CHAPTER II

LITERATURE REVIEW

A. *Artocarpus lakoocha* Roxb.

Artocarpus lakoocha Roxb. is a tropical tree belonging to the family Moraceae and locally known as Ma-Haad (Figure 1). It is widely distributed in the northern, northeastern and central part of Thailand as well as in South and Southeast Asian countries.

Ma-Haad is a large deciduous tree reaching 15-18 m in height with a spreading head; bark rough, grey; young shoots thin, densely clothed with a soft grey, tawny or rusty tomentum. Leaves cariaceous, 10-30 by 5-15 cm, oblong, elliptic or subovate, entire (the young ones sometimes serrate), obtuse, cuspidate, glabrous, and shining above, softly pubescent beneath, base broad or narrow, truncate or rounded; main nerves 6-12 pairs with reticulate venation between; petioles 1.3-2.5 cm long, lanceolate tawny-pubescent. Flower in auxiliary globose shortly pedunculate heads; bracteoles peltate. Male flower: Sepals 2-3, triangular, truncate, puberulous. Stamen 1; filament broad below, tapering upwards; anther exserted, short, broad, 2-called. Female flowers: Anthocarps completely unites. Fruit 5 - 7.5 cm diam., lobulate, smooth, velvety, yellow, edible. Seeds oblong, few, board, about 13 mm across (Kirtikar and Basu, 1980).

They are cultivated for medicinal use. The claimed efficacies in Thai traditional textbooks are as follows (Farnsworth and Bunyapraphatsara, 1992):

Roots: as an antipyretic, anthelmintic; for alleviation of toxic symptoms and treatment of urinary stones.

Wood: an antiflatulence, carminative and laxative; treatment of skin rash; chronic gastrointestinal ailments of children between the ages of 5 and 13 characterized by marked malnutrition and usually associated with intestinal parasitism; round and tape worm infestation; menstrual disorders; fainting; and any disorders or diseases which cause cachexia, disorders of flatulence and tendomyopathy.

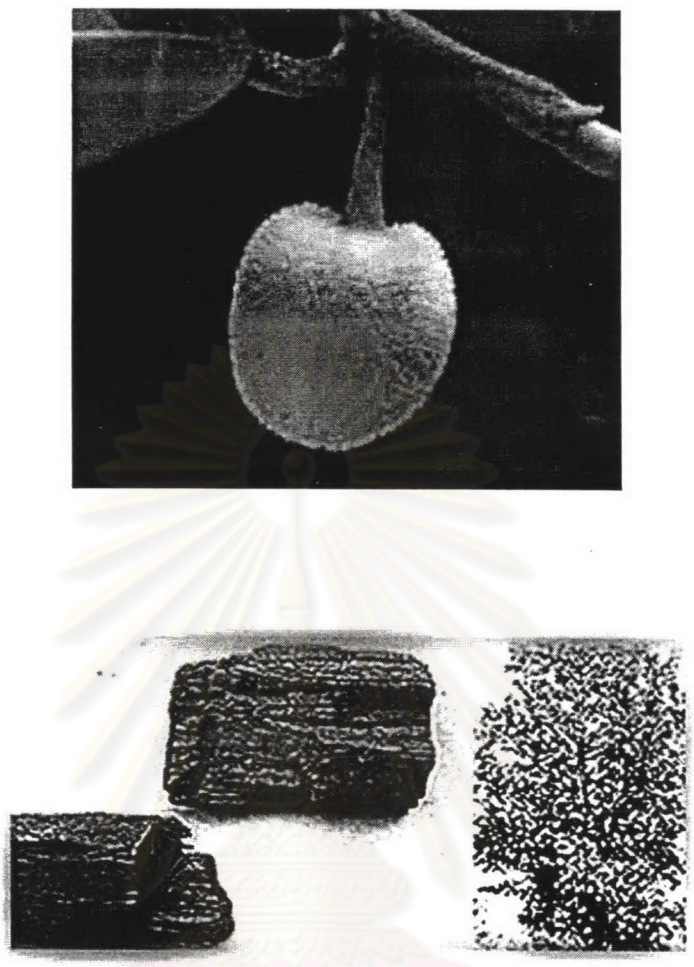


Figure 1. *Artocarpus lakoocha* Roxb. (Ma-Haad) (Joshee et al., 2002)

