CHAPTER II

LITERATURE REVIEW

A. *Raphanus sativus* Linn. (Radish)

Raphanus sativus Linn. belongs to family Brassicaceae (Crucifereae - Mustard family). The common names of this plant are radish, oriental radish, garden radish, long-podded radish (English), chinese radish, white radish or daikon (China and Japan). The name of this plant is suggested by its color. The genus is distinguished by its elongated pod, which has no longitudinal partition when ripe, but contains several seeds separated by a pithy substance filling the pod. It is originally from Europe and Asia. It grows in temperate climates at altitudes between 190 and 1240 m. It is 30-90 cm high and its roots are thick and of various sizes, forms, and colors (Figure 1). They are edible with a pungent taste. This plant is used popularly to treat liver and respiratory illnesses (George and Pandalai, 1949).

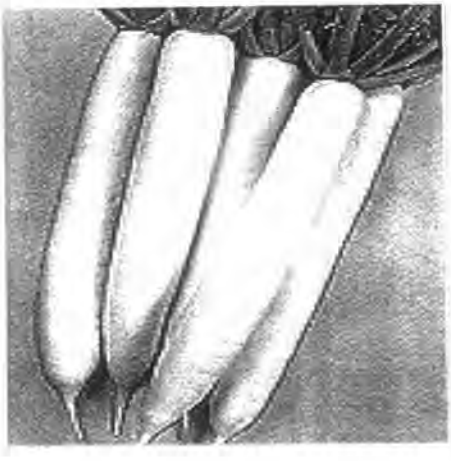


Figure 1. *Raphanus sativus* L. *var. longipinnatus*

1. Chemical constituents of *R. sativus* root

The chemical compounds presented in the root of *Raphanus sativus* Linn. can be classified into eight groups, namely alkaloids and nitrogen compounds, coumarins, enzymes, phenolic compounds, polysaccharide, sulfur, protein and other consituents as shown in Table 1.

Table 1. Chemical constituents in the root of *Raphanus sativus* L.

Compounds	Ref.
Alkaloids and Nitrogen compounds	
1. Raphanusamide	Hasegawa *et al.*, 1986
Coumarins	
Scopoletin	Harborne and Baxter, 1993
Enzymes	
1. Glutathione reductase	Thompson, Turner and Gering, 1964
2. Peroxidase c	Yuhei, 1973
3. β-Amylase	Shigeo and Yuhei, 1976
4. Cysteine synthase	Tamura *et al.*, 1976
5. Myrosinase	Shyamala and Singh, 1987
6. Cationic isoperoxidases: C1 and C3	Lee and Kim, 1995
and anionic isoperoxidases: A1, A2,	
A3n and A3	Jin and Kyung, 2001
7. Superoxide dismutase	
Phenolic compounds	
1. Raphanusin	Harborne, 1963
2. Kaempferol	Isikura, Hoshi and Hayashi, 1965
3. Cyanidin	Narbut, Samorodova and Fedorov, 1972
4. p-Hydrobenzoic acid	Stoehr and Herrmann, 1975

Table 1. Chemical constituents in root of *Raphanus sativus* Linn. (Continued)

Compounds	Ref.
Phenolic compounds (Continued)	
5. Kaempferol-7-O-rhamnoside	Kamil and Kalina, 1977
6. Gentisic acid, hydrocinnamic acid, salicylic acid and vanillic acid	Strack *et al.*, 1985
7. Pelargonidin	Guisti, Ghanadan and Wroslstad, 1998
8. Quercetin, kaempferol	Stoehr and Herrmann, 1975
Polysaccaride	
Lipopolysaccharides (LPS)	Matsuura and Hatanaka, 1988
Protein	
1. Ferredoxin isoproteins	Keishiro, Oka and Hirozo, 1985
2. Arabinogalactan-proteins (AGPs)	Tsumuraya *et al.*, 1988
Sulfur	
1. *trans*-4-Methylthio-3-butenyl-isothiocyanate	Friis and Kjaer, 1966
2. Phenethylisothiocyanate	Creason, Madison and Thompson, 1985
Other constituents	
Vitamin C	Bulinski and Zhinda, 1962

2. Biological activities

2.1 Antimicrobial activity

This common plant may be an important source of antimicrobial substances (Abdou et al., 1972). Crude juice of the radish inhibited *in vitro* the growth of *Escherichia coli*, *Pseudomonas pyocyaneus*, *Salmonella typhi*, and *Bacillus subtilis*. The ethanolic and aqueous extracts showed activity against *Streptococcus mutans* and *Candida albicans* (Caceres, 1987). The pungent principle extracted from radish root is *trans*-4-Methylthio-3-butenyl-isothiocyanate (Friis and Kjaer, 1966). This compound possesses antimicrobial activity with the MIC against fungi and bacteria ranging from 50-400 μg/ml. The antifungal and antibacterial actions were due to sporicidal and bactericidal activities (Terras *et al.*, 1995).

2.2 Antitumor activity

A neutral fraction of kaiware daikon radish aqueous extract showed *in vitro* proliferation inhibition of mouse embryo fribroblast 3T3 cells and papovavirus SV40-transformed 3T3 cells with IC_{50} of 17.4 and 8.7 μg/ml, respectively. Aqueous extract of the roots showed antimutagenic activity against *Salmonella typhimurium* TA98 and TA100 (Caceres, 1987).

2.3 Antiviral activity

The lipopolysaccharides showed antiherpes activity. Aqueous extract of leaves showed antiviral effect against influenza virus (Strack *et al.*, 1985).

2.4 Intestine motility stimulation

The effect of radish aqueous extract at doses of 10 μg/ml to 2 mg/ml caused a dose-dependent increase in the contractions of duodenum, jejunum, and ileum (Yong *et al.*, 2000).

2.5 Cardiovascular disease prevention

Radish powder decreased the lipid levels by increasing the fecal excretion of total lipids, triglycerides, and total cholesterol. Superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-Px) activities in the liver were increased by radish powder. Xanthine oxidase (XOD) activities in the liver were decreased by radish. Flavonoids and vitamin C in radish may inhibit lipid peroxidation, promote liver and

RBC catalase, and inhibit XOD activities in animal tissues. Radish can be recommended for the treatment and prevention of diseases such as cardiovascular disease and cancer and for delaying aging (Jin and Kyung, 2001).

2.6 Other activities

Lipopolysaccharides (LPS) were isolated from radish having a macrophage activating activity with ED_{50} of 0.4-100 ng/ml. These compounds can be used as antidiabetic agents in pharmaceutical or veterinary fields. The LPS also showed analgesic activity (Matsuura and Hatanaka, 1988). In the radish, the allyl isothiocyanate was identified as a possible sensitizing substance. In some cases, it can produce allergic contact dermatitis (Mitchell and Jordan, 1974).

