (50-100 mg/kg) acted as an antioxidant in lard during 6 month of storage at fluctuating temperature (16-27 °C) and relative humidity (48-72 %). Milovanovic *et al.* (1996) found that the extract of *Anthriscus sylvestris* was superior to quercetin, apigenin, or a tocopherol mixture in reducing oxidation of lard. Purified antioxidant prepared from extraction of Rosemary in different solvents showed an excellent antioxidant activity when added at a concentration of 0.02 % into prime steamed lard and also effective when used with chicken fat, sunflower oil or corn oil (Chang *et al.*, 1977). Sant' Ana and Mancini-Filho (2000) reported that the tocopherol had the best protection against oxidation when compared with the effects of BHT and rosemary extracts.

Phospholipids showed the effective antioxidant activity in lard and had synergy with tocopherols and flavonoids (Hudson and Mahgoub, 1981). Von Schler and Löschner (1985) also found that phospholipids from Antarctic krill exhibited antioxidant activity in synergy with tocopherol in an animal fat system. Kashima *et al.* (1991) noted that the oxidative stability of perilla oil increased markedly after the addition of phosphatidylethanolamine (PE) and phosphatidylserine (PS), but phosphatidylcholine (PC) scarcely showed any antioxidative effect. King *et al.* (1992) reported antioxidant of

PC to be equivalent to that of PE in a salmon oil model system. PC was an effective synergist of ethoxyquin in menhaden oil (Olcott and Van Veen, 1963).

Marine oils containing large amounts of polyunsaturated fatty acids (PUFA) undergo rapid oxidation and undesirable off-odors are produced (Cho *et al.*, 1987). Addition of flavonoids to seal blubber oil (SBO) and menhaden oil (MHO) reduced their peroxide value, compared to those of the control. Among flavonoids used, flavonols were most effective in lowering peroxide value in both oils, followed by flavanones, flavones and flavonols (Cho *et al.*, 1987). Han *et al.* (1990) investigated the effect of ascorbic acid, rosemary extract and δ -tocopherol on the oxidation of the fish oil stored in uncovered Petri dish at 30 °C as shown in Figure 9. Rosemary extract and δ -tocopherol were ineffective in retarding the oxidation of fish oil. On the other hand, the peroxide value of the fish oil containing ascorbic acid remained remarkably low. The ascorbic acid seemed to act as a primary antioxidant in an open system by stopping the free radical chain reaction as well as in a closed system by absorbing free oxygen to prevent oxidation (Niki *et al.*, 1984).

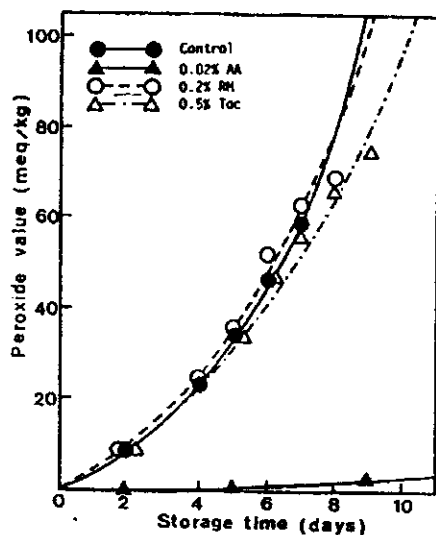


Figure 9 Effect of solubilized ascorbic acid (AA), rosemary extract (RM), and δ -tocopherol (Toc) on the oxidation of fish oil stored in uncovered Petri dish at 30 °C

Source: Han *et al.* (1990)

9. Low-Density Lipoprotein (LDL) oxidation and its prevention.

9.1 Lipoprotein structure

The main lipid in blood plasma are cholesterol, cholesterylesters, phospholipids, triglycerides and unesterified fatty acids. The majority of fatty acids are transported by albumin. Because of their minimal solubility in water, the other lipids are packaged into high molecular weight structures called lipoproteins. The principle function of lipoproteins is to distribute lipid among their sites of synthesis, storage, utilization and excretion (Patsch and Gotto, 1995). The lipoproteins are macromolecular complexes of lipids and protein. The general structure of lipoprotein molecular is globular (Figure 10).