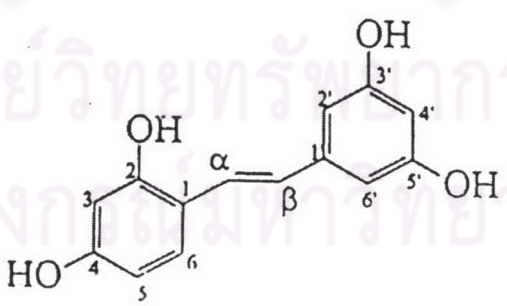


Figure 2. Chemical structure of oxyresveratrol or 2, 4, 3′, 5′-tetrahydroxystilbene (Sritularuk, 1998)

Bark: as antipyretic

Pith: treatment of menstrual disorders; any disorders or disease which cause cachexia; nephropathy; distension of abdomen due to peritonitis or paralytic ileus; insomnia; malnutrition syndrome in children due to intestinal parasitism; splenomegaly; eye irritation; dissipate hematoma; oropharyngeal symptom from gastroenteric disease; dyspepsia caused by wind element; cramps; clouded mind; incontinent urination; as antidiarrheal, anthelmintic, taenifuge, antituberculosis, analgesic and for increasing appetite.

In Thai traditional medicine, a dried aqueous extract of *A. lakoocha* heartwood, locally known as "Puag-Haad", has been used as an anthelmintic and antipruritic. The main component of the extract or Puag-Haad powder is 2, 4, 3′, 5′-tetrahydroxystilbene, which is also known as oxyresveratrol (Figure 2) (Mongkolsuk et al., 1957). Yodhabandu (1960) and Poopyruchpong et al., (1978) found oxyresveratrol in 51 and 70 percent yield of Puag-Haad. Tiptabiankarn (1967) reported that the main constituent of Puag-Haad (oxyresveratrol) is considered to be an effective antioxidant (delaying rancidity of lard) compared to Tenox II (Tenox II contains 20% BHA, 6% Propyl gallate, 4% Citric acid and 70% Propylene glycol). Oxyresveratrol has been reported to exert an anthelmintic activity (Charoenlarp et al., 1981; Preuksaraj et al., 1983) and exhibit good safety profile in cytotoxicity test (Nilvises et al., 1985; Ngamwat et al., 1987). Moreover, the pharmacokinetic properties in human studies have also been investigated (Tanunkat, 1990).

Recently, Sritularak et al., (1998) reported a potent inhibitory effect of the methanolic extract of *A. lakoocha* on enzyme mushroom tyrosinase in vitro using L-DOPA as a substrate. Further comparison of its active constituent, 2, 4, 3′, 5′-tetrahydroxystilbene (oxyresveratrol), showed that the compound had a concentration causing 50% enzyme inhibition (IC_{50}) of about 1.5 μM, which was 17.9 times higher than kojic acid (Sritularak, 1998; Sritularak et al., 1998). The IC_{50} value of oxyresveratrol was in agreement with Shin et al. (1998) and Kim et al. (2002), who reported the value of 1.0 and 1.2 μM, respectively. Following, the in vitro study, the in vivo skin whitening efficacy of the extract was evaluated in guinea pigs and human volunteers (Tengamnuay et al., 2003). The result of the study clearly demonstrated that the heartwood extract of *A.*

lakoocha could reduce melanin formation in both guinea pigs and humans. Comparing to other tyrosinase inhibitors commonly used in commercial whitening products such as kojic acid and licorice extract, the data were in agreement with the in vitro tyrosinase inhibitory effect which showed that oxyresveratrol demonstrated the highest anti-tyrosinase activity (Sritularak, 1998). Also, the anti-HIV and Herpes simplex virus activity has recently been reported (Sritularak, 1998; Likhitwitayawuid et al., 2003). Despite the above findings about *A. lakoocha* heartwood extract, however, its many other beneficial properties, especially for cosmetics and dermatological applications are not widely known or studied.

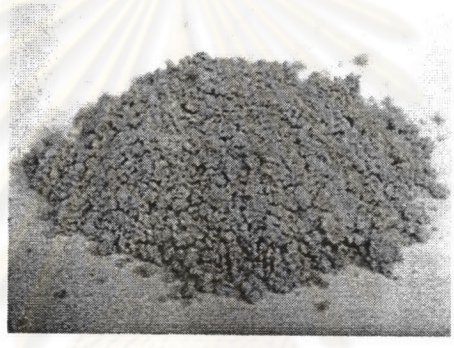


Figure 3. Puag-Haad

Puag-Haad

Puag-Haad (Figure 3) is a dried aqueous extract of the heartwood of *A. lakoocha* and its activities come from 2, 4, 3′, 5′-tetrahydroxystilbene, the major constituent (Poopyrunchpong et al., 1978; Farnsworth and Bunyapraphatsara, 1992). Puag-Haad usually appears in the local herb market as a brown lump, which can be ground to give a light yellow powder. It is prepared by boiling chips of *A. lakoocha* wood in water and the aqueous extract is concentrated by gentle heat. On cooling a yellow-brown powder of Puag-Haad is separated. The precipitate is filtered and dried near the fire (Mongkolsuk, et al., 1957).