Bulinski and Zhinda (1962) reported the content of vitamin C in aqueous radish juice was 17.95 - 27.86 mg%.

Stoehr and Herrmann (1975) reported that two major flavonoids in methanol extract of white radish were quercetin(17.5 mg/kg) and kaempferol (38.5 mg/kg).

Recently, Yoshiaki et al. (2003) reported the scavenging effect of methanol extract of radish sprout (Japanese name "kaiware-daikon") compared with L-ascorbic acid on hydroxyl radical using the bleomycin-Fe method. The methanol extract of radish sprout exhibited the highest potency (1.8 times that of L-ascorbic acid).

In addition to the above finding about radish, women in Thailand have used fresh white radish root as a traditional treatment of melasma.

Various effects of the chemical compounds in radish have been reported in term of food. However, its many other beneficial properties, especially for cosmetic and dermatological applications are not widely known or studied.

B. Phytochemical background

Historically, most of the food plants (including fruits and vegetables) has been used as medicinal agents due to the number of phytochemicals (bioactive non-nutrient plant chemicals) presented in varying levels of different plants. They are natural compounds of low molecular weight with none or minimum toxicity. The primary action of all these substances are mostly concerned with their antioxidant activities. Moreover, the search for cheap, renewable and abundant sources of antioxidant compounds is gaining worldwide interest. Among these several phytochemicals in food plant, a lot of attention has been focused on plant polyphenols due to their positive health benefits.

(1) Plant polyphenols

Plant polyphenols are a group of chemical compounds, which are secondary plant metabolites and are the most numerous and widely distributed in the plant kingdom. They are abundant in diet and have a wide range of structures and functions. Basically, the common structure possesses an aromatic ring bearing one or more hydroxyl substituents. They have been classified into major groupings distinguished by the number of constitutive carbon atoms in conjunction with the structure of the basic skeleton. Distinctions are thus made between the phenolic acids, flavonoids, stilbenes, and lignans (Figure 2).

Plant polyphenols possess several common biological and chemical properties, namely, antioxidant activity, the ability to scavenge both active oxygen and nitrogen species, to chelate metal ions, the potential sources for auto-oxidation, as well as the capability to modulate certain cellular enzyme activities (Robards et al., 1999). Moreover, the consumption of polyphenols in the diet is associated with a lower incidence of diseases, particularly of degenerative diseases, such as cancer, heart disease and improved human health and well being.

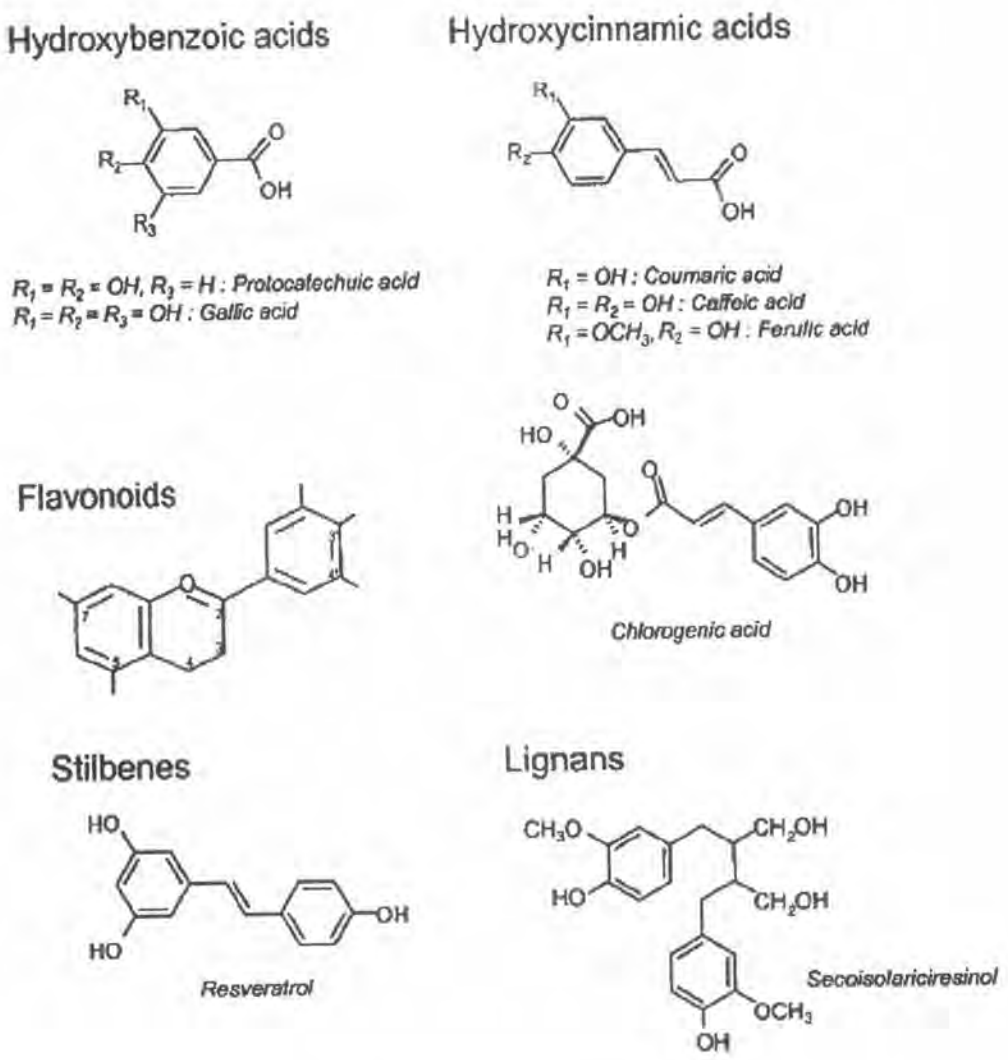


Figure 2. Chemical structures of polyphenols

(2) Flavonoids

Among the polyphenols, flavonoids are a large class of compounds, ubiquitous in plants and are one of the most common and naturally occurring compounds with low molecular weight (Rice-Evans, Miller and Paganga, 1997). They are widely distributed in fruits and vegetables. The basic structure is (C_6-C_3-C_6), the two aromatic rings linked through three carbons that usually form an oxygenated heterocycle (Leighton, 1999).