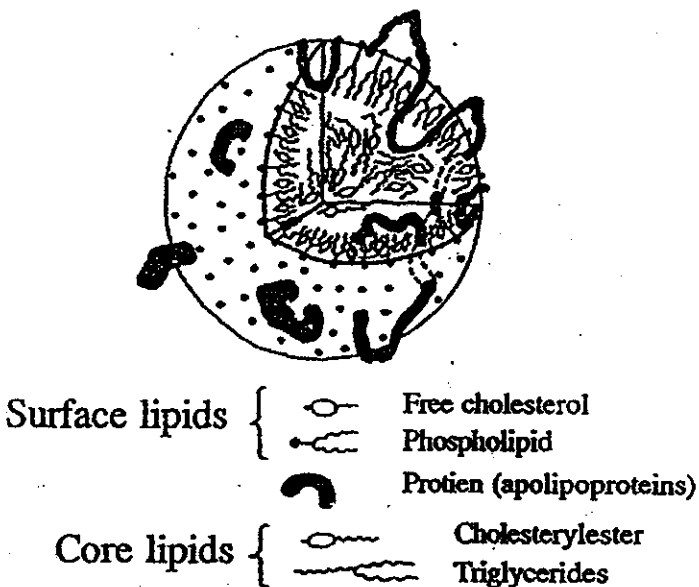


Figure 10 Lipoprotein structure

Source: Durrington (1995)

In the core of lipoprotein particle, more hydrophobic lipids, are localized and esterified cholesterol and the triglycerides are surrounded by polar lipids groups. Within the outer part of the lipoprotein, the more polar lipids, namely the phospholipids and free cholesterol, with their charged groups pointing out towards the water molecules are found. The protein components of lipoproteins are apoproteins, a group of proteins

of immense structural diversity. Apoproteins are anchored by their more hydrophobic regions exposed at the surface (Durrington, 1995). This construction protects the hydrophobic core from the watery surroundings and allows transport of large amounts of cholesterol triacylglycerols through the blood vessels (Mensink *et al.*, 1998). Lipoproteins are a heterogeneous group, which can be divided into four major classes: chylomicron, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) (Mensink *et al.*, 1998).

9.2 Plasma lipoproteins and coronary heart disease

LDL and HDL have different effects on the risk for coronary heart disease. High concentrations of LDL are atherogenic, while high levels of HDL are negatively associated with the risk for coronary heart disease (Mensink *et al.*, 1998). The modification of LDL, especially oxidation of this lipoprotein, has become recognized as important to many of the atherogenic actions ascribed to LDL. Thus, the "oxidation hypothesis" of atherosclerosis proposes that oxidative modification of LDL is an important event in the initiation and progression of atherosclerosis (Patsch and Gotto, 1995; Cox and Cohen, 1996).

9.3 Characteristics of oxidized LDL

In vitro oxidation of LDL results in alterations in both lipid and protein components of LDL. The unsaturated cholesteryl-ester content decreases, especially cholesteryl-arachidonate and cholesteryl-linoleate (Mensink *et al.* 1998). In addition, phosphatidylcholine, the main phospholipids in LDL, is converted into lysophosphatidylcholine after cleavage of a fatty acid from the *sn*-2 position by phospholipase A₂. It has been postulated that the released fatty acid is readily oxidized and may then become responsible for the propagation of the lipid peroxidation chain reaction, inasmuch as inhibitors of phospholipase A₂ block not only the generation of lysophospholipids but also of lipid peroxides. After peroxidation, lipid peroxides are decomposed and breakdown products such as malondialdehyde (MDA) are formed.

Peroxidation products are cytotoxic, and chronic irritation of endothelial cells results in lesions of the endothelial cell layer (Mensink *et al.*, 1998).

The formation of an atherosclerotic plaque was shown in Figure 11. LDL enters the intima layer and can be oxidized by several factors, such as lipoxygenase or reactive oxygen species (1). Oxidized LDL was cytotoxic and causes endothelial cells damaged (2), which results in the expression of adhesive glycoproteins to which monocytes and T-lymphocytes attach (3). The damaged endothelial cells excrete chemoattractants, causing a continuous recruitment of monocytes and T-lymphocytes (4). These cells pass the endothelial cell layer, and monocytes may become macrophages (5). Oxidized LDL prevents return of macrophages back to the lumen (6), where the arrested macrophages absorb large amounts of oxidized LDL via the scavenger receptors and become foam cells, which may eventually lead to the formation atherosclerotic plaques. (7).