Due to its polyphenolic structure, one study has been carried out to determine the antioxidant property of oxyresveratrol and its derivatives from *A. lakoocha* (Tiptabiankarn, 1967). The extract was evaluated in terms of its anti-rancidity in lard using the active oxygen method and Wheerer method. It was found that oxyresveratrol

can increased the stability of lard by delaying rancidity and is considered to be an effective antioxidant compared to Tenox II. Recently, the antioxidant and free radical scavenging effects of oxyresveratrol have been reported (Lorenz et al., 2003). They found that oxyresveratrol was a more potent scavenger of DPPH (2, 2-diphenyl-1-picryl-hydrazyl) and nitric oxide radicals than resveratrol, a related substance well known for its strong antioxidant activity. They thus suggested that it may have important therapeutic applications such as in neuropathologies where oxidative/nitrosative stress is involved. Others have reported about the inhibitory effect of oxyresveratrol on clyclooxygenase (Shin et al., 1998b) and rat liver mitochondrial ATPase (Nimmanpisut et al., 1976). However, little is still known about the many aspects of the antioxidative/free radical scavenging activities of the extract or oxyresveratrol, especially regarding the cosmetic applications. Since there are many possible anti-oxidative mechanisms, particularly those involving free radical scavenging pathways, the anti-oxidative capacity of the *A. lakoocha* extract should be investigated in more detail and compared with the commercial antioxidants used for cosmetic purposes.

B. Aging and the Skin

1. Aging theory

Aging is an integral part of the process of growth and development. It may be defined as the sum of all the changes that occur in man with the passage of time and lead to functional impairment and death. An alternative definition might be a decreasing ability to survive stress (Kenny, 1982). Such a definition directs attention to the defense systems of the body, i.e., the finely regulated mechanisms that control the internal environment to produce homeostasis as well as the defense mechanisms of the immune system. When the process of aging occurs, the changes in the body are expressed not only in its functions but also in the anatomy.

Some of the better-known theories of biological aging, as well as selected relevant theories of psychosocial aging, are describe d as below (Kenny, 1982; Saxon, 2002)

1.1 The genetic theory

The "genetic" theory has a variety of names, but the essential concept centers on the belief that maximum life span is controlled by the genetic material, DNA and therefore is fixed in time.

1.1.1 Cellular aging as a programmed phenomenon (the program theory of aging)

This is one of the earliest of genetic theories, proposed by Hayflick in 1961. During embryonic development, tissues and organs undergo extensive and continuous remodeling. This is brought about by the orderly death of some cells and the activation of other cell lines controlled by genetic means. It is proposed that all cells, except the germ cells and transformed cells, bear specific "death" genes which are programmed to switch off some cellular processes in a sequential fashion to produce in the tissue the aggregate sign of aging. In this way, cellular aging and death are the ends of cellular differentiation. This theory state that the life span of animals is predetermined by a genetic program, or a so-called biologic clock (Hayflick and Moorhead, 1961; Kenny, 1982; Saxon, 2002).

1.1.2 The error theory

This theory is also based on the genetic information systems of the cell, DNA and RNA. It is proposed that the conversion of the information borne by these molecules into enzyme and protein synthesis becomes increasingly subject to errors, thus leading to the accumulation of inappropriate molecules that are unable to support the cell's metabolism. This theory has been invoked as the mechanism underlying the fact that the life span of a species is inversely correlated with the rate of metabolism. The faster rate of metabolism affects to the faster rate of material turnover and thus the greater chance for biochemical errors (Kenny, 1982). Thus, aging and death are presumed to be the result of errors that occur and are transmitted at the cellular level. Research has not yet provided support for this theory, and it is generally no longer accepted. However, it has stimulated a great deal of research (Saxon, 2002).

1.1.3 Repair failure

Errors in the transcription of DNA, such as may be caused by experimental irradiation of the cell or by in vivo production of free radicals, can be corrected by repair processes. Two lines of evidence support the notion that aging is rooted in this mechanism: (1) the rate of DNA repair is related to the life span of the species, and (2) in cultured human cells, the rate of repair decreases as the cells age. The consequence, therefore, would again be the production of inappropriate molecules that are unable to support cell metabolism (Kenny, 1982).

1.1.4 Redundancy failure

The genetic message borne by the DNA molecule has a high degree of redundancy. Less than 1% of the information carried by the DNA is used by the cell, and gene sequences are repeated many times along the molecule. The theory supposes that as errors occur in gene synthesis, a supply of correct genes is available to take over from the ones damaged by error. As the cell ages, the supply of redundant (and correct) genes becomes exhausted and errors are then free to express themselves (Kenny, 1982).