Flavonoids can be subdivided into groups depending on the structural variations within the rings; such as unsaturation and oxidation of the three-carbon

segment, the number and nature of substitute groups attached to the rings (Robards et al., 1999) . They are (1) flavonols (eg. quercetin and kaempferol), with the 3-hydroxy pyran-4-one C ring; (2) flavones (eg. apigenin and luteolin), lacking 3-hydroxyl group; (3) flavanols (eg. catechins), lacking the 2,3-double bond and the 4-one structure; (4) isoflavones (eg. genistein), in which the B ring is located in the 3 position on the C ring; (5) flavanones (eg. naringenin and hesperetin), with the presence of a saturated 3-C chain and a keto carbon in 4-position; (6) anthocyanidins, the water soluble pigments responsible for red, blue and violets color (eg. pelargonidin and cyanidin) (Rice-Evans, Miller and Paganga, 1997) (Figure 3).

Diet rich in flavonoids are flavonols (quercetin) found in apples, onions and broccoli, flavanols (catechins) in green tea and red wine, flavanones primarily found in citrus fruits, and anthocyanins presented in cherries, berries and grapes.

Various assay methods for phenolic compounds have been developed. These assays can be classified as either those which determine total phenolics content, or those quantifying a specific group or class of phenolic compounds. Folin-Denis assay and Prussian blue test are examples of method used for total phenol determination. Vanillin test is used as an assay method for catechin and proanthocyanins, while protein precipitation methods are employed for biologically active phenols. A number of chromatographic techniques have also been developed to identify and quantify specific phenolics (Shahidi and Naczk, 1995).

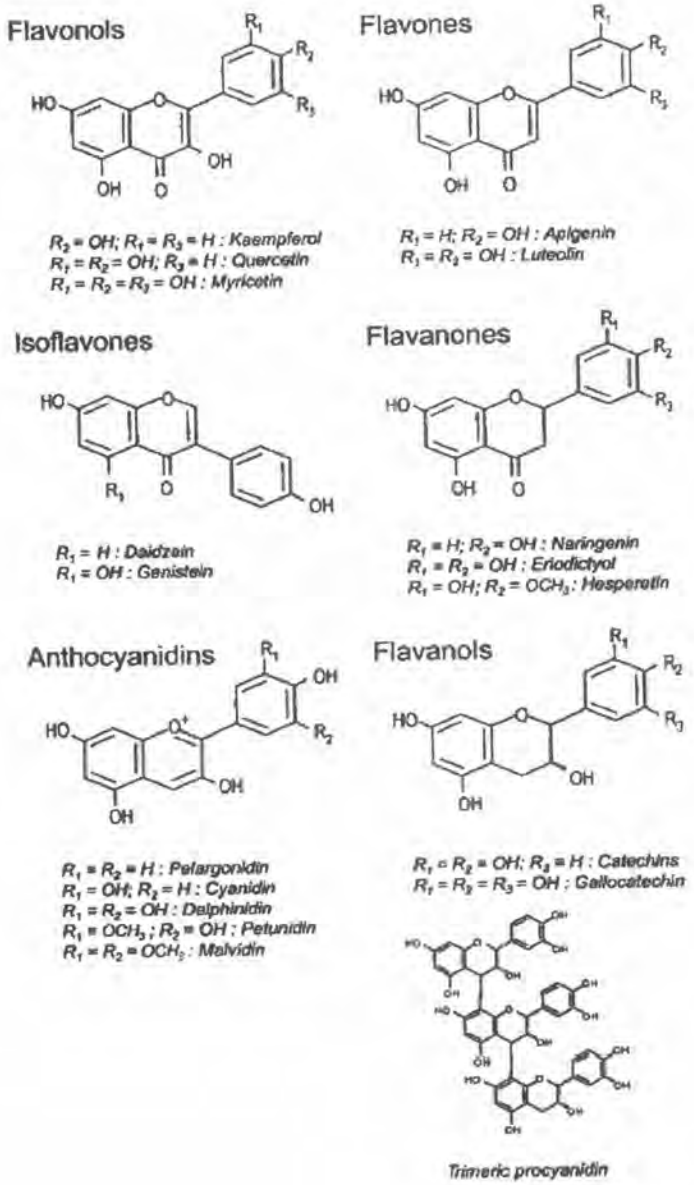


Figure 3. Chemical structures of flavonoids

C. Melanin synthesis.

Melanocytes constitute 2 to 5% of the total epidermal cell population. These cells, which produce the pigment melanin, and their neighboring keratinocytes, are closely associated. Keratinocytes are the pigment recipients. Melanin synthesis, or the production of pigment within melanocytes, is a multi-step enzymatic process.

There are basically two types of melanin in mammals, the eumelanins and pheomelanins. The former are brownish-black and the latter range in color from yellow to reddish-brown. Raper in the 1920's laid the ground work that was later extended by Mason for understanding the mechanisms of tyrosine conversion into melanin by the actions of the enzyme tyrosinase. The most significant outcome of their work was the derivation of the Raper-Mason pathway of melanogenesis (Figure 4). The two initial steps involve the tyrosinase-mediated hydroxylation of tyrosine to 3, 4-dihydrophenyalanine (DOPA), and the oxidation of DOPA to Dopaquinone (DQ). It has been presumed that the backwards reactions from DOPA and Dopaquinone are so small compared to the forward reactions that they can be neglected.

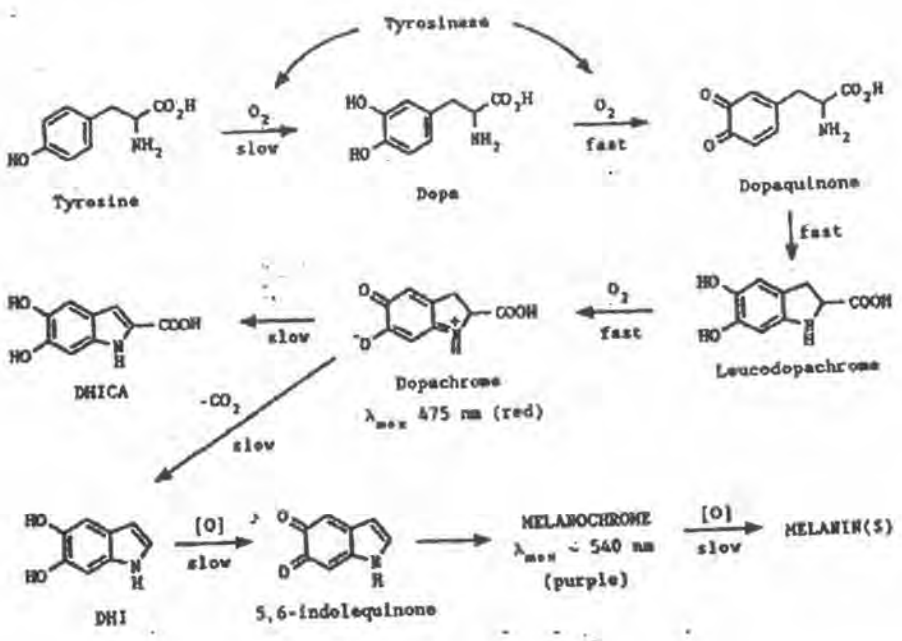


Figure 4. The Raper-Mason pathway of melanogenesis

The Raper-Mason pathway has been further updated as ongoing studies developed new elucidations into metabolic pathways of melanin synthesis. An updated comprehensive pathway (Figure 5) depicts the two major pathways of synthesis that are divided into eumelanin and pheomelanin pathways.

