CHAPTER II

REVIEW OF RELATED LITERATURES AND RESEARCH

Free radicals and reactive oxygen species

Free radicals are the atoms, molecules or ions with unpaired electrons on an open shell configuration (Ternay and Sorokin, 1997, p. 3). The free radicals can carry both an odd electron (hydroxyl radicals, $HO^{\cdot}$, peroxyl radicals, $HOO^{\cdot}$) and a charge which may either be radical cations or radical anion (superoxide anion, $O_2^{\cdot -}$) (Kaiser and Kevan, 1968). Free radical formation can occur by the loss of a single electron from a non-radical, or by the gain of a single electron by non-radical (Halliwell and Gutterige, 1989, p.11). The reactions common to many radicals are (a) abstraction of a hydrogen atom from a nearby molecule and (b) addition to molecular oxygen to form peroxyl radicals (Figure 1) (Ternay and Sorokin, 1997, p. 3). A continuing reaction becomes a chain reaction which can damage the cells by affecting the functions of biomolecules (Acworth, 1997, p. 23).

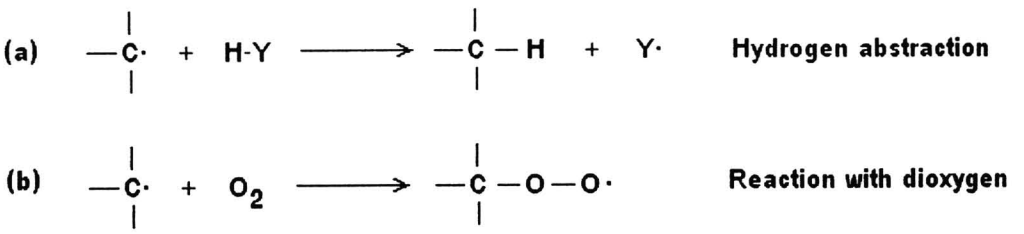


Figure 1 Free radical reactions

Source: Ternay and Sorokin, 1997, p. 4)

There are many kinds of molecules (may have net charge of zero or have unpaired electrons) which can produce free radicals or play the same role as free radicals such as hydrogen peroxide (H_2O_2), nitric oxide (NO), peroxynitrous acid (ONOOH) and hypochlorous acid (HOCl) (Acworth, 1997). These molecules are

called reactive species depending on the kinds of reactive atoms such as reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive chlorine species (RCS) (Halliwell, 2008).

The radical nature of molecular oxygen permits oxidation/reduction chemistry (Figure 2). The oxygen number of oxygen, superoxide, hydrogen peroxide, hydroxyl radical and water are 0, -1/2, -1, -1 and -2, respectively. There are many enzymes for the conversion of dioxygen into superoxide radicals in the body such as xanthine oxidase, NAD(P)H oxidase and enzymes in the electron transport chain. The superoxide can convert to hydrogen peroxide using the enzyme superoxide dismutase (Figure 3). And then hydrogen peroxide can generate the hydroxyl radical by the Fenton reaction (Figure 4). The reduction via hydroxyl radical to water occurs non-enzymatically (Bienert, et al., 2006).

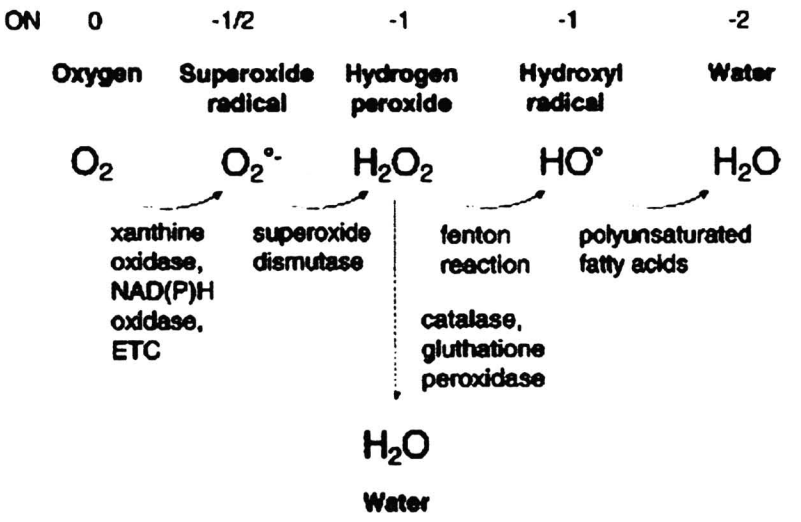


Figure 2 Consecutive reduction of dioxygen yields reactive oxygen species

Remark: ON= oxidation number of oxygen, ETC = electron transport chain.

Source: Bienert, et al., 2006

Hydrogen peroxide production, probably mainly via superoxide, has been frequently observed from mitochondria and microsomes. There are also several enzymes that produce hydrogen peroxide including glycollate oxidase, D-aminoacid oxidase, and urate oxidase. Hydrogen peroxide is also known to be produced during phagocytosis, and hydrogen peroxide vapor has been detected in humans expired air (Halliwell and Gutterige, 1989, pp.79-80).

The oxidation of hydrogen peroxide with organic substrates generally is slow. However hydrogen peroxide, when combined with ferrous iron known as Fenton reaction (Figure 4), could be quite hazardous due to hydroxyl radical formation (Ternay and Sorokin, 1997, p. 6). Fenton reaction was as follows:

$$Fe^{+2} + H_2O_2 \longrightarrow Fe^{3+} + HO^- + HO^\cdot$$

Ferrous iron Hydrogen peroxide Ferric iron Hydroxyl ion Hydroxyl radical

The hydroxyl free radical ($HO^\cdot$) is one of the most aggressive radicals found in the body reacting at a diffusion-controlled rate with almost every molecule in the living cells including DNA, lipid, proteins and carbohydrates (Acworth, et al., 1997, p. 27) However, $HO^\cdot$ does not travel far due to its short half-life.

The roles of free radicals and reactive oxygen species in the body

Human cells produce free radicals every day. Some of them are the byproduct of the non-complete reaction from the electron transport chain (ETC) process of mitochondria (Sjodin, et al., 1990), while others come from reaction of enzyme in the cells such as xanthine oxidase, aldehyde dehydrogenase, nitric oxide synthase and enzymes in arachidonic acid metabolism (Martínez-Cayuela, 1995). The exogenous sources of free radicals are ionizing radiation, tobacco smoke, pesticides, pollutants or some medications (Martínez-Cayuela, 1995).

In our body, free radicals play a dual role to be both useful molecules and worsening molecules. To their advantage, the free radicals play important roles in signal transduction (Demple, 2003, pp. 191-195), sensing of oxygen tension (Ehleben, et al., 1998), energy generation, (Raha and Robinson, 2000) and the essential molecule

synthesis (Marco-Contelles, et al., 1993). Free radicals are produced by macrophage during phagocytosis to destroy infected bacteria, and they can activate the immune system (Räisänan, et al, 2005). This process is due to an enzyme named reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase which is located on the external surface of the plasmic membrane (Martínez-Cayuela, 1995).

To their disadvantage, free radicals accumulate in the body in an out of balance way resulting in a condition called oxidative stress, and they cause damage to cells (Zwart, et al., 1999). The high reactivity of free radicals results in their having a short half-life. However, when these molecules react with non-radical compounds, new free radicals can be formed which again react. In this way, long chains of propagation are established causing biological effects far from the system which produced the first radical (Martínez-Cayuela, 1995). The damage caused by free radicals could lead to cell membrane dysfunction, protein denaturation, enzyme inactivation, mutation and cell death.

ROS have shown to oxidize lipids. Hydroxyl and hydroperoxyl radicals, but not $O_2^{\cdot-}$ nor H_2O_2 are able to attack unsaturated fatty acids of phospholipids resulting in lipid peroxidation process (Martínez-Cayuela, 1995). In this process (Figure 3) the primary free radical abstracts a hydrogen atom from a methylene bond of the carbon chain yielding a carbon-centered lipid radical. The radical produced is stabilized by a molecular rearrangement that produces a conjugated diene, which readily reacts with molecular oxygen to form a peroxyl radical. The peroxyl radical may remove a hydrogen atom from another molecule setting up a propagation chain of peroxidative damage. Some lipid peroxy radicals form endoperoxides, which give rise to short chain products and extra free radicals. Lipid peroxidation causes severe damage to the membrane structure and, consequently, alters its fluidity and ability to function correctly (Martínez-Cayuela, 1995).

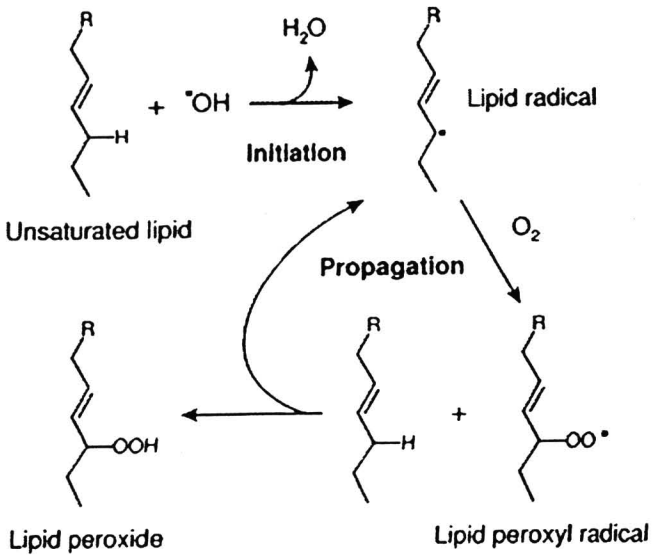


Figure 3 Initiation and propagation reactions in lipid peroxidation by hydroxyl radicals.

Source: http://www.websters-online-dictionary.org

The proteins are modified by ROS in different ways. The free radicals may react with amino acids containing unsaturated or sulfur groups such as tyrosine, phenylalanine, tryptophan, histidine, cysteine and methionine. These reactions give rise to structural disturbances in proteins as well as cross-linking and aggregation phenomena. In general, oxidized proteins increase their hydrophobicity and sensitivity to proteolysis (Martínez-Cayuela, 1995).

Carbohydrates are also targets of ROS. Consequently, glycosylated proteins are more sensitive to oxidative damage. The free radicals may oxidize monosaccharides but they can also react with polysaccharides such as hyaluronic acid inducing their depolymerization (Martínez-Cayuela, 1995).

The ROS-mediated pathogenesis processes of human diseases are indicated by biomarkers of oxidative damage to lipids, protein, or DNA (Acworth *et al.*, 1997, p. 23). Molecular mechanisms of ROS toxicity and ROS-mediated disease, include: (a) oxidation of vital thiol compounds to disulphides, (b) loss of reduced glutathione

(GSH), (c) impairment of energy generation, (e) inhibition of calcium transport and electrolyte homeostasis, (f) oxidation of cytochromes (such as hemoglobin), (g) DNA strand cleavage, and (h) the initiation and promotion of mutations and carcinogenesis (Parke, 1999).

ROS have been implicated in many diseases such as aging (Romano, et al., 2010), diabetic (Giacco and Brownlee, 2010), atherosclerosis (Halliwell, 1994), neurodegenerative diseases (Evan, 1993) and cancer (Witz, 1991; Ames, et al., 1993). In many of these cases, it is unclear if oxidants trigger the disease, or if they are produced as a secondary consequence of the disease (Valko, et al., 2007).

Antioxidant

The damaging of free radicals, ROS and RNS were inhibited by the molecules called "antioxidant". Since such reactions frequently involve radicals, "antioxidant" has taken on a more mechanisms and now generally refers to any substance which inhibits a free radicals reaction (Ternay and Sorokin, 1997, p. 6). Halliwell (1989) has defined an antioxidant as "any substance that, when present at low concentrations compared to those of an oxidizable substrate (e.g., lipids, proteins and DNA), significantly delays or prevents oxidations of that substrate". Antioxidant can give the electron to free radicals and make them stable while antioxidant themselves would be stable too (Seifried, et al., 2007).