1.1.5 The "killer hormone"

This theory invokes a hormone derived from the pituitary gland that depresses the responsiveness of peripheral cells to the thyroid hormone. Two systems for which adequate thyroid activity appears to be necessary are the immune and the cardiovascular systems. Depression of the peripheral effects of the thyroid hormone by the pituitary factor may produce the decline and ultimate failure of these two major systems. This putative killer hormone appears to begin to be secreted at puberty, at which time it may buffer the tissues against the endocrine surge that occurs and it may restrain what otherwise would be an excessive metabolic response and burn out. Starvation, which when started before puberty delays it, extends the life span and also delays the appearance of this factor (Kenny, 1982).

1.2 The environmental theory or free radical theory

The free radical theory, first proposed in the mid 1950s (Harman, 1956), continues to provide the basis for much of the current research on aging. Free radicals are proposed as a central agent in the changes seen with aging in the tissue, cellular, and subcellular levels. These molecules are highly reactive and commonly have a brief half-life. They are capable of attacking other molecules because they possess an extra electric charge, or free electron. They rapidly interact with and damage cellular components such as lipids, proteins, and nucleic acids (Kenny, 1982).

The free radical theory is the most viable and the most important concept in aging mechanism yet proposed (Gordon, 1974; Brocklehurst, 1987; Jay and Berthon, 1998). The free radical concept may be classified as an environmental cause of aging as opposed to the genetic cause. The major difference between the two concepts is that the genetic concept assumes a fixed and relatively immutable life span, while the environmental concept sees adverse aging as a result of exogenously produced damage to the cell systems resulting in impairment of normal functions (Pugliese, 1987). The free radical theory is described in more detail in subsequent sections.

2. **Skin and aging**

2.1 Skin

The skin is divided into three layers called epidermis, dermis, and the subcutaneous layer (Figure 4).

The epidermis is composed of several cell layers about 0.1-0.3 mm thick. From the external surface inwards, these layers are called stratum corneum, stratum spinosum, stratum lucidum, stratum granulosum, and stratum basale (Figure 4). The principle cells of the epidermis are keratinocytes whose main purpose is to produce the fibrous protein keratin, which protects against frictional forces. The basal keratinocytes undergo cell division. One of the newly-divided cells remains at the basal layer and others move towards the outer epidermis, beginning the keratinization process. The horny cells are created continuously, the oldest cells are shed from the outer surface of the skin but they are replaced from below thereby maintaining the thickness of the horny layer. This type of continuous replacement of the cell layer is call "turnover". The turnover rate varies

with the site and age, but it has been estimated to be approximately 26-28 days (Mitsui, 1997; Rongone, 1997). When the skin is penetrated by a foreign object or the honey layer is damaged, the division of the cell in the basal layer increases in response causing the turnover rate to increase thereby expelling the foreign object and promoting recovery. In addition, repeated chemical or physical stimulation increases the thickness of the horny layer. These responses protect the epidermis from external stimuli.

In addition to these keratinocytes, the epidermis also contains melanocytes which produce the pigment melanin. The melanocytes are scattered between the basal cells at the basal layer. Melanocytes produce melanin for skin pigmentation, which is partially protective against UV radiation. Melanosomes (pigment containing granules produced within the melanocytes) are present in melanocyte dendrites and are transferred to surrounding keratinocytes. Melanin synthesis begins with the oxidation of tyrosine by the enzyme tyrosinase to form 3, 4-dihydroxyphenylalanine (dopa) within the melanosomes. A second oxidation, also under the control of tyrosinase, forms dopaquinone, which undergoes additional non-enzymatically mediated oxidation and polymerization leading to the formation of the final product, i.e., either eumelanin (brown or black) of phaeomelanin (red, yellow) pigment. Phaeomelanin is formed by the addition of cysteine to dopaquinone. The epidermis also contains Langerhans cells which have immune response functions as a protective mechanism against invasion of foreign materials (Fenske and Lober, 1986; Thody, 1986; Marieb, 1995).