The major steps in eumelanin formation are the cyclization of dopaquinone to leucodopachrome which is immediately oxidized to form dopachrome (DC). DC is a relatively stable intermediate with a half-life of approximately 30 minutes, after which it is automatically rearranged to form 5, 6-dihydroxylindole-2-carboxylic acid (DHICA). DC may also spontaneously decarboxylate to 5, 6-dihydroxyindole (DHI) which will rapidly oxidize to form indole 5, 6-quinone. The final step is the polymerization to eumelanin.

The pheomelanin pathway is initiated by the addition of thiols such as glutathione and cysteine to DQ. The glutathionyldopa is rapidly converted to cysteinyldopa by γ-glutamyl transpeptidase (GTP). Oxidation of cysteinyldopa to cyclic quinine-imine intermediates that rearrange into benzothiazine derivatives occur. The benzothiazine derivatives rearrange into pheomelanin and trichochromes (melanin pigment of low molecular weight) through a series of not clearly understood reaction (Figure 5). The interaction between the eumelanin and pheomelanin compounds gives rise to a heterogeneous pool of mixed type melanins (Sanchez-Ferrer, Rodriquez-Lopez and Garcia-Canovas, 1995).

The most important regulator of melanin synthesis is UV radiation, which can stimulate melanocytes directly or indirectly by inducing paracrine secretion of various agents by keratinocytes. UV radiation increases the activity of tyrosinase and results in tanning. It can lead to pigmentation problems and increased risks of skin cancer. UV- induced DNA photoproducts increase melanin synthesis.

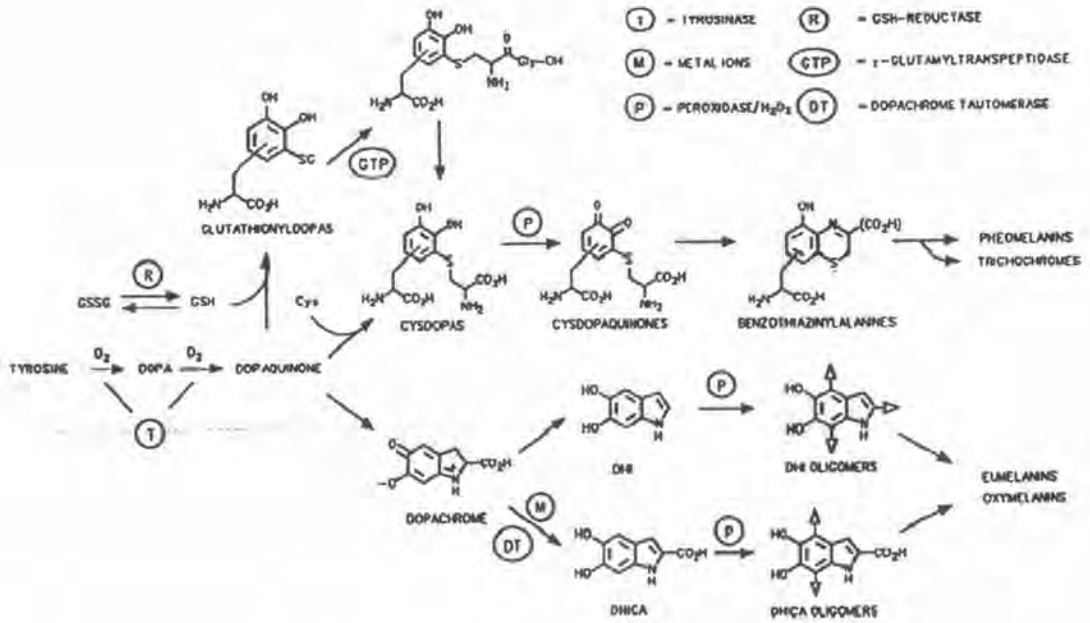


Figure 5. The scheme of melanogenesis leading to melanins and related metabolites

Tyrosinase plays a key role in melanin synthesis. However, many other cellular factors regulate melanin biosynthesis, including the enzymes dopachrome tautomerase (also known as TRP-2, a tyrosinase-related protein), peroxidase, catalase and glutathione reductase, metallic ions like Cu^{2+}, Zn^{2+}, Fe^{2+} as well as hormone and histamine (Lee and Kim, 1995).

Whitening agents are used for the treatment of skin hyperpigmentation. Since hyperpigmentation or the overproduction of melanin pigment is one of the most commonly found skin disorders, several agents had been evaluated for their possible depigmenting or skin whitening effects. Some of these agents are listed in Table 2 together with their mechanisms of action.

Skin lightening formulations have been important in Asia. Many herbs have been reported to exhibit lightening activity, generally due to inhibition of tyrosinase activity (Masuda, Tejima and Suzuki, 1996). Skin-lightening agents can be classified as either phenolic (hydroquinone and related compounds such as arbutin) or non-phenolic (i.e. azelaic acid, kojic acid, licorice extract, paper mulberry extract, etc).

Table 2. The action mechanisms of some depigmenting materials (Lee and Kim, 1995)

Mechanism	Depigmenting materials	Remark
Inhibit tyrosinase activity	Licorice extract, mulberry root extract, *Scutelleria baicalensis* extract, kojic acid, arbutin, glutathione, vitamin C derivatives	-
Inhibit melanosome transfer from melanocyte to keratinocyte	Niacinamide (vitamin B_3)	-
Inhibit tyrosinase synthesis	Glucosamine, galactosamine, monosamine, tunicamycin, linoleic acid	not specific to tyrosinase, potent cytotoxic effect
Scavenge free radicals	Tocopherol	less whitening effect
Stimulate epidermal turnover	Butylated hydroxyanisole (BHA), lactic acid	-
Interrupt intermediates in melanin biosynthesis	Kojic acid	vague mechanism
Cytotoxic effect on melanocytes	Hydroquinone	high toxicity to the skin
Reduce melanin formed and inhibit auto-oxidation	Tocopherol, vitamin C derivatives	obscure effect
Stimulate melanin elimination through the keratinocytes	Placental protein, azelaic acid	obscure effect

Examples of whitening agents commonly used in the commercial preparations are:

1. Phenolic compounds

Arbutin (Hydroquinone-beta-D-glucopyranoside)

Arbutin, a hydroquinone derivative isolated from the bearberry shrub. Arbutin inhibits melanin synthesis by inhibiting tyrosinase activity. The effective concentration for treating hyperpigmentation has not been established in a clinical trial, but manufactures have reported efficacy at 1% (Chakraborty, Funasaka and Komoto, 1998).