There are many types of natural antioxidants such as vitamins (ascorbic acid, tocopherol), enzymes (superoxide dismutase, catalase, glutathione peroxidase), low-molecular-mass molecules which have chain-breaking activity (glutathione, uric acid), proteins used to sequester metals capable of hydroxyl radical production (transferring, ferritin, ceruloplasmin and albumin), trace elements (zinc, selenium), Iron chelator (lactoferrin), hormone (melatonin) and phytochemicals (polyphenols, flavonoids) (Acworth, et al., 1997, p. 25; Halliwell, 2008). The water-soluble antioxidants react with oxidants in the cell cytosol and the blood plasma, while lipid-soluble antioxidants protect cell membranes (Sies, 1997). Enzyme and protein antioxidants are synthesized in the body but antioxidants which are minerals, vitamins and polyphenols must be obtained from the plant-based diet.

Many kinds of enzymes in the body have antioxidative activity such as superoxide dismutase (SOD), catalase and glutathione peroxidase. The catalase has been shown to consist of four protein subunits, each of which contains a haem (Fe^{3+}-protoporphyin) group bound to its active site. The catalase is the mainly enzyme which can remove hydrogen peroxide in erythrocytes. This enzyme is localized to peroxisomes in most eukaryotic cells. The reaction of catalase on hydrogen peroxide was as follows:

$$\underset{\text{Hydrogen peroxide}}{2H_2O_2} \longrightarrow \underset{\text{Water}}{2H_2O} + \underset{\text{Oxygen}}{O_2}$$

The superoxide dismutases (SOD) can convert superoxide to hydrogen peroxide. SOD enzymes are present in almost all aerobic cells and in extracellular fluids (Johnson and Giulivi, 2005). SOD contains different metal ion cofactors such as copper, zinc, or manganese. In humans the copper/zinc SOD is present in the cytosol while manganese SOD is present in mitochondrion. The reaction of superoxide dismutase on superoxide radicals was as follows:

$$\underset{\text{Superoxide}}{2\,O_2^-} + \underset{\text{Proton}}{2H^+} \longrightarrow \underset{\text{Hydrogen peroxide}}{H_2O_2} + \underset{\text{oxygen}}{O_2}$$

Glutathione is essential for maintenance of the thiols of protein (and other compounds) and of antioxidant (e.g. ascorbate, a-tocopherol), (b) reduction of ribonucleotides to form the deoxyribonucleic precursors of DNA, and (c) protection against oxidative damage, free radical damage, and other types of toxicity (Meister, 1991). The glutathione (glutamylcysteinylglycine) which have thiol group at cysteine is substrate of glutathione peroxidase. Most free glutathione in vivo is present as reduced glutathione (GSH) rather than oxidized glutathione (GSSG). Glutathione peroxidase catalysed the oxidation of GSH to GSSG at the expense of hydrogen peroxide. The reaction of glutathione peroxidase on reduced glutathione was as follows:

$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O$$

Reduced glutathione Hydrogen peroxide Oxidized glutathione Water

The ratio of GSH/GSSG in normal cells is kept high so there must be a mechanism for reducing GSSG back to GSH. This is achieved by glutathione reductase enzymes, which catalyse the reaction (Halliwell and Gutteridge, 1989, p.94). The reaction of glutathione reductase on oxidized glutathione was as follows:

$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$$

Oxidized glutathione Reduced glutathione

The dietary antioxidants are considered to be a supplement in the hopes of maintaining health and preventing disease. People who eat dietary antioxidants from fruits and vegetables have lower risk of heart disease, some neurological diseases (Esposito, et al., 2002), and cancers (Stanner, et al., 2004). The health benefits of fruit and vegetables may come from individual substances (e.g. flavonoids), or from a complex mix of substances (Shenkin, 2006).

The mechanisms of antioxidants

The three major mechanisms of antioxidants are their effects on radical formation, radical scavenging reaction and the repairing processes (Strube, et al., 1997). Some kinds of antioxidants such as the one with chelating activity can inhibit the initiation of free radicals. Some of them terminate the chain reaction of the free radicals by removing free radical intermediates. The other antioxidants play a role in the repairing processes. However, since free radicals do have useful functions in cells, the function of antioxidant systems is not to remove free radicals entirely, but instead to keep them at an optimum level. The required characteristics for effective antioxidant molecules include (a) the presence of hydrogen-/electron-donating substituent, (b) the ability to delocalize the resulting radical and (c) the transition metal-chelating potential (Rice-Evans, 1999).

However the antioxidant activities of some known antioxidant compounds are up to their concentrations (Vertuani, et al., 2004). Low concentrations of ascorbic acid and gallic acid (a kind of non-flavonoids polyphenol) act as prooxidant due to the strong reducing power and weak metal chelating ability, while high concentrations act as antioxidant compounds (Yen, et al., 2002). Most plant-derived polyphenolic antioxidants including tannins and flavonoids are known to act as prooxidants either alone or in the presence of transition metals (Khan, et al., 2000). For example, it is known that flavonoids can either enhance or inhibit the formation of hydroxyl radical by Fenton-type reactions (Puppo, et al., 1992). It has been reported that a phenolic antioxidant is converted into a phenoxyl radical which can be the basis of a cascade of prooxidant events (Kahl, 1991, pp. 245-273). However, the prooxidant mechanisms of polyphenol are still unclear.

Healthy cells have an antioxidant balance system to control all kinds of free radicals; however, if they are out of balance, they can cause oxidative stress (Figure 4). The purpose of antioxidants in a physiological setting is to prevent ROS concentrations from reaching a high-enough level within a cell that damage may occur (Seifried, et al., 2007).

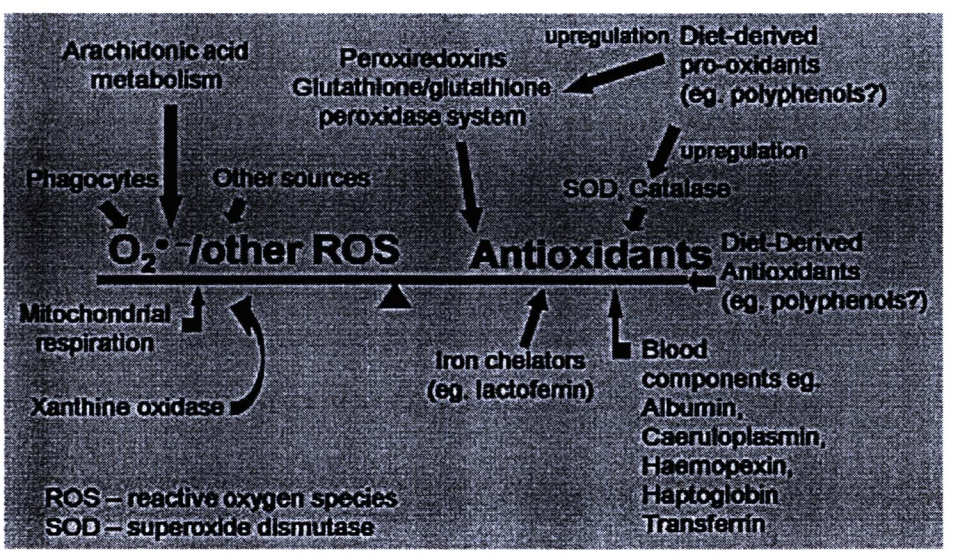


Figure 4 Balance of antioxidants and reactive species *in vivo*

Source: Halliwell, 2008.

Methodology for antioxidant activity

The ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) radical scavenging method was developed by Rice-Evans and Miller in 1994 and was then modified by Re *et al.* (1999). The method derives from the principle that ABTS, when incubated with hydrogen peroxide and metmyoglobin, oxidized ABTS is formed (Figure 14). The antioxidant can inhibit this reaction. The modification is based on the activation of metmyoglobin with H_2O_2 in the presence of $ABTS^{\cdot+}$ to produce a radical cation. The reactions occurring in ABTS/metmyoglobin/H_2O_2 method (Modified from Re, et al., 1999) were as follows:

Inhibited by antioxidant

Metmyoglobin (Fe^{3+}) + H_2O_2 ⟶ Ferryl myoglobin + Hydroxyl radical ($HO^{\cdot}$)

Hydroxyl radical ($HO^{\cdot}$) + ABTS ⟶ ABTS radical (blue-green colour)

This improved method generates a blue/green $ABTS^{\cdot+}$ chromophore via the reaction of ABTS and potassium persulfate and is now widely used. The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate (Figure 5), and its reduction in the presence of hydrogen-donating antioxidants is measured. This decolourisation assay measures the total antioxidant capacity in both lipophilic and hydrophilic substances (Krishnaiah, et al., 2010). The formation of covalent adducts obtained from the reaction of the polyphenolic, catechin, with ABTS radicals was reported (Osman, et al., 2006).

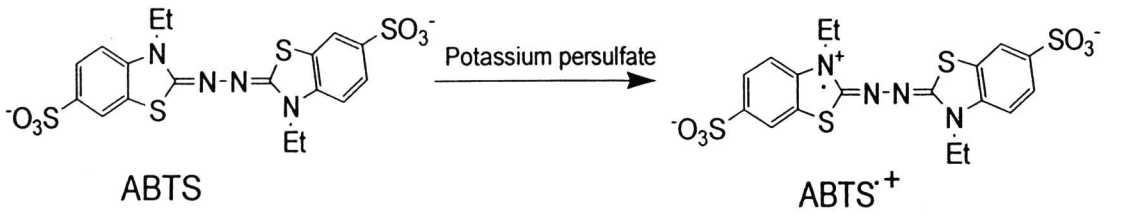


Figure 5 The ABTS radical cation formation by the oxidation of ABTS with potassium persulfate.

Source: Modified from Kadnikova and Kostic, 2002.

The 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging assay was firstly described by Blois in 1958 and was later modified slightly by numerous researchers. DPPH is a stable free radical that reacts with compounds that can donate a hydrogen atom. This method is based on the scavenging of DPPH through the addition of a radical species or antioxidant that decolorizes the DPPH solution. The scavenging reaction between (DPPH) and an antioxidant (A-H) can be written as Figure 6. Both ABTS and DPPH methods are the widely used antioxidant assays for plant samples (Krishnaiah, et al., 2010).

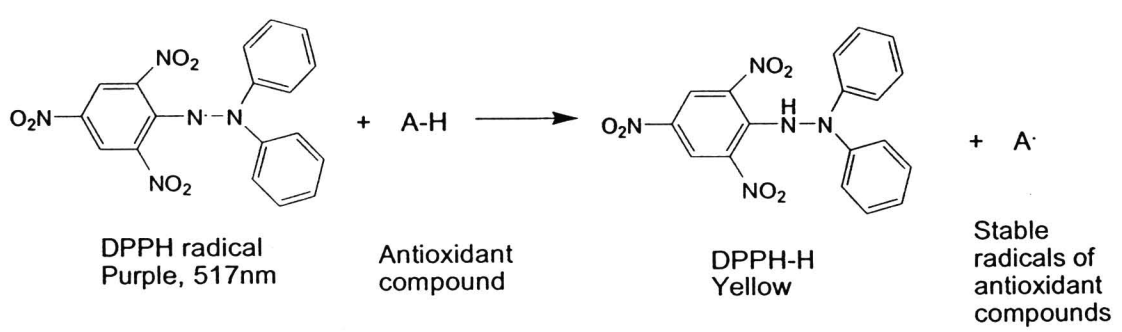


Figure 6 The reaction between DPPH and antioxidant compounds

Remark: A-H = Antioxidant compound (A = oxidizing structure, H = hydrogen)

Source: Nikhat, et al., 2009.

Chemical structures of flavonoids, proanthocyanidins and tannin

Flavonoids are polyphenolic substances based on a flavan nucleus. The molecular structure of flavonoids consists of two aromatic rings (A ring and B ring), that are linked through three-carbon frequently formed as an oxygenated heterocyclic C ring (Figure 7-8). Flavonoids constitute the largest and most important group of polyphenolic compounds containing a number of phenolic hydroxyl groups attached to ring structures conferring a strong antioxidant activity (Haslam, 1989). Different arrangements of hydroxyl and/or carbonyl groups, and carbon-carbon double bonds in the basic structure define the different subgroups of flavonoids as shown in Figure 13a. The important subgroups of flavonoids families are anthocyanidins, flavanols (proanthocyanidins), flavanones, flavones, and flavonols (Figure 7a). Flavonoids often occur in nature as glycosidic derivatives, which have been reported to be more readily absorbed than the simple form (Hollman, et al., 1995).