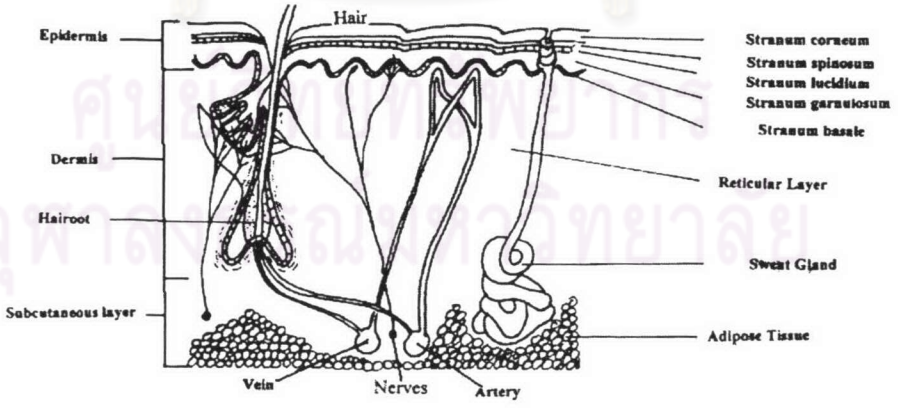


Figure 4. Basic structure of the skin (Burton, 1997)

The dermis is composed predominantly of collagen and elastin fibers which are embedded in an interfibrillar gel of glycosaminoglycans. Collagen is the major component and is secreted by fibroblasts which are the principal cells of the dermis. The dermis is divided into the superficial papillary dermis which interlocks with the rate ridge of the epidermis, and a deeper zone called the reticular dermis. The former is generally the thinner, being composed of finer collagen and elastin fibers which allow the dermis to mould to the contours of the overlying epidermis in such a way that its interface represents an exact mirror image of the undersurface of the epidermis. The dermal papillae which dovetail into the rate ridges of the epidermis have a rich blood supply and contain many of the sensory nerve endings of the skin. The reticular dermis, on the other hand, is relatively avascular and acellular. Its collagen and elastin fibers are much thicker than those in the papillary dermis and form a denser lattice meshwork, which depending upon its degree of packing, confers great strength and flexibility. This enables the skin to adapt to the various movements of the body and in addition, to resist mechanical damage. There are profound regional variations in the dermal texture rendering it appropriate to the local requirement – thus it is thin and flexible over joints but very thick and tough on the back (Fenske and Lober, 1986; Marieb, 1995).

Beneath the dermis, there is subcutaneous layer or hypodermis which contains many adipose cells in and between the connective tissue. The subcutaneous tissue protects the skin from blunt and pressure – related trauma and serves as an insulator of heat loss. The loss of this protective padding results in an increase in problems of weight-bearing and pressure-prone surfaces, and other injuries, as well as the risk of hypothermia (Montagna and Parakkal, 1974; Balin, 1992).

2.2 Skin aging

As a person ages, skin undergoes significant changes (Potts, 1984; Yamauchi, 1988; Gilchrest, 1991):

- The cells divide more slowly, and the inner layer of the skin (the dermis) starts to thin.

- Fat cells beneath the dermis begin to atrophy.

- The underlying network of elastin and collagen fibers, which provides scaffolding for the surface layers, loosens and unravels.

- Skin loses its elasticity. When pressed, it no longer springs back to its initial position but instead sags and forms furrows.

- The sweat and oil secreting glands atrophy, depriving the skin of their protective water lipid emulsions. The skin's ability to retain moisture then diminishes and it becomes dry and scaly.

- Brow lines (those between the eyebrows) and crow's feet (lines that radiate from the corners of the eyes appear to develop because of permanent small muscle contractions. Habitual facial expressions also form characteristic lines.

- Gravity exacerbates the situation, contributing to the formation of jowls and drooping eyelids. (Eyebrows, surprisingly, move up as a person ages, possibly because of forehead wrinkles).

Some of the skin changes that accompany aging are natural and inevitable, and together make up the process called intrinsic aging or sometimes chronological aging. More significant for most people are the changes arising from external causes called extrinsic aging. Both the intrinsic (or chronological) and the extrinsic aging overlap during lifetime and both are more or less responsible for dysfunction of the skin's natural self-protection and repair.

In intrinsic aging, the skin becomes thinner and loses much of its elasticity, while the normal expression line deepens. The boundary between the epidermis and the dermis is flatted, and the dermis starts to wither (atrophy). The number of blood vessels in the dermis begins to fall. All at the same time the hair often loses its color, and within the skin there are fewer hair follicles and fewer sweat glands. The collagen, elastin and ground substance also decrease in amount, but the proteins remain in a reasonably stable state. Fine lines and shallow wrinkles begin to develop in this type of aging but these wrinkles will disappear easily on stretching (non-permanent wrinkle) (Gray, 2000).