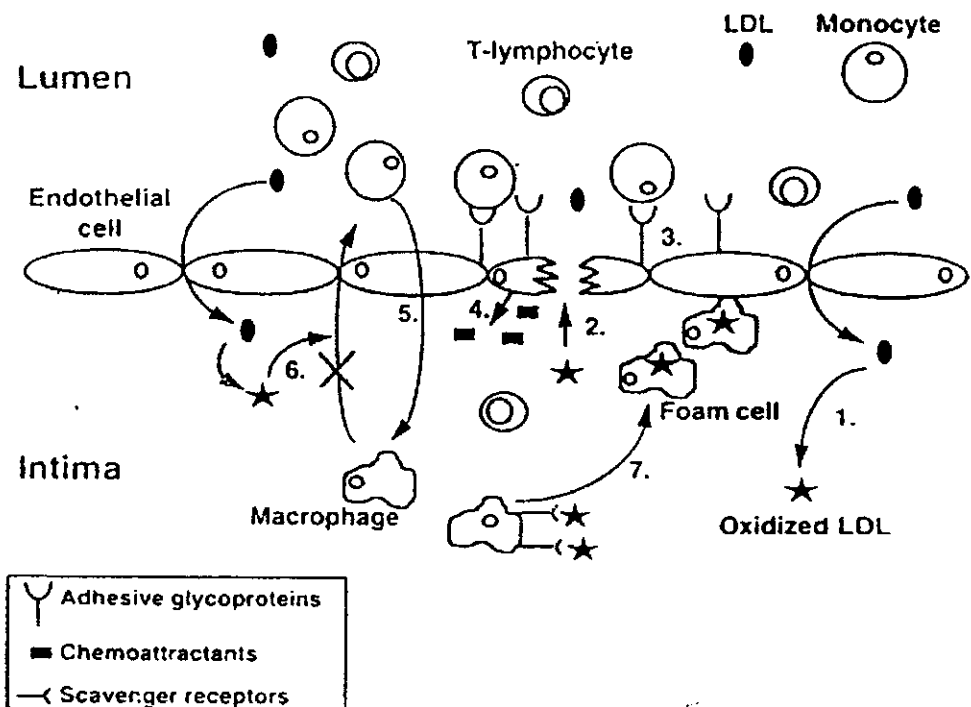


Figure 11 Schematic representation of seven steps in the formation of an atherosclerotic plaque

Source: Mensink *et al.* (1998)

9.4 Antioxidants and their protection against LDL oxidation

LDL itself contains several antioxidants such as α -tocopherol, γ -tocopherol, β -carotene, lycopene, and ubiquinol. When these antioxidants are exhausted, oxidative modification will occur, leading to the generation of macrophage-derived foam cells (Kalyanaraman, 1995). Lipid foam cells are characteristic of atherogenesis and it is widely accepted that oxidative modification of LDL is necessary for foam cell formation (Yang and Koo, 2000). This hypothesis is supported by the observation that oxidatively modified LDL is present in atherosclerosis plaque but absent in normal artery wall (Zhang *et al.*, 1997). Some compounds and their mechanisms on inhibition of LDL oxidation are shown in Table 4 (Kalyanaraman, 1995).

Table 4 Exogenous antioxidants and their protection against oxidation of LDL

Exogenous antioxidants	Mechanism
Probucol and Butylatedhydroxytoluene	Peroxy radical scavenger
Ascorbic acid	Regeneration of vitamin E, probucol and BHT
Probucol diglutarate	Water-soluble, intracellular peroxy radical scavenger
Flavonoids	Peroxy radical scavenger, metal-ion chelator
Ebselen (\pm GSH)	Lipid hydroperoxide and phospholipids-hydroperoxide scavenger
Hydroxamates	Peroxy radical scavenger, metal-ion chelator
α -Phenyl- <i>tert</i> -butyl N-nitrone	Lipid-derived radical scavenger
Trolox	Good scavenger of peroxy and alkoxy radicals, giving a Trolox radical that can be regenerated by ascorbate.
Curcumin	HO radical scavenger and Metal-ion chelator