2. Non-phenolic compounds

Licorice extracts

Glabridin is the ingredient in licorice extract. Its inhibitory effect on skin pigmentation had been investigated and the results showed that glabridin inhibited tyrosinase activity of melanocytes without cytotoxicity. A 0.5% concentration has been shown to inhibit UVB-induced pigmentation and erythema. It is also anti-inflammatory due to inhibition of superoxide anion production and cyclooxygenase activity (Yokota, Nishio and Kubota, 1998).

Kojic acid

Kojic acid or 5-hydroxy-2-(hydroxymethyl)-4-pyrone is a substrate extracted from the fermentation fluid of koji mold. It inhibits tyrosinase activity by chelating the copper in the tyrosinase molecule (Maeda and Fukada, 1991). In cultured human melanocytes, tyrosinase activity per well was slightly reduced by kojic acid at the concentration range between 0.1 and 0.5 mM, but was rapidly and dose-dependently reduced at higher concentrations.

Ascorbic acid (vitamin C)

In ascorbic acid-treated cells, tyrosinase activity per well was slightly reduced at final concentrations between 0.05 and 0.5 mM, but rapidly and dose-dependently reduced at higher concentrations. Ascorbic acid was oxidized rapidly in the aqueous phase, with loss of activity in time and very limited transcellular potency owing to its being hydrophilic. Some ascorbic derivatives were considered stable and

transcutaneous with regard to antipigmenting function. For example, magnesium ascorbic acid phosphate, which is a stable ascorbic acid derivative, prevented erythema and post-inflammatory hyperpigmentation following UV irradiation in humans. Lipophilic ascorbic acid was also reported to prevent freckles and melanin spots on the skin. Ascorbic acid is a potent antioxidant in addition to its anti-enzymatic properties. It may prevent melanin synthesis by suppressing inflammation and by inhibiting the auto-oxidation of DOPA and dopaquinone (Maeda and Fukada, 1991).

D. Free Radical and Oxidation Reaction

1. Oxidation mechanism

Oxygen is the most prevalent element on earth and constitutes a large amount in the atmosphere as oxygen molecule (O_2). It plays a pivotal role for all aerobic organisms by generating energy and activates enzymes for normal body metabolic functions. Oxygen is relatively non-reactive. However, during respiration at a cellular level, some oxygen molecules are converted into "free radicals" as a consequence of oxidation.

Oxidation is the chemical process and is the part of the normal metabolism in which oxygen adds to and withdraws energy from carbon-based molecules resulting in loss of electron from an atom or ion. Indeed, this reaction is useful for the synthesis of nucleic acids, hormones and proteins. Besides this reaction, reactive oxygen species (ROS) are evolved as by-products (Harman, 1956).

2. Free radicals

Free radicals are atoms or molecules that are generally stable in the ground state. An atom is considered to be "ground" when every electron in the outermost shell has a complimentary electron that spins in the opposite direction. By definition, a free radical is any atom (e.g. oxygen, nitrogen) with at least one unpaired electron in the outermost shell, and is capable of independent existence. A free radical is easily formed when covalent bond between entities is broken and one electron remains with each newly formed atom (Karlsson, 1997). For example, oxygen centered free radicals contain two unpaired electrons in the outer shell. When free radicals steal an electron from a surrounding compound or molecule, a new free radical is formed in its place. In turn, the newly formed radical then looks to return to its ground state by stealing electrons with antiparallel spins from cellular structures or molecules.

Any free radical involving oxygen can be referred to as a reactive oxygen species (ROS) or reactive nitrogen species (RNS) if nitrogen is involved. The types of radicals are shown in Table 3 (Halliwell, 1997). Besides the free radicals, there are other species that are non-radical in nature but also very reactive such as singlet-oxygen. Thus, the free radicals and the non-radicals are often collectively called reactive oxygen or reactive nitrogen species (ROS and RNS). These species are unstable, highly reactive molecules and capable of reacting with each other or with

other molecules to equilibrate its charge and to form more or less reactive molecules. It is believed that free radicals are one of the causes of many diseases.

Table 3. Reactive oxygen and nitrogen species

Radicals	Nonradicals
Reactive oxygen species (ROS)	
Superoxide, $O_2^{\cdot -}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $HO^{\cdot}$	Singlet oxygen, $^1O_2$
Peroxyl, $RO_2^{\cdot}$	Hypochlorous acid, HOCl
Hydroperoxyl, $HO_2^{\cdot}$	
Reactive nitrogen species (RNS)	
Nitric oxide, $NO^{\cdot}$	Peroxynitrite, $ONOO^-$
Nitrogen dioxide, $NO_2^{\cdot}$	Nitrous acid, HNO_2

In biological systems, free radicals are continuously produced in the body as the result of the normal metabolic processes from mitochondria, phagocytes, inflammation and enzyme action. External environment stimuli such as toxic substances, microbial attacks, ozone, UV radiation, cigarette smoke, or intensive exercise are other sources of free radical formation (Dufresne and Farnsworth, 2001).

Reactive oxygen species (ROS) formation is of great concern and interlink with oxidative stress. Oxidative stress is induced by an overproduction of ROS, leading to an improper balance between the formation and the destruction of free radicals in organisms.

The ROS comprise molecules with oxygen-centered radicals such as superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($HO^{\cdot}$), and non-radicals such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), etc.