In extrinsic aging, the skin has a different texture; it looks dry, rough and coarse. It may appear thicker. It loses elasticity due to hypertrophy of the elastin tissue and changes in collagen fibers. The skin presents as a deep wrinkle which does not disappear on stretching (permanent wrinkle) (Gray, 2000). Extrinsic aging is due to outside factors

that have affected the skin. Reactive oxygen species (ROS) such as free radicals are considered to be the most active agents in this aging (Jay and Berthon, 1998). ROS initiate lipid peroxidation, oxidation changes of proteins and DNA and other disturbing mechanisms (Billek, 1996). The sources of these free radicals can be endogenous such as those associated with metabolic reactions (oxidation reaction in mitochondria with disruption of electron transport, excessive phagocytosis, activation of arachidonic acid metabolism) and exogenous due to UV radiation, pesticides, air pollution, antitumoral drugs and unhealthy lifestyles as summarized in Figure 5 (Jay, 1998).

From Figure 5, oxidative stress leads to many changes in both the cellular and extracellular systems as well as a decrease in the endogenous defense system. The body naturally protects against free radicals by chemical or enzymatic detoxification mechanisms. Nevertheless, the protective capacity of these systems decreases during the extrinsic aging. Many exogenous compounds such as enzymes, antioxidants (including some vitamins and metals) and phenolic compounds (including some flavonoids and tannins from plants) can reinforce the body's natural protection by limiting oxidation reaction (Jay, 1998).

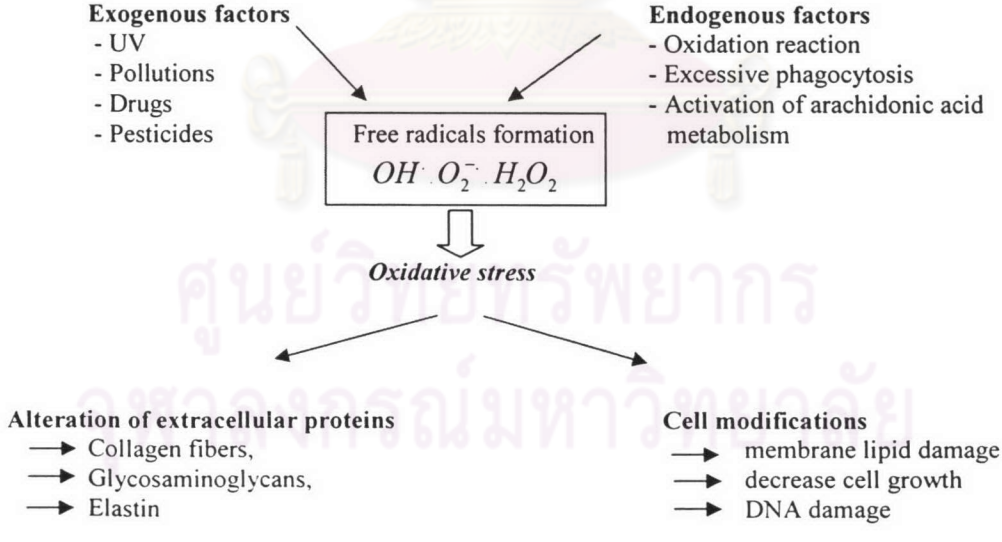


Figure 5. Free radicals formation and its deleterious effects

C. Free Radicals 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 electrons from an atom or ion. Indeed, this reaction is useful for the synthesis of nucleic acids, hormones and proteins. Beside this reaction, reactive oxygen species (ROS) are evolved as by-products (Cho, 2002).

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 a 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 a reactive nitrogen species (RNS) if nitrogen is involved. The types of radicals are shown in Table 1 (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 or 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 1. 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)	Peroxynitrite, $ONOO^-$
Nitric oxide, $NO^{\cdot}$	Nitrous acid, HNO_2
Nitrogen dioxide, $NO_2^{\cdot}$	

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

Reactive oxygen species (ROS) formations are 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 like 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. The superoxide anion is created from molecular oxygen by the addition of an electron as shown below:

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

It 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 membrane as easily as H_2O_2, so $HO_2^{\cdot}$ could conceivably produce damage. The formation of superoxide takes place spontaneously, especially of 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 $HO^{\cdot}$. 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).

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

(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).

(c) Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is not a free radical but is nonetheless highly important much 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 a 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, 1894; Dombi, 2000). Hydrogen peroxide can be generated by divalent reduction of oxygen or by enzymatic dismutaion 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 a presence of a photosensitiser or the absorption of energy from photo-excited photosensitizer molecules of oxygen. Photosensitiser, 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 photosensitiser 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; McVean, 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 radicals 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 2, 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 phospholipases, 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 2. 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	Stabilization of heme
Ceruloplasmin, albumin	Sequestration of copper
(c) Quenching of active oxygen species	
Superoxide dismutase (SOD)	Disproportionation of superoxide
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.