Quantitatively, flavanols represent a major group of flavonoids in cocoa (Figure 7b). Generally, when viewed as a composite, the flavanol (flavan-3-ol) monomers epicatechin and catechin are approximately 10% of the combined monomer and oligomer total. Heiss, et al.(2007) suggest that the pooling of monomers and procyanidins when presenting dietary intake data is inappropriate, given that while monomers and dimers are absorbed in the small intestine, the longer oligomers are not absorbed (see the detail in further topic).

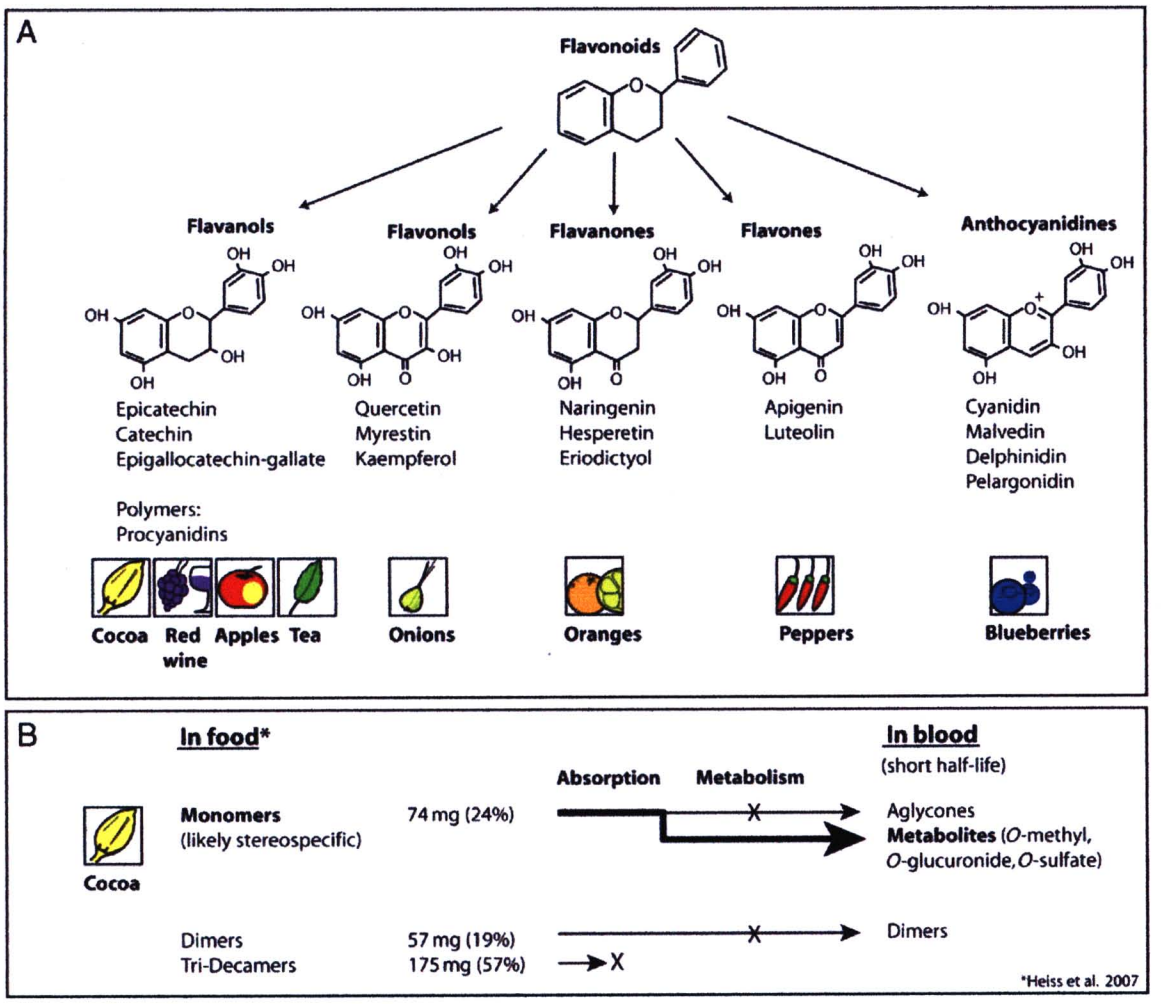
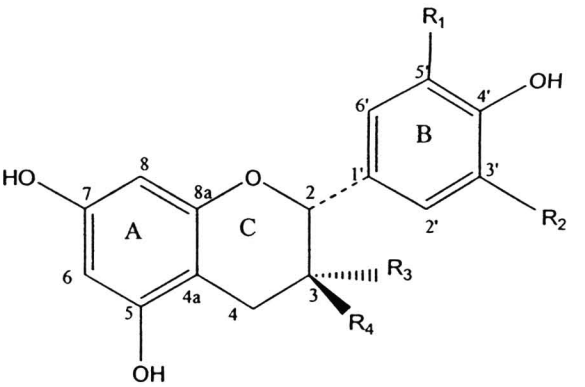


Figure 7 (A) Basic structure and examples of the main subclasses of dietary flavonoids. (B) Whereas the majority of flavanols are present as oligomer in food (i.e. cocoa), metabolized flavanol monomers are the dominant flavanols in blood.

Source: Heiss, et al., 2007.



Flavan-3-ol compounds	R1	R2	R3	R4
Afzelechin (Propelargonidin)	H	H	H	OH
Epiafzelechin(Propelargonidin)	H	H	OH	H
Catechin (Procyanidin)	H	OH	H	OH
Epicatechin (Procyanidin)	H	OH	OH	H
Gallocatechin (Prodelphinidin)	OH	OH	H	OH
Epigallocatechin(Prodelphinidin)	OH	OH	OH	H

Figure 8 Basic chemical structure and numbering system of flavan-3-ol (flavanols) compounds known as proanthocyanidins

Source: Modified from Shoji, et al., 2006.

Proanthocyanidins, composed of a flavan-3-ol structure of flavonoids (Figure 8), are the most abundant and diversified flavonoids in plants after lignins. Proanthocyanidins can be divided into several groups such as procyanidins, prodelphinidins and propelargonidins (Appeldoorn, et al., 2009). The procyanidins have exclusive (epi) catechin units which have 3′, 4′- dihydroxyl groups at B-ring of flavonoid. Besides procyanidins, prodelphinidins have heterogenous group consisting of at least one (epi) gallocatechin units which have 3′,4′,5′- trihydroxyl group at B-ring of flavonoids (Figure 15). Propelargonidins are the heterogenous group consisting of at least one (epi) afzelechin unit and additional epi (catechin) unit. The monomeric subunit of proanthocyanidins can be linked mainly through C4-C8 and/or C4-C6 linkages (B-type), which can coexist with an additional C2-O-C7 linkage (A-type) (Appeldoorn, et al., 2009).

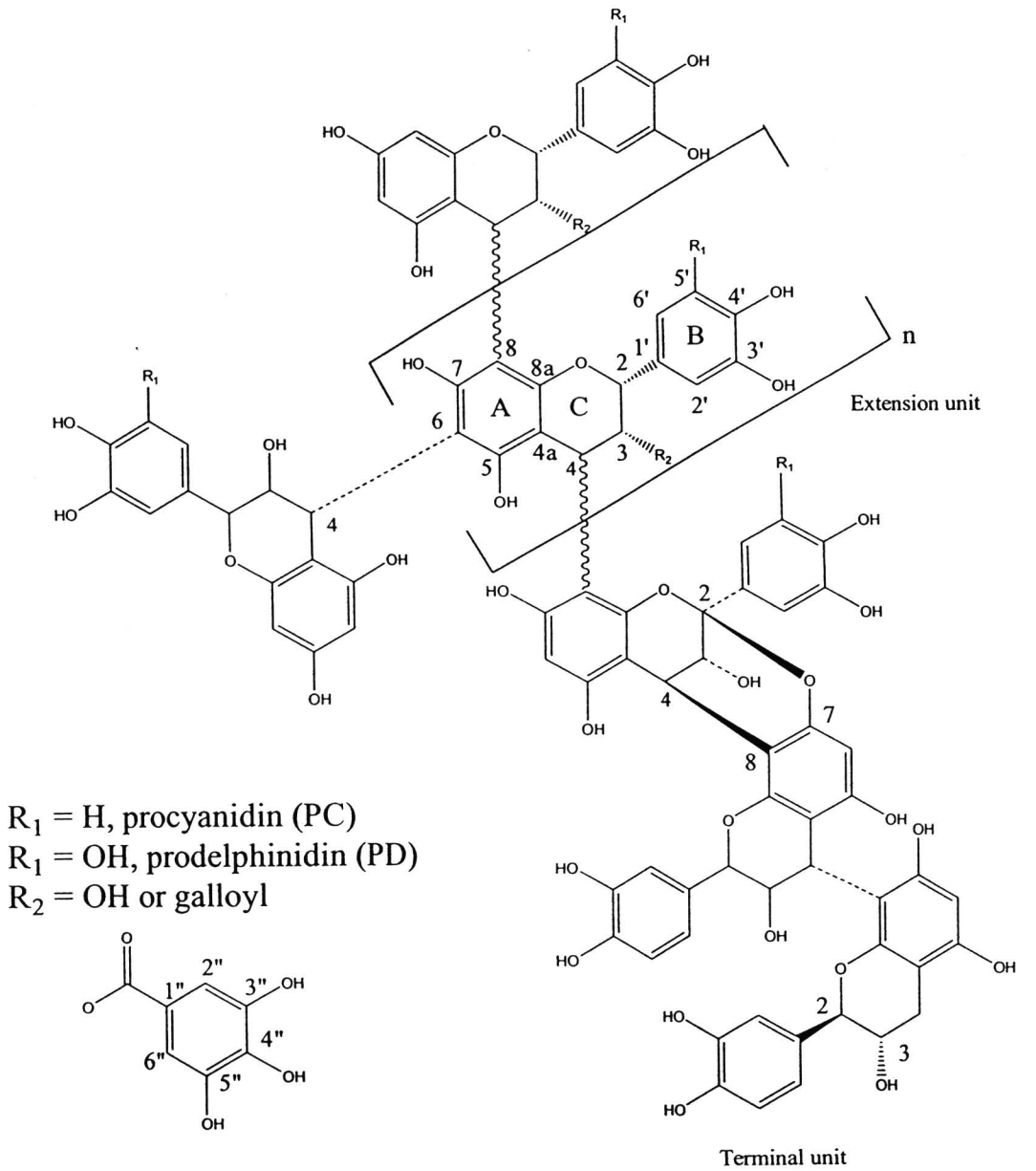


Figure 9 An exampling structure of oligomeric or polymeric proanthocyanidins showing flavan-3-ols which are mainly bonded by C4-C8 linkage. The C4-C6 and C2-O-C7 linkages are an alternative interflavan bond. If R_1 = H or OH then the structure represents a procyanidin or prodelphinidin, respectively. The terminal unit is at the bottom of such a multi-unit structure.

Source: Modified from Kashiwada. et al., 1990 and Schofield, et al., 2001)

The further addition of flavan-3-ol units results in the formation of larger proanthocyanidin oligomers and polymers (Figure 9). Each proanthocyanidin oligomer contains one terminal unit of which the C-ring is not connected to another monomeric unit. According to the presence of different monomers, linkages and subunits in proanthocyanidins, this complex compounds contain at least 83 different molecular species as reported by Appeldoorn (Appeldoorn, et al., 2009). Condensed tannin composed of proanthocyanidins subunits are linked mainly through a C4-C8 (or C4-C6) bond, and this give rise to a degree of polymer branching. The main difficulty in studies on proanthocyanidins is probably that of obtaining them in an individual molecular form. The complete purification of a proanthocyanidins with a DP above five is almost impossible (Jerez, 2007). Moreover, synergistic effects of active mixtures make plant extracts and fractions more interesting than pure compounds for functional food application (Jerez, 2007).

Tannins have been defined as polyphenols of high molecular weight which are water soluble and capable of precipitating proteins (Bryant, et al., 1992). By definition, tannins have the ability to react with protein, forming stable water insoluble co-polymers (Harborne, 1998). Chemically, there are two main types of tannin: condensed tannin and hydrolysable tannin.