(a) Superoxide anion ($O_2^{\cdot -}$)

Superoxide anion is the most common intracellular radical. Generally, *in vivo* $O_2^{\cdot -}$ radicals can be generated by phagocytic cells such as macrophages during defense against foreign organism. The superoxide anion is created from molecular oxygen by the addition of an electron as shown below:

$$O_2 + e^- \longrightarrow O_2^{\cdot -}$$

Superoxide anion lacks the ability to penetrate lipid membranes and is therefore enclosed in the compartment where it was produced. The protonated form of superoxide anion, hydroperoxyl radical $HO_2^{\cdot}$, is somewhat more reactive than superoxide anion itself. $HO_2^{\cdot}$ should be able to cross membranes as easily as H_2O_2. Thus, $HO_2^{\cdot}$could conceivably produce damage. The formation of superoxide takes place spontaneously, especially in the inner mitochondrial membrane with the respiration chain. Superoxide is also produced endogenously by flavoenzymes, e.g., xanthine oxidase activated in ischemia-reperfusion. Other superoxide-producing enzymes are lipoxygenase and cycloxygenase. Superoxide is involved with several damages such as lipid peroxidation, cellular toxicity and single strand breaks of DNA. Many toxic effects attributed to $O_2^{\cdot -}$ could be due to its metal-catalyzed interaction with H_2O_2 to produce hydrogen radical $HO^{\cdot}$. However, superoxide anion radical life span depends on the presence of enzyme superoxide dismutase (SOD), which catalyzes it to H_2O_2 and molecular oxygen (Jay and Berthon, 1998; Nordberg and Arner, 2001; Cho, 2002).

$$2O_2^{\cdot -} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

(b) Hydroxyl radical ($HO^{\cdot}$)

Hydroxyl radical is a very energetic, short-lived and toxic oxygen species. Due to its strong reactivity with biomolecules, hydroxyl radical is probably capable of doing more damage to biological systems than any other ROS. It can react with molecules which are able to give an electron such as enzymes, sugars, aminoacids, nucleic acids or membrane phospholipids. Hydroxyl radical has a large destructive and mutagenic potential in biological systems. It mainly reacts with fatty acids of membranes which lead to membrane disorganization. As the membrane plays a major role in cellular functions, the effects can be cellular destruction or a wrong transmission of messages inside the cell.

Hydroxyl radical is produced by many mechanisms such as radiolysis of water, superoxide-driven Fenton reaction (Haber-Weiss) and metal-catalyzed decomposition of hydrogen peroxide (Fenton reaction), etc. (Fenton, 1984; Jay and Berthon, 1998; Nordberg and Arner, 2001).

$$H_2O_2 + O_2^{\cdot -} \longrightarrow O_2 + OH^- + HO^{\cdot}$$ (Haber-Weiss reaction)

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + HO^{\cdot}$$ (Fenton reaction)

(c) Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is not a free radical but is nonetheless highly important because of its ability to penetrate biological membrane (Halliwell, 1997). It plays a radical forming role as an intermediate in the production of more reactive ROS molecules including hypochlorous acid (HOCl) by the action of myeloperoxidase, an enzyme present in the phagosomes of neutrolphils, and most importantly, the formation of hydroxyl radical via oxidation of transition metals. Transition metals may cause the so-called heterolysis of H_2O_2, the result of which is a split of the molecule into regular HO^- ion, and a hydroxyl free radical ($HO^{\cdot}$). The iron-induced reaction of H_2O_2 is called Fenton reaction: it has been empirically described as the most potent oxidative mixture already toward the end of the 19th century (Fenton, 1984; Dombi et al., 2000). Hydrogen peroxide can be generated by divalent reduction of oxygen or by enzymatic dismutation of superoxide anion by SOD. Hydrogen peroxide is removed (decomposed) by at least three antioxidant enzyme systems, namely, catalase, glutathione peroxidase, and peroxiredoxins (Jay and Berthon, 1998; Nordberg and Arner, 2001).

(d) Singlet oxygen ($^1O_2$)

Singlet oxygen is obtained by several processes such as the irradiation of "normal" oxygen in the presence of a photosensitizer or the absorption of energy from photo-excited photosensitizer molecules of oxygen. Photosensitizer, such as flavins, tryptophan, tyrosine, quinine, porphyrin, NADH, NADPH, and nucleotide, etc., is excited by a photon (Fuchs, 1998). It transfers its energy to oxygen which is photon-excited. A new reactive species is formed, i.e., singlet oxygen, which is not a radical: there are no unpaired electrons. It can react with chromophores and attack the photosensitizer itself, which leads to a photodynamic effect. Singlet oxygen reacts with several compounds containing carbon-carbon bond such as polyunsaturated fatty acids in membranes to form lipid peroxidation. Production of $^1O_2$ has been shown to cause lipid peroxidation in human dermal fibroblasts, collagen cross-linking, and

matrix metalloproteinase production in human dermal fibroblast (Scharffetter, 1997; Fuchs, 1998; Jay and Berthon, 1998; Mc Vean, Stickland, and Liebler, 1999).

Indeed, free radicals are parts of the immune system which intercepts the challenge of invaders like microbes and viruses, but in certain condition they tend to attack the body by altering the cell membranes, tamper with DNA, and accumulate oxidized LDL which lead to coronary heart disease and in worst case may lead to cancer and cell death. Although regular exercise builds up body defense systems, an increase in demand and utilization of oxygen increases the free radical formation.

Normally, aerobic organisms are protected from oxidative stress induced by free radicals and non-radicals by an array of defense systems. As summarized in Table 3, various kinds of antioxidants with different functions play an important role in these defense mechanisms. The preventive antioxidants acting in the first defense line suppress the formation of free radicals and reactive oxygen species. The radical scavenging antioxidants are responsible in the second defense line and inhibit chain initiation and/or break the chain propagation. The repair enzymes such as phospholipase, protease, DNA repair enzymes and transferases act as the third line of defense (Noguchi and Niki, 1999).

With increasing experimental, clinical, and epidemiological evidence which shows the involvement of oxidative stress in a variety of diseases, cancer, and aging, the role of antioxidants has received increasing attention.

Table 4. Defense systems *in vivo* against oxidative damage

1. Preventive antioxidants: suppress the formation of free radicals	
(a) Non-radical decomposition of hydroperoxides and hydrogen peroxide	
Catalase	Decomposition of hydrogen peroxide
Glutathione peroxidase	Decomposition of hydrogen peroxide and free fatty acid hydroperoxides
Phospholipid hydroperoxide, glutathione peroxidase	Decomposition of phospholipid hydroperoxides
Peroxidase	Decomposition of hydrogen peroxide and lipid hydroperoxides
Glutathione D-transferase	Decomposition of lipid hydroperoxides
(b) Sequestration of metal by chelation	
Transferrin, lactoferrin	Sequestration of iron
Haptoglobin	Sequestration of hemoglobin
Hemopexin	Sequestration of heme
Ceruloplasmin, albumin	Sequestration of copper
(c) Quenching of active oxygen species	
Superoxide dismutase (SOD)	Disproportionation of superoxide anion
Carotenoids, vitamin E	Quenching singlet oxygen
2. Radical-scavenging antioxidants: scavenge radicals to inhibit chain initiation and break chain propagation*	
Hydrophilic: Vitamin C, uric acid, bilirubin, albumin	
Lipophilic: Vitamin E, ubiquinol, carotenoids, flavonoids	
3. Repair enzymes: repair the damage and reconstitute membranes	
Lipase, protease, DNA repair enzymes, transferase	

*Lipid peroxidation consists of three steps, namely, chain initiation, chain propagation, and chain termination.