D. 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, 1995). The term "oxidizable substrate" include 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" (Cho, 2002). Basically, the mechanisms of antioxidants involve in three different ways as previously shown in Table 2; (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). More recently, one has provided a convenient summary of the sequence of events involved in free radical damage and antioxidant mechanism as shown in Figure 6 (Ternay and Sorokin, 2000).

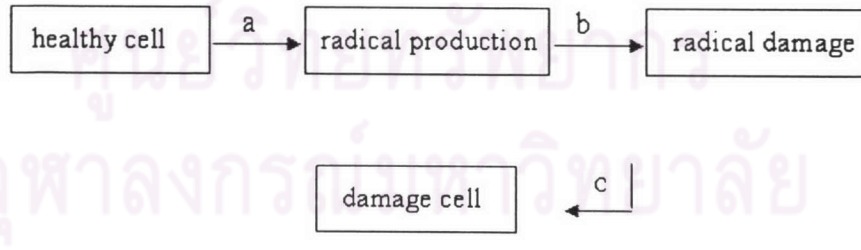


Figure 6. Diminishing radical-induced cell damage; a = radical formation prevention; b = radical scavenging; c = repair of radical-induced damage

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 naturally occurring materials that can protect against ROS and their antioxidant activities have been identified (Cho, 2002).

Vitamin C (l-ascorbic acid)

Vitamin C has long been known to be essential for the protection against scurvy in humans. The ascorbic 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 7. 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 cells or thiols such as homocysteine. Therefore, both ascorbic acid and dehydroascorbic acid are biologically active forms 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 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 as the preventing lipid peroxidation and modulating 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 and, on current hypothesis; 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. Bowrey, Ingold, and Stocker (1992) point out recently that tocopherol might be come a prooxidant via the so-called tocopherol mediated peroxidation, α-tocopheroxyl radical, in LDL particles in the absence of other endogenous antioxidants

such as vitamin C and uniquinol-10 (Sies and Stahl, 1995; Giacosa and Filiberi, 1996; Jacob and Burri, 1996; Punchard and Kelly, 1996).

Trolox®

It is a water-soluble form of α-tocopherol with the hydrophobic side-chain replaced by a hydrophilic –COOH group. Its structure is shown in Figure 7. It 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 plastics, 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 antioxidant 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 like some flavonoids and polyphenols have chain-breaking antioxidant activity. Examples are curcumin, catechin, quercetin, kaempferol and caffeic acid, etc. Several of these compounds, such as quercetin and catechin, also have metal-binding capacity.

EGCG

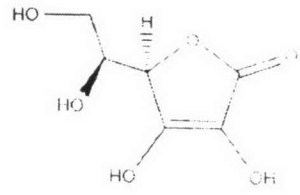
EGCG or (-)-Epigallocatechin gallate is the main polyphenolic component of catechins in green tea with a vast range of activity and is the major catechin present in the

green tea extract. Its structure is shown in Figure 7. EGCG has been represented as a powerful radical scavenger, as investigated by many in vivo and in vitro techniques (Hatano et al., 1989; Yoshida et al., 1989; Nanjo et al., 1996; Agarwal, 2000; Katiyar and Elmets, 2001; Nakagawa and Yokozawa, 2002; Geetha et al., 2004; Hsu, 2005). Moreover, EGCG is an excellent antioxidant agent against lipid peroxidation and it is functional as antioxidant at relatively low concentrations. While at higher concentrations or under some condition used, EGCG itself is also susceptible to oxidation and thus, it can behaved as a pro-oxidant (Furukawa et al., 2003; Geetha et al., 2004).

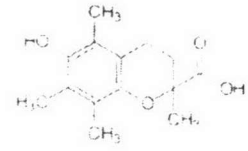
Pine bark extract

Pine bark extract is a substance obtained from the bark of *Pinus maritime* (French maritime pine) or *Pinus pinaster*. The main constituents of pine bark extract are known to be the mixture of procyanidins or proanthocyanidins, polyphenol, and phenolic acid (such as caffeic, ferulic, and p-hydroxybenzoic acids) as minor constituents (Grimm, Schafer and Hogger, 2004). The proanthocyanidins, mainly components, are biopolymer comprising catechin or epicatechin monomer units in varying chain lengths. When the number of connected catechins (referring to both catechins and epicatedchins) is 10 or less they are called oligomers and hence the term oligomeric proanthocyanidins (OPCs). When the number of connected catechins is more than 10 the term condensed tannins is generally used. Many names refer to this compound including luecoanthocyanin, anthocyanidin and still other. According to many studies, it has demonstrated a potential as an active free-radical scavenger and anti-inflammatory activity (Guo, Zhao, and Packer, 1999; Packer, 1999; Grimm, Schafer and Hogger, 2004) and has been used in food supplementary products in order to help promote blood circulation, retina malfunction and inflammatory collagen disease (Robbers et al., 1996).