Condensed tannins, which are flavonoid-based tannins, can be regarded as being formed biosynthetically by the condensation of single flavan-3-ol (proanhtocyanidins) to form dimer and then higher oligomers and polymers. The name proanthocyanidins is used alternatively for condensed tannins because on treatment with hot acid, some of the carbon-carbon linking bonds are broken and anthocyanidin monomers are released (Harborne, 1998). Procyanidins and prodelphinidins are also known, as are mixed polymers of condensed tannin with yield cyanidin and delphinidin on acid degradation. An increase in the prodelphinidin/procyanidin ratio in condensed tannin increases the ability of that tannin to complex with proteins (Aerts, et al., 1999)

Hydrolysable tannins, which are non-flavonoids compounds, are mainly of two classes. The simplest is the galloylglucose, in which a glucose core is surrounded by five or more galloyl ester groups. A second type occurs where the core molecule is

a dimer of gallic acid, namely hexahydroxyfiphenic acid, again with glucose attachment (Harborne, 1998).

The hydrolysable tannins, commonly called tannic acid (Figure 10), exist in various forms and configurations and could be hydrolyzed into glucose, gallic acid or ellagic acid units (Chen, et al., 2009). Use of tannic acid in food application is far more widespread and significant amounts are used as process aids in beer clarification, aroma compound in soft drinks and juices (Wikipedia).

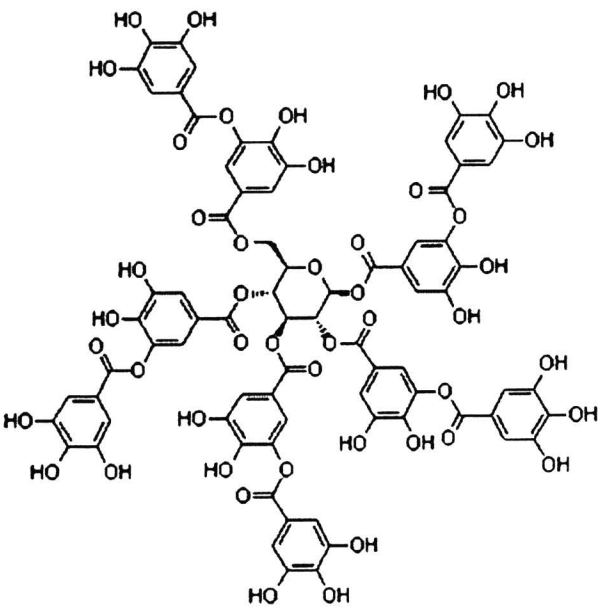


Figure 10 A structure of tannic acid

Source: www.sigmaaldrich.com.

Antioxidant activity of flavonoids and relating compounds

Flavonoids which have high antioxidant activity are attractive and provide increasing interest as nutritional compounds recognized to promote human health. The ability of flavonoids to act as antioxidants by electron donation depends directly on the reduction potentials of their radicals and inversely on the reactivity of the flavonoid molecules with oxygen (Rice-Evans, et al., 1999, p. 241). Three criteria for effective radical scavenging by the flavonoids are :(a) the o-dihydroxy structure in the B ring which confers higher stability to the radical form and participates in electron

delocalization ; (b) the 2,3 double bond in conjugation with 4-oxo function in the C ring (in case of flavones) responsible for electron delocalization from the B ring; and (c) the 3- and 5-OH groups with 4-oxo function in A and C rings for maximum radical scavenging potential (Rice-Evans, et al., 1996) (Figure 11).

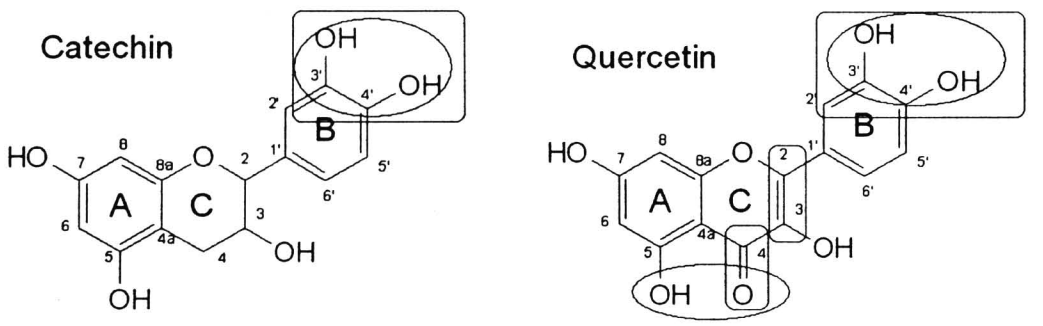


Figure 11 Structure of the catechin (flavan-3-ol) and quercetin (flavonol) showing features important in defining the classical antioxidant (in square shape) and chelation (in cyclic shape) potential of flavonoids. The most important of these is the catechol or dihydroxylated B-ring. Other important features include the presence of unsaturation in the C-ring and the presence of a 4-oxo function in the C-ring. The catechol group and other functions may also ascribe an ability to chelate transition metal ions such as copper and iron.

Source: Modified from William, et al., 2004.

In general, the more hydroxyl substitutions on the ring of flavonoids exhibit the stronger the antioxidant activity (Cao, et al., 1997). Areias, et al. (2001) suggested that the higher antioxidant activity of the flavonoids in cultured retinal cells is not correlated with the presence of a double bond at C2-C3 and/or a hydroxyl group at C3 on the C ring, but rather may depend on the capacity to inhibit the production of ROS to interact hydrophobically with membranes.

The mechanism of the antioxidant action of the flavonoids is a subject of considerable debate. As polyphenolic compounds, flavonoids have the ability to act as antioxidants by a free radical scavenging mechanism with the formation of less reactive flavonoid phenoxyl radicals. On the other hand, through their known ability to chelate transition metals, these compounds may inactivate iron ions through complexation, thereby suppressing the Fenton reaction (Figure 4), which currently believed to be the most important route to active oxygen species (Arora, et al., 1998). The antioxidant reactions of flavonoids according to Arora *et al.* (1998) were as follow:

(1) $ROO^{\cdot}$ + Fl-OH ⟶ ROOH + $Fl\text{-}O^{\cdot}$

Peroxyl radicals Flavonoids Peroxide Flavonoid phenoxyl radicals

(2) $HO^{\cdot}$ + Fl-OH ⟶ H_2O + $Fl\text{-}O^{\cdot}$

Hydroxyl radicals Flavonoids Water Flavonoid phenoxyl radicals

The flavonoids were reported to have antioxidant effects as free radical scavengers, hydrogen-donating compound, singlet oxygen quenchers, and metal ion chelators, properties attributed to the phenolic hydroxyl groups attached to the ring structures (Rice-Evans *et al.*, 1996 and Kandaswami, et al., 1994). The structure and degree of polymerization (DP) of proanthocyanidins were related to their antioxidant activity (Tourino, et al., 2005). The chemical properties of proanthocyanidins in terms of the availability of the phenolic hydrogens as hydrogen donating radical scavengers and singlet oxygen quenchers predict their antioxidant activity (Ohshima, et al., 1998).

The oxidation of the catechin ring by DPPH-derived radicals may occur at the B-ring of catechin, forming the corresponding O-quinones, or even at the C-2 position of the C-ring (Kondo, et al., 2000). But Osman, et al. (2006) have reported that the oxidation of catechin by ABTS radical cations occurs at the A-ring, which is also the modified part of the molecule. A possible explanation for the difference in the site of oxidation is probably due to the inaccessibility of the antioxidant to the radical centre,

especially when both the antioxidant and the radical are bulky as is the case with DPPH and tannins (Yokozawa, et al., 1998; Osman, et al., 2006).

However, dietary polyphenols including proanthocyanidins may have non-antioxidant roles in low concentrations that affect cell-to-cell signaling, receptor sensitivity, inflammatory enzyme activity or gene regulation (Williams, et al., 2004; Virgili, et al., 2008). Numerous enzymes such as hydrolases, oxidoreductases, DNA synthetases, RNA polymerases, phosphatases, protein phosphokinases, oxygenase and amino acid oxidases are inhibited by flavonoids (Havsteen, 2002). The presence of gallate ester linking to proanthocyanidins was proved to regulate the cell cycle (Liberto and Cobrinik, 2000).

Generally the dimer-, trimer- and tetrameric proanthocyanidins, referred to as extractable proanthocyanidins, or bioflavonoids, have been shown to be highly bioavailable and provide excellent health benefits (Bagchi, et al., 2000). Oligomeric proanthocyanidins (OPCs), naturally occurring antioxidants widely available in fruits (Perez-Jimenez, et al., 2009), flowers (Svedstrom, et al., 2002), seeds (Ling, et al., 2005), red and blak rice (Finocchiaro, et al., 2010), nuts (Appeldoorn, et al., 2009) and bark (Packer, et al., 1999; Jerez, et al., 2007). The popular and well-known sources of OPCs are grape seed (Bagchi, et al., 2000) and pine bark (Packer, et al., 1999; Jerez, et al., 2007). The OPCs which are found in grape seed or pine bark have been reported to be a powerful antioxidative supplement.

The oligomeric proanthocyanidins of grape seed and other natural sources have been reported to exhibit a variety of biological activities, including antiulcer (Saito, et al., 1998), antibacterial (Kusuda, et al., 2006), anticancer (Kim, et al., 2005), and the preventive properties in relation to the development of multiple human diseases, such as atherosclerosis (Yamakoshi, et al., 1999), and cardiovascular diseases (Facino, et al., 1999). The grape seed extract also shown to demonstrate cytotoxicity towards human breast, lung and gastric adenocarcinoma cells, while enhancing the growth and viability of normal human gastric mucosal cells (Bagchi, et al., 2000). The dose-dependent free radical scavenging ability of grape seed proanthocyanidin extract was determined both in vitro and in vivo models (Bagchi, et al., 2000). Moreover, this grape seed proanthocyanidin extract can protect the free radical-induced DNA fragmentation and lipid peroxidation (Bagchi, et al., 2000).

Pycnogenol is a standardized extract from pine (*Pinus maritima*) bark composed of a mixture of flavonoids, mainly oligomeric procyanidins (Packer, et al., 1999; Jerez, et al., 2007). Pycnogenol has strong free radical-scavenging activity against ROS and RNS (Packer, et al., 1999). The other species of bark such as *Pinus pinaster* and *Pinus radiate* have been reported to be source of procyanidins which have high antioxidant activity (Jerez, et al., 2007); although, pine bark extract is used as an herbal medicine to overcome many degenerative disorders (Rohdewald, 2002). There are many aspects of pine bark extract, but only in vitro biological studies have been done so far (Maimoona, et al., 2011). A few studies which are on human beings are limited either by their poor design or fewer numbers of subjects included in the trial (Maimoona, et al., 2011). Pine proanthocyanidins are devoid of gallate esters, which appear to interfere with crucial cell functions; they may be innocuous chemopreventive agents of choice for many applications (Tourino, et al., 2005).

The polymeric proanthocyanidins were found in bark of *Hamamelis* virginiana (Dauer, et al., 2003), pine bark (Jerez, et al., 2009), pear juice (Es-Safi, et al., 2006), birch leaves (Karonen, et al., 2006) and grape skin (Souquet et al., 1996). The cell-proliferation-stimulating effects are reported for high molecular proanthocyanidins from the bark of Hammamelis virginiana L. (Deters, et al., 2001).

Chelating activity of polyphenols

The variable valency of transition element especially iron allows them to undergo changes in the oxidation state involving one electron (Halliwell and Gutterige, 1989, p.15). About two-thirds of body iron is found in hemoglobin, with smaller amounts in myoglobin, various enzymes, and the transport protein transferring (Halliwell and Gutterige, 1989, p.34).

The simple mixture of an iron salt and hydrogen peroxide by Fenton reaction (Figure 4) can provoke a whole series of radical reactions (Halliwell and Gutterige, 1989, p.18). Some compounds contribute to antioxidant defense by chelating iron and preventing them from catalyzing the production of free radicals in the cells. The various natural or synthetic antioxidants have been reported to be beneficial in the prevention and attenuation of metal induced biochemical alterations such as vitamin, N-acetylcysteine, melatonin and dietary flavonoids (Flora, 2009).