E. Antioxidant Mechanisms

An antioxidant is any substance that when present at low concentrations compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate (Halliwell and Gutteridge, 1989; Halliwell, 1997). The term "oxidizable substrate" includes almost everything found in living cells, including proteins, lipids, carbohydrates, and DNA. On the other hand, antioxidants are molecules that interact with the "free radicals" thereby neutralizing them, which results in protecting normal tissue and DNA from potential damage. Because of the seriously damaging potential of reactive oxygen species, cells depend on elaborate defense mechanisms to effectively neutralize or metabolize these toxic intermediates and to prevent significant free radical-induced injury. Fortunately, the normal body mechanism has its own antioxidants to neutralize "free radicals" (Harman, 1956). Basically, the mechanisms of antioxidants involve three different pathways as previously shown in Table 3; (1) act as preventive antioxidant which reduces the rate of initiation of free radicals, (2) act as chain-breaking antioxidant which interacts rapidly with the radicals after chain-reaction is initiated, and converted to the stable free radicals and inhibit the propagation phase, (3) repair compounds to their original state or degrade them to non-functional compounds (apoptosis) where enzymes reaction are also involved (Bidlack et al., 1998).

In a biological system, a complex antioxidant defense system normally exists to protect its cellular system against the injurious effects and the cellular damages caused by free radical production. Cells possess enzymatic and non-enzymatic internal defense systems for protection against ROS, and consequently prevent cellular damages. For instance, enzymatic antioxidants comprise certain enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase whereas non-enzymatic antioxidants are antioxidant vitamins (vitamin C, vitamin E) and some trace elements like zinc, copper and selenium.

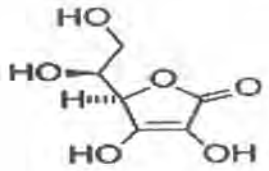
The first line of defense against the superoxide radicals are the superoxide dismutase enzymes (SOD). They catalyze the reduction of superoxide radical to H_2O_2. Although H_2O_2 which is formed during superoxide dismutation is also toxic, it can be removed by enzyme catalase. This whole mechanism is necessary for the cell survival.

$$O_2 + e^- \xrightarrow[\text{Glutathione peroxidase}]{} O_2^{\cdot -} \xrightarrow[\text{SOD}]{} H_2O_2 \xrightarrow[\text{Catalase}]{} H_2O + O_2$$

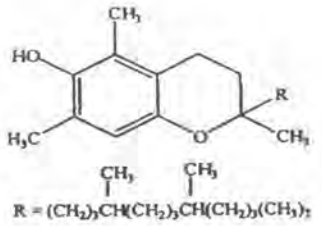
Generally, the body's natural antioxidant systems can effectively neutralize the radicals or oxidized products up to a certain limit. However, massive oxidative stress and aging induced by an overproduction of reactive oxygen species (ROS) can lead to a disruption of cellular functions. Under these circumstances, there is an imbalance between oxidants and antioxidants necessitating the addition of exogenous antioxidants. Therefore, diets rich in antioxidants such as vitamin C, vitamin E, vitamin B_2, B_6, β-carotene and flavonoids have played an important role. Moreover, considerable attention has been emphasized on other naturally occurring materials such as Coenzyme Q or ubiquinone, which is a naturally occurring, lipid-soluble substance found in high levels in the epidermis that can protect against ROS and their antioxidant activities.

Vitamin C (L-ascorbic acid)

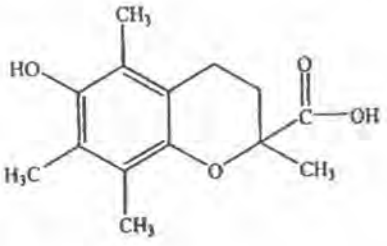
Vitamin C has long been known to be essential for the protection against scurvy in humans. The activity of vitamin C lies in the role of ascorbic acid (the reduced form of vitamin C), which is known as an essential cofactor in hydroxylation reactions involved in the biosynthesis of stable cross-linked collagen. This and other metabolic functions of ascorbate depend on its strong reducing potential, and its structure is shown in Figure 6. The same property makes this vitamin an excellent antioxidant, capable of scavenging a wide variety of different oxidants. For example, ascorbate has been shown to effectively scavenge superoxide, hydrogen peroxide, hyperchloric acid, aqueous peroxyl radicals, and singlet oxygen and seems to have a protective effect for many kinds of cancer and carcinogenesis (Sies and Stahl, 1995; Giacosa and Filiberi, 1996; Jacob and Burri, 1996). During its antioxidant action, ascorbate undergoes a two-electron oxidation to dehydroascorbic acid (the oxidized form of vitamin C). Although dehydroascorbic acid is relatively unstable and readily hydrolyzed to l-2, 3-diketogulonic acid, it can be reduced back to ascorbate by a variety of cellular thiols such as homocysteine. Therefore, both ascorbic acid and dehydroascorbic acid are biologically active form of vitamin C. Ascorbate is able to interact synergistically with membrane-bound and lipoprotein confined α–tocopherol, i.e., it can readily reduce α-tocopherol (Sies and Stahl, 1995).



Vitamin C



Vitamin E



Trolox®

Figure 6. Structure of Vitamin C (L-ascorbic acid), vitamin E (α–tocopherol) and Trolox®

Vitamin E (α-tocopherol)

Alpha-tocopherol is the main component and the most active form of vitamin E. It is well accepted as the major endogenous lipid-soluble, chain breaking antioxidant in human plasma and LDL (Liu et al., 2000). The structure is shown in Figure 7. Moreover, it serves to prevent lipid peroxidation and modulate the metabolism of the arachidonic acid cascade initiated by lipoxygenase and/or cycloxygenase, and an increased intake of vitamin E is recommended for heart disease prevention. Thus is also a current hypothesis that it could be protective against cancers where *N*-nitroso compounds are implicated. Other isomers of vitamin E, such as β-, γ- and δ-tocopherols, are either present in very low concentrations or not detectable at all. Judging by their rate of reaction with peroxyl radicals, the antioxidant activity decreases in the order of $\alpha > \beta > \gamma > \delta$, in analogy with the biological potencies of these different forms of vitamin E. Bowery, Ingold, and Stocker (1992) pointed out recently that tocopherol might become a prooxidant via the so-called tocopherol mediated peroxidation, α-tocopherol radical, in LDL particles in the absence of other endogenous antioxidants such as vitamin C and ubiquinol-10 (Sies and Stahl, 1995; Giacosa and Filiberi, 1996; Jacob and Burri, 1996; Punchard and Kelly, 1996).