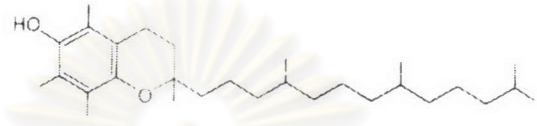
In addition, many of these components are also found in commonly ingestsed fruits and vegetables, in plant derived substances from grapes and berries, and in beverages such as green and black tea and red wine (Packer, 1999). Pine bark extract used in this study was obtained from China and according to the quality control certificate from the manufacturer, it was claimed to have an amount of the active compounds, total proanthocyanidins, of 96.88%, which is considered to be of high purity.



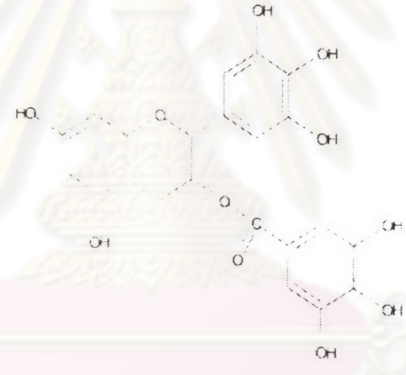
Vitamin C



Trolox®



Vitamin E



EGCG

Figure 7. Structure of Trolox®, vitamin C, vitamin E, and EGCG

E. 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 dimethylpyrrolidine-N-oxide (DMPO), which is a hydrophilic compound and traps free radicals in an aqueous environment. However, this technique also has limited applications. In another method such as the fluorescence probe, dichlorofluorescein deacetate is used and the oxidative process in the 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 proportional 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 ($ABTS^{+\cdot}$), and 2, 2-diphenyl-1-picrylhydrazyl radical ($DPPH$), 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.

For the oxidative damage, DNA damage and lipid peroxidation was always used as a model. Especially for lipid peroxidation test, a direct test of antioxidant activity ability toward lipid is examined whether a substance inhibits peroxidation of artificial lipid system such as lipoprotein, tissue homogenates, fatty acid/ester emulsion,

liposomes, food systems or biological systems such as erythrocytes, lipoproteins, tissue homogenates or microsomes (Halliwell et al., 1995; Halliwell, 1997).

F. Stability of Cosmetic Preparations and Role of Antioxidants

The stability of a cosmetic product may refer to both the physical and chemical stability (Mithal, 1980). The most common goal is to preserve the products that are particularly susceptible to oxidation which causes the formation of objectionable degradation products with unpleasant odor or color. Many active components in cosmetic preparations are claimed to have an anti-aging effect through their antioxidant activities on the skin. Most of these compounds are phytochemicals and plant extracts. Their chemical structures are often flavonoids or polyphenols in nature. However, these substances are also prone to oxidation, especially from the atmospheric oxygen. Fortunately, this aspect can be taken care of by inclusion of appropriate synthetic antioxidants. These additives have appreciable benefits by providing protection for other oxygen sensitive compounds like those phytochemicals that are the active components of the products (Carter, 1975).

In general, the antioxidants used as a product stabilizer can be categorized into:

1. True antioxidants (by breaking the free radical chain). They are effective against oxidation by atmospheric oxygen (autooxidation). For example, 0.01-0.1% butylated hydroxyanisole (BHA), 0.005-0.15% propy gallate and 0.01-0.1% tocopherol (Boylan, Chowhan and Cooper, 1896; Smolinske, 1992).

2. Reducing agents; they are not preferentially oxidized but act by blocking an oxidative chain reaction. For example, 0.01-0.15% sodium metabisulfite and 0.01-0.02% sodium sulfite (Carter, 1975).

3. Synergists; these antioxidants generally have little effect by themselves but enhance the action of the true antioxidants either by removing pro-oxidant metals or by regenerating the antioxidant by reduction. For example, ethylene diaminetetraacetic acid (EDTA) and calcium. EDTA is a chelating agent. It forms stable water-soluble complexes (chelates) with alkaline earth and heavy metal ions. The chelated form has few of the properties of the free ions, and for this reason chelating agents are often

described as "removing" ions from solution (also called sequestering). The stability of the metal-EDTA complex depends on the metal ion involved and also on the pH. Antioxidant synergists have been used both alone and in combination with true antioxidants. Concentrations in the range of 0.005-0.1% have been employed (Boylan et al., 1986).