The catechol group and other functions of flavonoids may also ascribe an ability to chelate transition metal ions such as copper and iron (Figure 17) (William, et al., 2004). The metal chelation has often been considered as a minor mechanism in the antioxidant action (Yoshino and Murakami, 1998). The flavonoids exhibited higher antioxidant efficacies against metal-ion-induced peroxidations than peroxyl-radical-induced peroxidation in a lysosome system, suggesting that metal chelation may play a larger role in determining the antioxidant activities of these compounds than has previously been believed (Arora, et al., 1998).

Iron may easily oscillate between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) states. Whereas ferric ions are the relative biologically inactive form of iron, ferrous ions are responsible for formation of a part of ROS that can lead to lipid peroxidation, nucleic acid or protein damage (Chvátalová, et al., 2008). One-electron reduction of oxygen by ferrous ions generates superoxide anion, which is itself an oxidant of many ferrous complexes causing a formation of hydrogen peroxide (Figure 19). According to Yoshino and Murakami (1998), one-electron reduction of oxygen by ferrous ion generates superoxide anion (equation 1 as follow), which is itself an oxidant of many ferrous complexes causing a formation of hydrogen peroxide (equation 2 as follow). Hydroxyl radical is further generated from hydrogen peroxide through the metal-catalyzed Fenton reaction.

(1) $Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2^{\cdot-}$

Ferrous ion Oxygen Ferric ion Superoxide

(2) $Fe^{2+} + O_2^{\cdot-} + 2H^+ \longrightarrow Fe^{3+} + H_2O_2$

Ferrous ion Superoxide Ferric ion Hydrogen peroxide

All phenolic acids bearing 3,4-dihydroxy (catechol) or 3,4,5,-trihydroxy (galloyl) moiety formed chelates with ferric (Fe^{3+}) iron and significantly increased the rate of ferrous (Fe^{2+}) autooxidation (Chvátalová, et al., 2008). Polyphenols can exert antioxidant activity by enhancing the autooxidation of ferrous to ferric ion (Yoshino and Murakami, 1998). This prevents radical propagation since Fe^{2+} is required to react

with superoxide to form H_2O_2, and to further convert H_2O_2 to hydroxyl radical and hydroxide ion. The natural polyphenols can be classified according to the antioxidant effect using iron redox reaction into two groups (Yoshino and Murakami, 1998): flavonoids enhanced the autoxidation of ferrous ion, resulting in the inhibition of the formation of ROS and non-flavonoid polyphenolics formed an inactive ferrous-polyphenolic complex by reducing ferric iron.

Polyphenols in tamarind seed husk (TaSH)

The tamarind (*Tamarindus indica* L.) belongs to the Leguminosae, Caesalpiniaceae family grows naturally in tropical and subtropical regions. The ripened fruit pulp is edible. Fresh stem bark and fresh leaves have been used as a traditional herbal medicine. Tamarind seeds (TS) are also of important use. The polysaccharides mainly consisting of xyloglucans prepared from the tamarind seed kernel have been used for cosmetics (Burgalassi, et al., 2000). The germ obtained from the tamarind seed is used for manufacturing tamarind gum (Tsuda, et al., 1994). The product prepared by extracting the tamarind seed has been used as a food additive (Kumar and Bhattachrya, 2008). Tamarind seed husk is used as fuel and as cattle feed (www.akproducts.in/tamarind-seed-husk.htm). An ethanol extract prepared from tamarind seed husk (TaSH) exhibited antioxidative activity, but there was no activity in the extract prepared from tamarind seed germ (Tsuda, et al., 1994). The TaSH was shown to have a very high antioxidative activity among the number of seeds, herbs and plants as reported by Soong, et al., 2004 and Maisuthisakul, et al., 2008.

Several studies on the extraction from TaSH and the antioxidant activity of the extracts from TaSH have been reported. Tsuda, et al. (1994) used ethanol and ethyl acetate as an extraction solvent and reported the isolation of 2-hydroxyl-3',4'-dihydroxyacetophenone, methyl 3,4-dihydroxybenzoate, 3,4-dihydroxyphenyl acetate, and (-)-epicatechin. The antioxidative activity of the TaSH extract was stable against the heat treatment at 100°C for 2 hours, and lower at pH 5.0 than at pH 3.0, 7.0 and 9.0 (Tsuda, et al.,1995).

Chemical analysis as well as UV absorption and IR spectra of the ethanolic extract compared with oligomeric proanthocyanidins (OPC), commercially extracted from pine barks and grape seeds, showed similar results indicating that the extract

of tamarind seed coat contained polyphenolic compounds, so-called OPC (Pumthong ,1999). The extract could also protect Ca^{2+} - ATPase in red blood cells membrane from oxygen free radical damage (Pumthong, 1999).

The condensed tannin in TaSH at low concentration (75g/kg body weight) has beneficial effect on the performance of crossbred lactating cows (Bhatta, 2000). Gu, et al. (2003) found that the tamarind seeds contain 29.32 g/kg of procyanidin oligomers and 101.89 g/kg of high molecular weight tannins.

Komutarin, et al. (2004) extracted, with ethanol/water (7:3, v/v), followed by extracting with the ethyl acetate, and studied the ethyl acetate fraction. They did not specify the structural characterization of the extract but reported them as a polyphenolic flavonoid. They found that polyphenolic flavonoids from TaSH could inhibits nitric oxide production in macrophages. While TaSH extract had no effect on macrophage cell viability as assessed by the MTT (3-[4, 5-dimethylthiazol-2-yl] - 2,5-diphenyl tetrazolium bromide) assay.

Luengthanaphol, et al. (2004) extracted (-)-epicatechin from sweet Thai TaSH with supercritical CO_2 and reported the high antioxidant activity of the TaSH extract. Aengwanich, et al. (2009) extracted with ethanol/water (19:1, v/v), followed by the precipitation with non-fat milk. They did not specify the structure and used the extract-protein complex for the study.

Sudjaroen, et al. (2005) reported the detailed study on the isolation and the structure elucidation of phenolic antioxidants from tamarind seed and pericarp. They used methanol and acetone/methanol/water (70:29:0.5, v/v) as an extracting solvent. The profile of polyphenolics in tamarind pericarp was dominated by proanthocyanidins (73.4) in various forms such as catechin (2.0), procyanidin B2 (8.2), epicatechin (9.4), procyanidin trimer (11.3), procyanidin tetramer (22.2), procyanidin pentamer (11.6), procyanidin hexamer (12.8) along with taxifolin (7.4), apigenin (2.0), eriodictyol (6.9), luteolin (5.0) and naringenin (1.4) of total phenols (polymeric tannin not included in total), respectively. The content of tamarind seeds comprised only procyanidins, represented (%) mainly by oligomeric procyanidin tetramer (30.2), procyanidin hexamer (23.8), procyanidin trimer (18.1), procyanidin pentamer (17.6) with lower amounts of procyanidin B_2 (5.5) and epicatechin (4.8).

They studied and elucidated the chemical structures of the minor ingredients but did not study the polymeric tannin, which was the major ingredient of tamarind extracts.

Siddhuraju (2007) reported the antioxidant activity of polyphenolic compounds extracted from TaSH. He dealt with the polyphenolic compounds as the tannins which could form the precipitates with polyvinylpyrrolidone and did not take notice of the structural properties of the polyphenolic compounds.

Some biological activities of TaSH extract have been investigated by Suksomtip and Pongsamart (2008). They reported protective effects of TaSH extract against Cu^{2+}- induced human low-density lipoprotein oxidation and oxidative damage of plasmid DNA induced by Fenton reactant.

The polyphenols from tamarind seed husk could reduce oxidative stress *in vivo* and improve the growth rate of heat-stressed broilers (Aengwanich and Suttajit, 2010), reduce heat stress in broilers (Aengwanich, et al., 2009).

Methodology for chemical characterization of flavonoids and proanthocyanidins

The preparation of a sample for analysis can present a problem since flavonoid glycosides are predominantly polar structures and, hence, water-soluble, whereas the aglycones are non polar. The latter, therefore, must be extracted by nonpolar solvents. Methanol is often a useful compromise that permits the extraction of the majority of the flavonoids (Havsteen, 2002).

Many solvent extraction systems were reported to separate phenolic, polyphenolic and flavonoids which found in TaSH such as ethyl acetate (Tsuda, et al., 1995), methanol (Sudjaroen, et al., 2005) and ethanol (Pumthong, 1999). The ethyl acetate can extract the non-flavonoids phenolic compound from TaSH (Tsuda, et al., 1995). The methanol and ethanol can extract the flavonoids and proanthocyanidins from TaSH (Sudjaroen, et al., 2005, Pumthong, 1999).

Solvent and method of homogenization affect both recovery of extraction and selectivity of extraction. Some solvents are incompatible with some methods of analyses. In particular, acetone inhibits all protein precipitation assays except the radial diffusion assay (Hangerman, 1988). Acetone/ water (7:3, v/v) has been reported to be the best solvent system for the extraction of phenolic compounds (especially procyanidins) from grape seed (Kallithraka, 1995). Aqueous acetone was a better

solvent than aqueous methanol for condensed tannins and total phenolics, but condensed tannins were less stable in aqueous acetone than aqueous methanol (Cork and Krockenberger, 1991). In many plants, there is a large fraction (sometimes>50%) of tannin that cannot be extracted (insoluble tannins) (Hagerman, 1998).

Freezed-drying may be not inappropriate for phenolic glycosides, unless done in a manner to prevent thawing during the drying process (Orians, 1995). Another commonly employed method, air-drying, is appropriate for phenolic glycosides but inappropriate for condensed tannin (Orians, 1995). However, Lindroth and Koss (1996) indicated that freeze-drying did not alter the concentrations of phenolic glycosides or tannins, relative to vacuum-drying. Lindroth and Koss (1996) suggest that the vacuum-drying method be used in studies in which carbohydrates are of no interest. The vacuum-drying allows researchers to quantify both phenolic glycosides and condensed tannins (Orians, 1995).

The commonly used assay for determination of total phenolics is the Folin-Ciocalteu assay. The chromophore produced is a blue phosphotungstic-phosphomolybdic complex of undefined structure and the underlying chemistry of this reaction is not well understood (Schofield, et al., 2001).

The study of condensed tannins has been difficult because of the structural complexity of these compounds. The simply and commonly used methods for condensed tannin determination are acid butanol assay and vanillin assay. The acid butanol assay (Figure 12) uses an acid-catalyzed oxidative depolymerization of condensed tannins to yield red anthocyanidins (Schofield, et al., 2001).

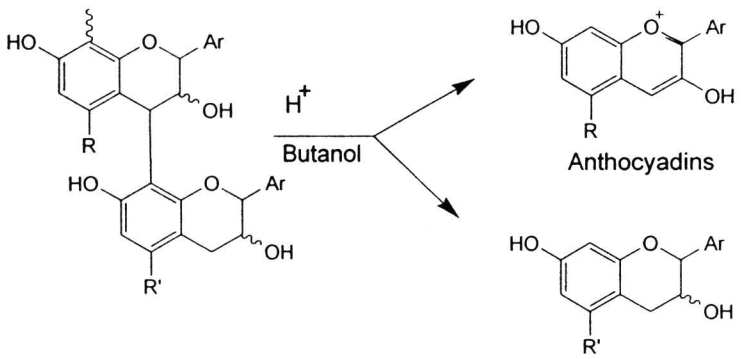


Figure 12 Chemistry of the acid-butanol reaction. Note that the reaction involves oxidation and that the terminal unit does not give a colored anthocyanidin product structure.

Source: Schofield, et al., 2001.

Vanillin reacts with the meta-substituted A-ring of flavanols (Figure 13) to form a chromophore. Therefore, the relative degree of polymerization can be estimated by comparing the absorbance obtained with the modified vanillin assay (due to terminal flavan-3-ol units) with the absorbance from the acid butanol method (due to total oligomeric flavan-3-ol residues) (Butler,1982;Hagerman,1998).