Trolox® (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)

Trolox® is a water-soluble form of α-tocopherol with the hydrophobic side-chain replaced by a hydrophilic-COOH group. Its structure is also shown in Figure 7. The compound is a good scavenger of peroxyl and alkoxyl radicals, giving a Trolox® radical that can be scavenged by ascorbate. Trolox® is commercially available for experimentation especially in an aqueous system.

Other antioxidants

In addition to those natural antioxidants, a huge range of synthetic antioxidants are available such as those used in the rubber industry to prevent copper-catalyzed oxidative degradation of polypropylene, or in the polymer industry to prevent UV-induced degradation of plastic, and for foodstuff to protect food lipid against oxidative damage (and consequent rancidity) during storage, in heat sterilization, or sterilization by ionizing radiation. Several synthetic antioxidants have long been used in biology and food technology such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate, etc. Many of these antioxidants also have properties other than a chain-breaking action. For example, most phenolic antioxidants have metal ion-complexing ability, especially those antioxidants with adjacent –OH group. However, the chain-breaking action is predominant in peroxidizing lipid systems, causing phenolic antioxidants to be powerful inhibitors of peroxidation process.

Several products of plant origin such as some flavonoids and polyphenols have chain-breaking antioxidant activity. Examples are curcumin, catechin, quercetin, kaempferol, caffeic acid, etc. Several of these compounds, such as quercetin and catechin, also have metal-binding capacity.

F. Measurement of Antioxidant Activity

The antioxidant activity of the test samples can be evaluated with different tests for different mechanisms. Many strategies have been developed and well established to evaluate the antioxidant activities of the test samples in terms of (1) detecting the free radicals to investigate the antioxidant's ability to inhibit/suppress free radical formation, (2) investigating the antioxidant's ability to scavenge free radicals and (3) studying its ability to prevent or reduce oxidative damage resulted from free radicals.

Free radicals can be detected by electron spin resonance, fluorescence and chemiluminescence. Electron spin resonance (ESR) spectroscopy is a well known method to measure the free radicals directly in conjunction with spin trapping agents such as dimethylpyrroline-N-oxide (DMPO), which is hydrophilic compounds, traps free radicals in an aqueous environment. However, this technique had limited application (Robards et al., 1999). In another method including fluorescence probe, for instance, dichlorofluorescein diacetate was used and oxidative process in living cells can be visualized.

Many different experimental methods have been developed for the determination of antioxidant activity. The most widely used methods are typically based on the generation of a radical species followed by monitoring its disappearance upon addition of an antioxidant. The extent of disappearance is proportionate to the amount of the added antioxidant and thus its free radical scavenging activity can be determined. Most of the reliable methods involved the measurement of the disappearance of the challenged free radicals such as superoxide radical, hydroxyl radical, 2,2'-azinobis (3-ethylbenzenthiazoline-6-sulphonic) radical ($ABTS^{+\cdot}$), 2,2-diphenyl-l-picrylhydrazyl (DPPH) radical, etc. The efficacy of an antioxidant is measured by monitoring the extent of decrease in oxidation with the help of chemicals and instruments with adequate sensitivity under standard conditions. Also, these methods should not be time-consuming but need to have an ability to screen for the radical scavenging activity of the test samples.

DPPH radical is not a naturally occurring compound, and is relatively stable compared to the highly reactive superoxide and hydroxyl radicals primarily responsible for oxidative damage in biological systems. Any molecule that can donate an electron or hydrogen to DPPH can react with it and thereby bleach the DPPH

absorption (Naik et al., 2003). Thus, DPPH is a commonly used reagent to screen for an electron-donating property of an antioxidant.

Some other well known methods for the determination of antioxidant activities are total radical trapping antioxidant parameter assay (TRAP), the spectrophotometric assay of the decline of peroxyl radicals generated by AAPH or ABAP (2,2'-azobis(2-amidinopropane)dihydrochloride). Trolox equivalent antioxidant capacity assay (TEAC) and hypoxanthine/xanthine oxidase assay. Trolox® equivalent antioxidant capacity assay (TEAC) is the spectrophotometric radical scavenging analysis with Trolox® as standard. In this assay the stable free radicals ($ABTS^{+\cdot}$ or $DPPH^{\cdot}$) which react with antioxidant were used.

Another strategy has been developed for measuring the total antioxidant activity (TAA) of the sample which has the ability to scavenge free radicals generated in the aqueous and lipophilic phases. Generated radical is coupled to oxidation of a substrate and the inhibitory effect of substrate is measured based on the detection of the radicals or the products of oxidation. This ability to scavenge specific radicals may be targeted for radicals such as hydroxyl radicals or nitric oxide radicals.

For the oxidative damage, DNA damage and lipid peroxidation were always used as model. Especially for lipid peroxidation test, a direct test of an antioxidant's ability toward lipid can be examined whether a substance inhibits peroxidation of artificial lipid systems, fatty acid/ester emulsions, liposomes, food systems or biological systems such as erythrocytes, lipoproteins, tissue homogenates or microsomes (Halliwell et al., 1995; Halliwell, 1997).

G. *In Vitro* Cytotoxicity Assay

In vitro cytotoxicity assays detect the degree to which a treatment is toxic to the cells by measuring the viability of a population of cells in culture. Cell viability can be reflected by variety of different parameters, but it is most often defined experimentally by MTT assay and LDH assay.

(a) MTT assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) is a water-soluble pale yellow substrate. Active mitochondrial dehydrogenase of living cells cleaves the tetrazolium ring and converts the yellowish MTT to an insoluble purple formazan crystal. This conversion does not take place in dead cells. This water-insoluble formazan crystal can be solubilized by using MTT solubilization medium, and give a homogenous blue solution, which is suitable for quantification by visible spectrophometry at 550 nm on microplate reader.

(b) LDH assay

LDH (lactate dehydrogenase) is a cytoplasmic enzyme. Normally, it is not secreted outside the cells, but upon damage of cell membrane LDH leaks out. The lactate dehydrogenase (LDH) assay is another method used to evaluate cell viability in terms of the degree of cell membrane integrity. The assay is based on the reduction of NAD (nicotinamide adenine dinucleotide) by the action of LDH. The resulting reduced NAD (NADH) is utilized in the stoichiometric conversion of a tetrazolium dye. The resulting red -colored compound is measured by spectrophotometry at 490 nm.