Source: Kalyanaraman (1995)

There is currently a lot of interest in the inhibition of low-density lipoprotein (LDL) oxidation by flavonoids and the possible protection against coronary heart. LDL oxidized locally in atherosclerotic lesions may be involved in the pathogenesis of atherosclerosis (Steinberg, 1997). Oxidized LDL is endocytosed rapidly by macrophages, activates proinflammatory genes, induces apoptosis, and has many other potentially atherogenic effects. Flavonoids were shown to be effective in protecting LDL from oxidation *in vitro* (Whalley *et al.*, 1990).

Some flavonoids at low concentration are active in inhibiting LDL oxidation (Whalley *et al.*, 1990). Flavonoids and other antioxidants in red wine and in black or green tea can inhibit LDL oxidation via chain-breaking reaction (Frankel *et al.*, 1993). They may also decrease the binding of iron and copper, which can catalyze LDL oxidation, to LDL particles (Brown *et al.*, 1998). In addition, flavonoids protect α -tocopherol (the main endogenous antioxidant in LDL) from being consumed during LDL oxidation (Whalley *et al.*, 1990) and can convert the α -tocopheroxyl radical back into α -tocopherol (Zhu *et al.*, 2000b). Pretreating cells with flavonoids decreases the ability of cells to oxidize LDL (Yoshida *et al.*, 1999).

It has been reported that antioxidants, e.g. β -carotene and α -tocopherol, prevented LDL oxidation and delayed the development of atherosclerotic plaques in animals (Yang and Koo, 2000). There is increasing interest in green tea polyphenols as dietary antioxidants against oxidation of LDL *in vivo*. In addition, increased consumption of green tea has been shown to be negatively associated with serum total cholesterol and triacylglycerols. Effect of drinking green tea on plasma lipoproteins is characterized by decreasing LDL cholesterol while increasing HDL cholesterol. In contrast, drinking black tea seems to have no effect on plasma total cholesterol. This suggests that the beneficial effect of drinking green tea over black tea is attributed to the content of green tea polyphenol because green tea polyphenol remain unchanged, whereas they are degraded by the fermentation process in black tea (Zhang *et al.*, 1997). Miura *et al.* (1995) have demonstrated that epigallocatechin gallate (EGCG), the major catechin in green tea, inhibited Cu^{2+} - ion induced LDL oxidation, and that tea catechins also acted

as radical scavengers against propagating lipid peroxy radical species. Catechin added to plasma inhibits the oxidation of lipids in the whole plasma, but high concentrations are required (Lotito and Fraga, 1998). The oxidizability of lipoproteins from cholesterol-fed rabbits or hamsters was reported to be decreased by tea ingestion (Vinson and Dabbagh, 1998). Consuming black or green tea has been reported to increase the total antioxidant activity of human plasma (Leenen *et al.*, 2000).

Flavonoids may act in the intestine to inhibit the formation of lipid hydroperoxides in the lumen during digestion and therefore reduce the uptake of lipid hydroperoxides into the circulation in the postprandial state (Ursini *et al.*, 1998). Uptake of flavonoids from the intestine may not be essential for their actions *in vivo*. Drinking black or green tea increased the concentrations of catechins in human plasma (Miura *et al.*, 2000). It is possible that flavonoids may protect against cardiovascular disease by inhibiting LDL oxidation, e.g., by being incorporated into cells and protecting the cells from the oxidative stress induced by oxidized LDL. (Negre-Salvayre and Salvayre, 1992).

Objectives

1. To study the antioxidant activities of extracts from 4 varieties of mulberry green tea cultivated in Thailand.
2. To study the extraction of antioxidants from selected mulberry green tea.
3. To study some properties and mode of action of antioxidants from selected mulberry green tea.
4. To study the application of mulberry green tea extracts in lard and partially purified fish oil.
5. To study the inhibition of Low-Density Lipoprotein (LDL) oxidation by mulberry green tea extract.