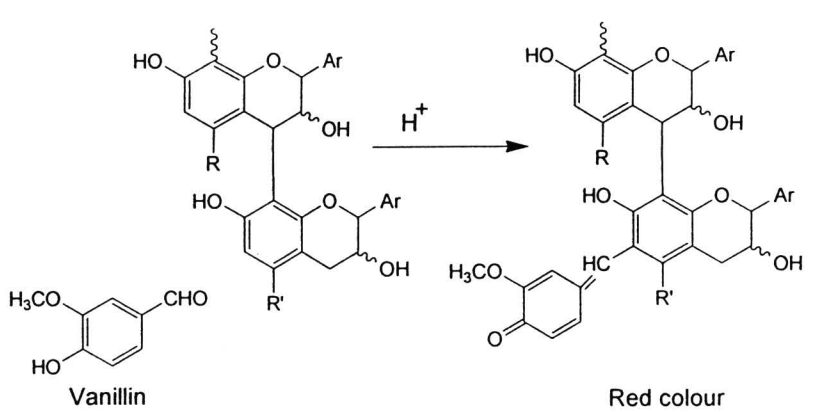


Figure 13 Chemistry of the vanillin assay for condensed tannins

Source: Schofield, et al., 2001.

The complete analysis of the absolute structure and configuration of flavonoids is usually a complicated task which requires the application of advanced techniques. Modern chromatographic techniques like HPLC have become standard equipment in biochemistry laboratories, and often yield not only an excellent resolution, but also retention times that can be very useful in the identification of flavonoids (Havsteen, 2002).

Many methods have been proposed for separating proanthocyanidins according to their degree of polymerization (DP). Normal-phase HPLC can separate proanthocyanidins according to DP up to decamers, but this method is specific for monomers and oligomers and results in a severe underestimation of proanthocyanidins, considering the prevalence of the polymers in nature (Czochanska, et al., 1980; Jerez, et al., 2007). Reversed-phase HPLC is the method commonly used for the separation of flavan-3-ol monomers and some small oligomers, especially dimers and trimers. However, there are difficulties in determining the DP using reversed-phase methods since oligomers elute in non-sequential order (Jerez, et al., 2007). Furthermore, analysis of higher oligomeric proanthocyanidins is not feasible, since the number of isomers increased with increasing DP. This effect results in a retention time overlap of isomer of differing DP, causing the higher oligomers to co-elute as a large unsolved peak (Santos-Buelga and Williamson, 2003). The technique of thin-layer chromatography (TLC), with a silica phase, permits the separation of oligomeric proanthocyanidins up to heptamer (Lea, 1978). This method can be used only for quantitative analysis.

The UV spectrums of general flavanoids have two peaks at 240-285 nm (band II) and 300-550 nm (band I) (Markham, 1982). The UV spectrum of each flavonoid families (Table 1) were reported by Markham (1982). However, most flavonoids also show the absorbance at 226 nm too (Markham, 1982).

Table 1 UV spectrums of flavonoids family (From Markham, 1982)

Band II (nm)	Band I (nm)	Types of flavonoids
250-280	310-350	Flavone
250-280	330-360	Flavonol (hydroxyl substitute)
250-280	350-385	Flavonol (hydroxyl group at C-3)
245-275	310-330 (tail)	Isoflavone
245-275	320	Isoflavone (5-deoxy-6,7-dioxygenate)
275-295	330-330	Flavanone and dihydroxy flavanone
230-270	340-390	Charcone
230-270	380-430	Aurone
270-280	465-560	Anthocyanidins

The reported IR spectrums of flavonoids were as follows. (Markham, 1982)

1. The hydroxyl groups found IR spectrum at 3,300-3,100 cm^{-1} (including hydroxyl groups with hydrogen bonded and phenoxyl groups).

2. The methyl groups found IR spectrum at 2,930 cm^{-1}

3. The carbonyl groups found IR spectrum at 1,685 cm^{-1}. Carbonyl groups of flavanone which have conjugated bond with ring A such as 5-hydroxy flavanone found IR spectrum at 1,650 cm^{-1}. Flavone which have conjugated bond with both ring A and ring B found IR spectrum at 1,655 cm^{-1}. 7-hydroxyflavone found IR spectrum at 1,630 cm^{-1}. In flavonol compounds, the IR spectrum of carbonyl group was decrease about 30 cm^{-1}.

4. The double bond of benzyl groups found IR spectrum at 1,500-1,610 cm^{-1}. And the second IR spectrum at 1,585 cm^{-1} was found in conjugated bond with benzyl groups.

5. The phenolic hydrogen found the intensive IR spectrum at 1,360 cm^{-1} and 1,200 cm^{-1}.

6. The meta-hydroxyl groups such as 5, 7-dihydroxyl found IR spectrum at 1,165 cm^{-1}.

7. The hydrogen atom of benzene found IR spectrum at 800 cm^{-1}.

Sephadex LH 20 columns are classically used to fractionate proanthocyanidins on the basis of molecular size (Jerez, et al., 2007). Purification of large quantities of tannins can be done by taking advantage of their adsorbtion on Sephadex LH-20 (Hagerman, et al., 1980). However size-based separation is not achieved on any Sephadex for tannin (Hangerman, 1998). Sephadex LH20 adsorbs condensed tannins in alcohol, and releases them in aqueous acetone (Hangerman, 1998). Chromatography on sephadex is very useful for separating tannin from nontannin phenolics (Hangerman, 1998). Because the Sephadex beads swell to different volumes in the alcohol and aqueous solvents, it is necessary to use mixtures of alcohol/water and acetone/water. Large molecular weight proanthocyanidins are then recovered with 60-70% acetone in water, with no further separation (Taylor, et al., 2003; Tourino, et al., 2005). However, the chromatographic separation of proanthocyanidins (condensed tannin) is complicated by the enormous variety of similar isomeric of them (Yanagida, et al., 2007).

The Sephadex LH20 column has been applied in conjunction with reversed phase HPLC for polymeric proanthocyanidins determination (Peng, et al., 2000; Waber, et al., 2007). Structural information for identification of polymeric proanthocyanidins can be obtained using nuclear magnetic resonance (NMR) (Schofield, et al., 2001; Kennedy, et al., 2001).

Bioavailibility of polyphenols and flavonoids

Little is known about polyphenol absorption, bioavailability, biodistribution and metabolism. The daily intake of flavonoids with normal food, especially fruit and vetgetables is 1-2g (Havsteen, 2002). Flavonoids occur in foods primarily as glycosides and polymer that are degraded to variable extents in the digestive tract (Heim, et al., 2002). The aglycones, that is, the non-conjugated forms, are generally absorbed intact from the digestive tract, while ester, glycosides, or polymers must be hydrolyzed before being absorbed (Petti and Scully, 2009). The best absorbed polyphenols are isoflavones, followed by flavanones, catechins and quercetin glycosides. The least well absorbed polyphenols are proanthocyanidins, anthocyanidins and galloylated catechins (Petti and Scully, 2009).

The studies on absorption, metabolism, and excretion of dietary procyanidins in rats have shown that dimeric procyanidins are present in the plasma (Baba, et al., 2002; Gonthier, et al., 2003). Caecal bacteria (Groenewoud, et al., 1986) and the low gastric pH (Spencer, et al., 2000) contribute to the hydrolysis of proanthocyanidin oligomers of 3 to 6 units into catechin dimers and free catechins after 3.5 hours in the gastric environment (Heim, et al., 2002). The low molecular proanthocyanidins are also known as sustained release antioxidant, and can remain in the plasma and tissues for up to 7-10 days and exert antioxidant properties, which is mechanistically different from other water soluble antioxidants (Bagchi, et al., 2000). On the contrary, high molecular weight polymeric non-extractable proanthocyanidins are very potent scavengers of peroxyl radicals, but these are not absorbable and bioavailable (Bagchi, et al., 2000). However, these high molecular weight proanthocyanidins can exert their antioxidant activity in the digestive tract and protect lipids, proteins and carbohydrates from oxidative damage during digestion and spare soluble antioxidants (Hangerman, et al., 1998). It is possible that the degree of polymerization is less predictive of antioxidant activity *in vivo* compared to *in vitro*, and the value of the latter research is limited in describing the role of proanthocyanidins in human nutrition (Heim, et al., 2002).

The high molecualar weights of condensed tannins have harmful effects on animal including human (Lu and Bennick, 1998). The highly polymerized proanthocyanidins (DP = 10), but not oligomeric procyanidins, were found to impair degranulation and membrane ruffling in cells (Kondo, et al., 2006). A major family of proteins secreted by the salivary glands of some animals constitutes the best characterized of the "defense mechanisms" against the possible toxic effects of dietary condensed tannins (Mehanso, et al., 1983). The parotid and submandibular salivary glands of some mammal synthesize a group of proteins which are unusually high in proline, the so-called salivary proline-rich protein (PRPs) (Carlson, et al., 1991). Recent evidence suggests that a primary role for these proteins may be protection against dietary condensed tannins.

Recently, Galleano, et al. (2010) reported that a disadvantage for flavonoids as antioxidants in humans is because of their very low bioavailability. Although, no definitive assessment about tissue and cellular concentrations of flavonoids can be

done at the moment, based on plasma bioavailability and cell culture experiments; it is expected to be relatively low (Galleano, et al., 2010). Flavonoids have been measured low μM or upper nM ranges after realistic consumption in human plasma (Table 2). Furthermore these concentrations are transient, peaking at 2-6 hours after their consumption in human and rodents (Rein, et al., 2000; Holt, et al., 2002). Even assuming the highest flavonoid concentration found in plasma (low mM range), the relative reaction rate with the more common oxygen radicals does not support the *in vivo* free radical scavenging effects in blood and in the vasculature (Galleano, et al., 2010).

Table 2 Estimated flavonoid presence in different body compartments

	Gastrointestinal tract	Extracellular/vascular compartments	Intracellular compartment
Total flavonoid content[a]	μM	≤ μM	< μM
Flavonoid oligomer or polymer	High concentration	Low concentration	Very low concentration of absence
Glycosylated/methylated flavonoids	High amount	??	??
Metabolites from flavonoids rupture	Principally from colonic microflora	Principally from enterocytes and extracellular metabolism	Principally from intracellular metabolism

Note: [a]Considered as the sum of the different forms (original compound and metabolites) in which each individual flavonoid can be measured in human tissues and fluids.

Source: Galleano, et al., 2010.

Effects of flavonoids on cell viability

Two types of cells used in this study were the retina ganglion cells (RGC-5) and hippocampal cells (HT22). The vertebrate retina has several features that make it vulnerable to damage from autoxidation. The photoreceptor membranes contain high levels of polyunsaturated fatty acids; abundant mitochondria are present which may leak activated oxygen species; and light exposure of the retina may cause photoxidation (Handelman and Dratz, 1986).

While previous in vitro studies often examined mechanisms of neuroprotection by the native forms of flavonoids, it has become increasingly clear that the use of biologically relevant metabolites of the flavonoids bioavailable to cells and tissues is advantageous (Pavlica and Gebhardt, 2010). The flavonoids penetration of the blood-brain barrier is dependent on the lipophilicity of the compounds (Youdim, et al., 2003)

Flavonoids are reported to exhibit a wide variety of biological effects on cells, relating to antioxidant and free radical scavenging activities (Horakova, et al., 2002, Chaudhuri, et al., 2007; Estany, et al., 2007). The uptake of flavonoids into cells is dependent on both flavonoid and perhaps more importantly the cells type (Spencer, et al., 2004). Many kinds of polyphenolic compounds, especially proanthocyanidins and other flavonoids, have been reported to protect against oxidative stress-induced cells death in neuronal (Choi, et al., 2002; Roychowdhury, et al., 2001), retina cells (Areias, et al., 2001), macrophage cells (Bagchi, et al., 2002), human normal gastric mucosal (Bagchi, et al., 2002), and rat pheochromocytoma (Horakova, et al., 2003). The antioxidant effect of quercetin improved the viability of oxidized endometrial cells when the oxidant and antioxidant were coincubated (Estany, et al., 2007).

In some of those studies, the cell viability assays relied on neutral red assay (Horakova, et al., 2003) and the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay (Ye, et al., 1999; Bagchi, et al., 2002). Some cell viability assay can detect death cells by determining the leakage of cytoplasmic enzyme lactate dehydrogenase (LDH) to extracellular medium (Areias, et al., 2001; Roychowdhury, et al., 2001). These studies have largely reported that flavonoids alone have little or no effect on the cell viability (Choi, et al., 2002; Areias, et al., 2001; Horakova, et al., 2003). Little attention has been reported that grape seed proanthocyanidin extract

enhanced the growth and viability of the normal cells whereas exhibited cytotoxity towards cancer cells using MTT assay (Ye, et al., 1999; Bagchi, et al., 2002).

Recently, the artifacts in cells-base studies have reported that many of flavonoids and condensed tannin interfered with MTT assay (Wisman, et al., 2008). Many studies of the biological effects of polyphenols in cell culture have been affected by their ability to oxidize in culture media, and awareness of this problem can avoid erroneous claims (Halliwell, 2008). The assessment of flavonoid cell uptake using in vitro cell culture models may not fully reflect uptake in vivo (Spencer, et al., 2004). The simple polyols such as mannitol, sorbitol and xylitol could clearly increase the apparent viability of hepatocytes by the uptake of neutral red up to approximately 60% but this false positive effect was not found in the MTT test (Olivier, et al., 1995).

Effects and mechanisms of oxidants and antioxidants on hemolysis

The erythrocytes were a suitable model for studying oxidative stress because of high oxygen tension, the presence of iron in hemoglobin (Hb), and a plasma membrane rich in polyunsaturated lipid (Chiu, et al., 1982, Puppo and Halliwell, 1988). Various factors such as aging, inflammation can lead to the generation of ROS such as $O_2^{\cdot-}$, H_2O_2 and $HO^{\cdot}$ in erythrocytes (Cimen, et al., 2008). A wide variety of drugs and xenobiotics that can undergo oxidation-reduction reactions have been found to cause red cell destruction and hemolytic anemia (Winterbourn, 1985).

The source of ROS in erythrocytes is the oxygen carrier Hb that undergoes autoxidation to produce. Occasional reduction of O_2 to $O_2^{\cdot-}$ is accompanied by oxidation of Hb to metHb, a rustbrown-colored protein that does not bind or transport O_2 (Cimen, et al., 2008). The superoxide ($O_2^{\cdot-}$) can convert to H_2O_2 using the enzyme SOD. H_2O_2 can easily cross cell membranes and this feature is potentially important because the extracellular environment possesses few antioxidant defense mechanisms (Al-Omar *et al.*, 2004). It has been known that H_2O_2 can react with $O_2^{\cdot-}$ (Haber-Weiss Reaction) or ferrous ions (Fenton reaction) to produce $HO^{\cdot}$, the most reactive ROS. However the mechanism of red cell $HO^{\cdot}$ formation is not as straightforward (Cimen, et al., 2008). Due to its charge, $O_2^{\cdot-}$ is concentrated in the intracellular compartment. As such, $HO^{\cdot}$ is produced predominantly from H_2O_2 by Harber-Weiss reactions whereas the Fenton reaction is more important extracellularly (Al-Omar, et al., 2004).

ROS initiate lipid peroxidation reactions in erythrocytes that lead to loss of membrane integrity, impairment of membrane protein and cell death (Dumaswala, et al., 1999). Haber-Weiss reaction was as follows:

$$O_2^{\cdot-} + H_2O_2 \longrightarrow O_2 + HO^{\cdot} + HO^{-}$$

Superoxide Hydrogen peroxide Oxygen Hydroxyl radicals Hydroxyl ion

The hemolysis induced by hydrogen peroxide was mainly decreased by catalase reaction. Ko *et al.* (1997) reported 45-65% hemolysis of human erythrocytes induced by 20 nm H_2O_2 in the presence of sodium azide which was a catalase inhibitor. The lists of journals using the hydrogen peroxide-induced hemolysis are shown in Table 3.

Table 3 The lists of journals using the hydrogen peroxide-induced hemolysis

Conc. of H_2O_2	Type of and %erythrocytes	Time	% hemolysis	Author, year
2.5% (750mM)	Uremic erythrocytes	N.R.	N.R.	Albright and White, 1982
N.R.	Rabbit	N.R.	0.8%	Horn *et al.*, 2010
1.2%	Human	N.R.	N.R.	Lubin *et al.*,1978
7.5mM	Human 2%	4h	N.R.	Paiva-Martins *et al.*, 2009
100mM	Human 10%	4h	N.R.	Paiva-Martins *et al.*, 2009
2% (600mM)	Infant human	90min	60%	Younkin *et al.*, 1971
1%	Pig 10%	N.R.	40%	Fontaine *et al.*, 1975
2.5%	Rat	4h	6%±0.5%	Kartha *et al.*, 1978

Table 3 (Cont.)

Conc. of H_2O_2	Type of and %erythrocytes	Time	% hemolysis	Author, year
100μM	Human 10%	N.R.	N.R.	Barreca *et al.*, 2009
40μM	Rat 5%	3h	N.R.	Ajila *et al.*, 2008
0.1mM-5mM	Mouse 2%	5& 30 min	N.R.	Masuoka *et al.*, 2006
0.4M	Rat	N.R.	N.R.	Catharine *et al.*, 1952
1mM	Rabbit 2.5%	N.R.	58.9%	Rafat *et al.*, 2010

Note: N.R.= Not reported

As H_2O_2 concentration is increased, a dose-dependent increase in metHb, lipid peroxidation, and spectrin-Hb complexes occurred (Cimen, et al., 2008; Snyder, et al., 1985). Synder et al. (1985) demonstrated that H_2O_2 induces a covalent complex of spectrin and Hb as well as a myriad of cellular changes that include alterations in cell shape, membrane deformability, phospholipid organization, and cell surface characteristics. The H_2O_2, in absence of catalase or superoxide dismutase, cause oxidation of hemoglobin. The reaction between hydrogen peroxide and oxyhemoblobin (Winterbourn, 1985) was as follows:

$$H_2O_2 + 2Hb\text{-}Fe^{2+}O_2 \longrightarrow 2Hb\text{-}Fe^{3+} + 2O_2 + 2OH^-$$

Hydrogen peroxide Oxyhemoglobin Methemoglobin Oxygen Hydroxyl ion

Not only ROS but also RNS play role in oxidative stress in erythrocytes. Substantial evidence supports the view that NO is a key component of the respiratory cycle, a third gas transported together with O_2 and CO_2 by erythrocytes (Cimen, et al., 2008). The erythrocytes possess endothelium-type nitric oxide synthase (NOS). Nitric oxide (NO) react with $O_2^{\cdot-}$ to yield peroxynitrite ($ONOO^-$). Intravascular $ONOO^-$

undergoes a fast reaction with oxyHb, resulting in metHb and nitrate (Romero, et al., 2006).

The erythrocytes damage due to oxidative stress is generally thought to be the end result of three processes (Lopez-Revuelta, et al., 2006). The first one is the oxidation of hemoglobin followed by the conversion of methemoglobin to hemichromes. For the second one, the increasing free radical attack on cells and induced lipid peroxidation. For the last one, a reduced glutathione (GSH) level was decreased resulting in an inability of the defense system. Each process was closely related to each other.

However, the normal erythrocyte is highly resistant to oxidative damage, because of its efficient protective mechanisms (Halliwell and Gutteridge, 1989, p.86). The erythrocyte interior is rich in superoxide dismutase, catalase, and glutathione. For extracellular hydrogen peroxide, catalase completely protected the cells, while the ability of glutathione peroxidase (GSHPx) to protect the cells was limited by the availability of glutathione (Nagababu, et al., 2003). Cohen and Hochstein (1963) have reported that GSHPx has a higher affinity for H_2O_2 than catalase, therefore, react with lower concentrations of H_2O_2. The overall metabolism of free radicals and defense system in erythrocytes are shown in Figure 14.

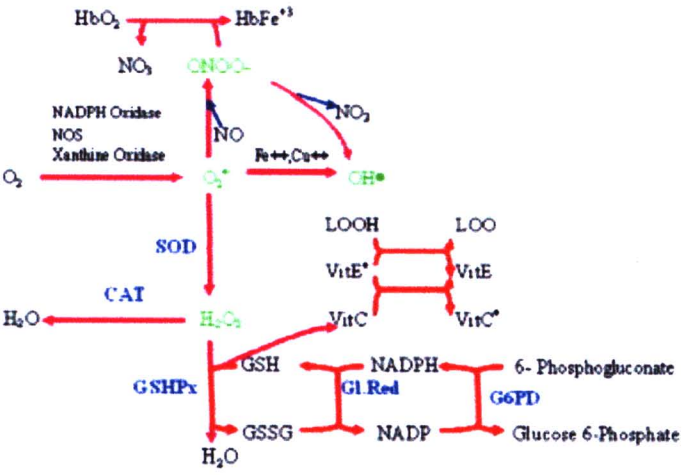


Figure 14 Main free radical metabolism pathways in erythrocytes. HbO_2= oxyhemoglobin (oxyHb), $HbFe^{+3}$= methemoglobin (metHb) , Fe^{++} = ferrous ions, NO= nitric oxide, NOS = nitric oxided synthase, LOOH= lipid peroxide, VitE = vitamin E, Vit C = vitamin C, CAT= catalase, Gl.Red= glutathione reductase and G6PD = glucose-6-phosphate dehydrogenase

Source: Cimen, et al., 2008.

Glutathione, which is synthesized in cells has antioxidant properties since the thiol group in its cysteine moiety, is a reducing agent. The reduced glutathione reacts directly with hydrogen peroxide using the enzyme glutathione peroxidase (Figure 7). Glutathione is maintained in the reduced form by the enzyme glutathione reductase in presence of NADPH (Figure 8). The NADPH required is mainly provided by the enzyme glucose 6-phosphate dehydrogenase which is the first enzyme in the oxidative pentose phosphate pathway of glucose metabolism. The GSH not only controls the H_2O_2 level but also protects hemoglobin sulfhydryl groups and red cell membranes from oxidation (Cimen, et al., 2008).

To bind oxygen, heme iron must be maintained in the reduced state (oxyhemoglobin). If these mechanisms fail, Hemoglobin (Hb) becomes non-function (Telen and Kaufman, 1999). As a result of this process, iron is released from Hb (or its derivatives) and the release is accompanied by methemoglobin (metHb) formation

(Cimen, et al., 2008). It has been suggested in particular that iron is released from iron stores under conditions in which oxidative stress is involved in both system in vitro and isolated perfuse organs (Ferrali, et al., 1992).

If erythrocytes are depleted of glutathione (GSH), the release of iron is accompanied by lipid peroxidation and hemolysis (Comporti, et al., 2002). However, GSH depletion by itself did not induce iron release, metHb formation, lipid peroxidation or hemolysis (Ferrali, et al., 1992). Rather, the fate of the cell in which iron is released depended on the intracellular availability of GSH (Ferrali, et al., 1992). Diethyl maleate, a thiol-depleting agent, can depleted reduced glutathione level in human erythrocytes (Palmen and Evelo, 1996) and induced hemolysis (Ferrali, et al., 1992). If the erythrocytes are severely depleted of glutathione (GSH) after preincubation with DEM with the other oxidants, the release of iron is followed by peroxidation of membrane lipids and hemolysis (Ferrali, et al., 1997).

Although the oxidative defense system in erythrocyte is powerful, a remarkable oxidant insult, xenobiotic drugs, or certain disorders, can increase hemolysis (Chiu, et al., 1982). If the rate of H_2O_2-production exceeds the capacity of the enzyme to generate NADPH, then GSH/GSSG ratios fall, and glutathione peroxidase stops working, leading to destruction of the erythrocytes (hemolysis), anemia, and jaundice due to the excessive degradation of released hemoglobin (Halliwell and Gutteridge, 1989, p.96).

The phenylhydrazine (PHZ, $PhNHNH_2$) is a redox reactive compound that reacts with hemoglobin (Hb) forming a number of bioactive radicals as shown in Figure 25 (Shetler and Hill, 1985). Ultimately, this process reduces the total functional Hb and results in cell lysis (Berger, 2007). The PHZ cause hemolysis in G-6-PD deficiency, as a result of methemoglobin and Heinz body formation (Hill and Thornally, 1982). Heinz bodies (also referred to as "Heinz-Ehrlich bodies" ,which are formed by damage to the hemoglobin component molecules, appear as small round inclusions within the erythrocytes. Heinz body causes an electron from the hemoglobin to be transferred to an oxygen molecule, which creates a ROS this can cause severe cell damage leading to premature cell lysis. Breakdown of the diazine (PhN=NH) is fast and $O_2^{\cdot-}$ dependent, but it is stabilized in the presence of methemoglobin by formation of hemichrome (Winterbourn, 1985). Mechanisms for

the autoxidation of phynylhydrazine catalysed by oxyhemoglobin (Modified from Hill and Thornally, 1982) were as follow:

Initiation:

(a) $PhNHNH_2 + HbFe^{2+}O_2 + H^+ \longrightarrow PhNHN{\cdot}H + HbFe^{3+} + H_2O_2$

Phenylhydrazine Oxyhemoglobin Methemoglobin Hydrogen peroxide

Superoxide-dependent propagation:

(b) $PhNHN{\cdot}H + O_2 \longrightarrow H^+ + O_2^{\cdot-} + PhN{=}NH$

(c) $O_2^{\cdot-} + H^+ + PhNHNH_2 \longrightarrow H_2O_2 + PhNHN{\cdot}H$

Termination:

(d) $2\ PhNHN{\cdot}H \longrightarrow PhNHNH_2 + PhN{=}NH$

(e) $2\ O_2^{\cdot-} + 2H^+ \xrightarrow{\text{Superoxide dismutase (SOD)}} H_2O_2$

Ferrali, et al. (1997) has reported that iron is released in a free form when erythrocytes are incubated with phenylhydrazine. This iron is released from hemoglobin and the release is accompanied by methemoblogin formation. Incubation of glutathione (GSH) depleted mouse erythrocytes with the oxidants phenylhydrazine resulted in the release of free iron and hemolysis (Ferrali, et al., 1997). The addition of the flavonoid, quercetin, which chelates iron and penetrates erythrocytes, resulted in remarkable protection against hemolysis (Ferrali, et al., 1997).

The flavonoids such as quercetin (Ferrali, et al., 1997), and red wine anthocyanins (Tedesco, et al., 2001) can protect red blood cells against the oxidant-induced hemolysis. The binding of the flavonoids to red blood cells membranes significantly inhibits lipid peroxidation (Chaudhuri, et al., 2007). However polyphenolics containing a phenol ring oxidized human erythrocyte oxyhemoglobin and caused erythrocyte hemolysis (Galati, et al., 2002). The preliminary study (Wuttisin, 2004) also found that tamarind seed husk protected normal erythrocytes

against oxidative damage by decreasing malondialdehyde (MDA) production and increasing GSH content.

Effects and mechanisms of oxidants and antioxidants on DNA damage

The ROS, when present in a high enough concentration, can damage DNA that may promote carcinogenic activity (Seifried, et al., 2007). Hydrogen peroxide, which crosses biological membranes, can penetrate to the nucleus and reacts with iron to form ROS (such as hydroxyl radical) by Fenton reaction (Halliwell, et al., 2000). The ROS generated by Fenton reaction can produce the lesions of DNA (Meneghini, et al., 1993, Patrzyc, et.al., 2001). ROS directly causes structure alteration in DNA e.g. base pair mutations, rearrangements, deletions and insertions.

However, neither $O_2^{\cdot-}$ nor H_2O_2 alone appear to cause strand breakage in DNA or modification of bases in DNA (Dizdaroglu, 1991). Intracellular iron plays a critical role in hydrogen peroxide-induced DNA damage by formation of single strand breaks using cultured Jurkat cells (Barbouti, et al., 2001). Iron and hydrogen peroxides are sources of hydroxyl radical which attack DNA by base modifications (Termini, 2000; Moriwaki, et al., 2008), strand cleavage (Meneghini, et al., 1997) and conformation change (Ambroz, et al., 2001; Moriwaki, et al., 2008). The attack by the hydroxyl free radical is the most common form of radical-induced DNA damage (Acworth, et al., 1997, p. 40). Iron-mediated formation of ROS leading to DNA damage appears to be important for the development of cancer and cancer cells (Kawanishi, et al., 2002; Valko, et al., 2006). The binding of intracellular iron by iron-chelating compounds is the most appropriate strategy to protect against H_2O_2-induced nuclear DNA damage (Melidou, et al., 2005).

The mechanism of DNA single-strand cleavage involves the attack of hydroxyl radical to the carbon-4 of the sugar moiety, generates a peroxyl radical then lead to sugar ring cleavage (Meneghini, et al., 1997; Moriwaki, et al., 2008). Box, et al. (2001) was hypothesized that strand breaks occurred via base radicals as intermediates (Figure 15). For the first step, the additions of hydroxyl radicals to base give the radical adduct. The next step involves the abstraction by the radical adduct of hydrogen from the C-4′ position of carbohydrate moiety leading eventually to a strand

break. Thus, the overall result is a base modification in conjunction with a strand break (Figure 15).

Attack on one of the strands of double-stranded DNA is not usually lethal, as the damaged DNA strand is usually held in place long enough for repair enzymes to rectify the damage. When attack by hydroxyl radicals is excessive, damage to both DNA strands can occur. This can lead to permanent strand scission of the DNA molecule and potentially to cell death (Acworth, et al., 1997, p. 41).

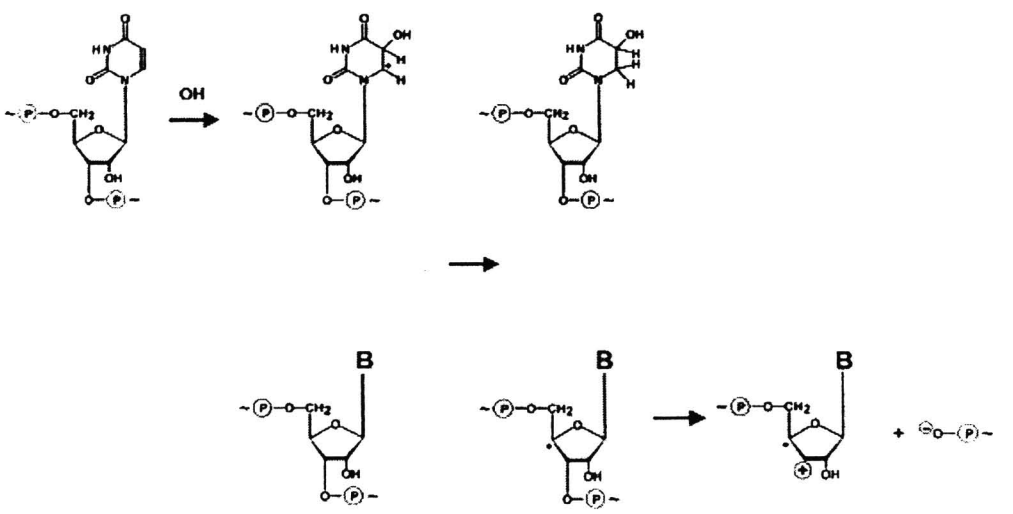


Figure 15 Mechanism proposed for free radical-induced formation of a double lesion consisting of a base modification and a strand break

Source: Box, et al., 2001.

When circular plasmid DNA is submitted to electrophoresis, the fastest migration will be observed for the supercoiled form. If the single strand of supercoid is cleaved, the supercoils will relax to produce a slower-moving open circular form (Moriwaki, et al., 2008). If double strands of supercoils are cleaved, the linear form will be generated and migrate in between. Extensive double-strand breakages lead to DNA degradation.

The non-flavonoids polyphenol such as tannic acid (TA), gallic acid (GA), propyl gallate (PA) and ellagic acid (EA) decreased the human lymphocytes DNA

strand breaks induced by H_2O_2 at concentrations of 0.1–10 μg/ml by dose dependent (Wu, et al., 2004). The low concentration of hydrolysable tannin at 0.04 and 0.1 μM significantly decreased the amount of DNA lesions induced by H_2O_2 (Labieniec, et al., 2003). The antioxidative properties of hydrolysable tannins may change to pro-oxidative activities at the higher concentrations (higher than 10 μM) which then contribute to the DNA damage (Labieniec, et al., 2003).

A number of flavonoids such as quercetin (Min and Ebeler, 2009), myricetin (Duthie, et al., 1997), and rutin (Aherne, et al., 1999) except catechin (Melidou, et al., 2005) can protect cellular DNA from hydrogen peroxide-induced strand breaks in cells containing iron (Melidou, et al., 2005). A number of flavonoids belonging to the flavone, flavonol, flavanone, and flavan-3-ol subclasses protect cellular DNA from H_2O_2-induced single-strand breaks by the comet assay (single-cell gel electrophoresis) (Melidou, et al., 2005).

The ability to protect DNA of flavonoids seems related with iron chelated ability of each flavonoids (Melidou, et al., 2005). The structure requirements of flavonoids for effective DNA protection are (a) the ortho-dihydroxy structure in either ring A or ring B, (b) the hydroxyl moiety on position 3 in combination with the oxo group at position 4, and (c) the presence of a C2, C3 double bond in ring C (Melidou, et al., 2005). Mathanolic extract from tamarind seed husk (1 mg/ml) protected supercoiled DNA strand in plasmid pBR322 against scission induced by Fenton-mediated hydroxyl radical (Suksomtip, et al., 2008).

Effects of flavonoids and tannic acid on cytotoxicity of cancer cells

Many studies have noticed increased ROS production in cancer cells that may be due to dysfunctional ROS generators and/or detoxifiers (Seifried, et al., 2007). Several studies also have assessed a number of tumor cell lines in which abnormally high levels of hydrogen peroxide are produced (Szatrowski and Nathan, 1991); other cell lines have been shown to have reduced catalase and glutathione peroxidase levels, suggesting an inability to detoxify hydrogen peroxide (Oberley, 1997). These observed abnormalities in ROS/RNS metabolism also have functional implications, as several lines of research have demonstrated, that experimental manipulation of ROS levels in cells can affect tumorigenicity (Seifried, et al., 2007).

The consumption of food products containing high amounts of flavonoids has been reported to lower the risk of various cancers (Brusselmans, et al., 2005). The cytotoxicity of flavonoids or proanthocyanidins on cancer cells have been reported (Miranda, et al., 1999; Ye, et al., 1999). In both prostate and breast cancer cells, a remarkable dose-response parallelism was observed between flavonoid-induced inhibition of fatty acid systhesis, inhibition of cell growth, and induction of apoptosis (Brusselmans, et al., 2005). The isoflavonoids stimulate the ATPase activity of the multidrug resistance protein in lung cancer GLC4/ADR cell membranes resulting in accumulation of anti-cancer drugs (Hooijberg, et al., 1997).

In normal cells, most of protein kinases are specific for the side chains of serine or threonine, whereas only a few of them are specific for the phenol group of tyrosine. In contrast, cancer cells have a strong tyrosine-specific protein kinase activity, especially in the cytoplasmic part of plasma membrane protein, growth hormone receptors and transport ATPases (Havsteen, 2002). Racker and colleagues (Suolinna, et al., 1974, 1975; Spector, et al., 1980) showed that the flavonoid, quercetin, removes the phosphate ester from the phenol group of tyrosine. Another effect of flavonoids on cancer cells is the inhibition of the glucose transporter in the plasma membrane, which furnishes such cells with glucose for glycolysis (Hume, et al., 1979).

Tannic acid, hydrolysable tannin, exerts chemopreventative activity against cancer in various animal models (Nepka, et al., 1999; Koide, et al., 1999; GaliMuhtasib, et al., 2000). When applied topically, injected or added to the diet or drinking water, tannic acid has been shown to decrease the risk of tumorigenicity in the lung (Altar, et al., 1989). Tannic acid has been shown to possess anti-cancer activity in leukemia (Chen, et al., 2009). The molecular mechanisms responsible for the cancer chemopreventative activity of tannic acid remain unknown.

A screening test using cancer cell lines is usually performed as an initial step in the evaluation of chemicals for cancer prevention and treatment (Skehan, et al., 1990). Those compounds that are found to be potent in killing cancer cells are then further screened for their ability to suppress the growth of tumors in animal models (Miranda, et al., 1999).