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 (Kartikar 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.

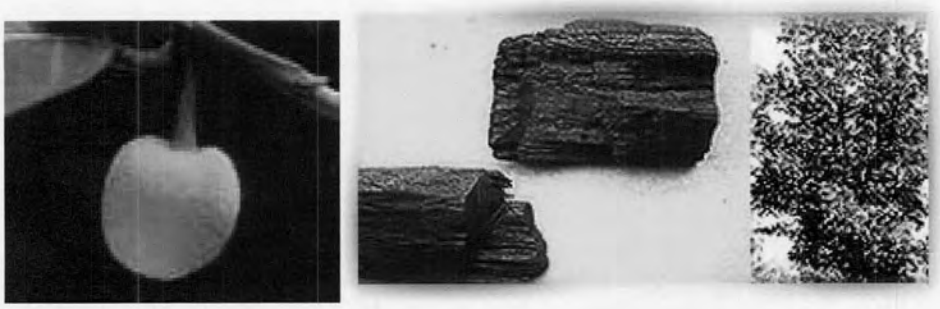


Figure 1. *Artocarpus lakoocha* Roxb. (Ma-Haad)

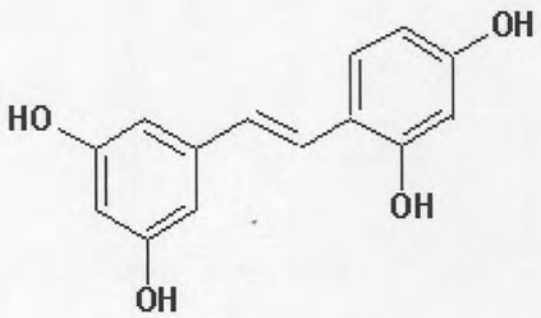


Figure 2. Chemical structure of oxyresveratrol or 2, 4, 3', 5' –tetrahydroxystilbene

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* was able to 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.

Puag-Haad and its possible antioxidant/anti-aging activities

Puag-Haad 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. Recenty, 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-picrylhydrazyl) 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 cyclooxygenase (Shin et al., 1998b) and rat liver mitochondrial ATPase (Nimmanpisut et al., 1976). However, the many aspects of the antioxidative/free radical scavenging activities of the extract or oxyresveratrol, especially regarding the cosmetic applications was known. The antioxidant properties of Puag-Haad was also evaluated the ROS scavenging effect on, DPPH radical, superoxide anion, hydroxyl radical and singlet oxygen. The results suggested they were capable of scavenging several reactive oxygen species (Wachiranuntasin, 2005). Since there are many possible anti-oxidative mechanisms, free radical scavenging pathways were investigated, those were interested in the anti-oxidative capacity. The study in cell culture of the *A. lakoocha* extract should be investigated compared with the commercial antioxidants in anti-aging 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.

Here are the four principal theories of aging. While none fully expresses the hows and whys for the process of aging, each accounts for some aspects of the process (Klatz and Goldman, 2003).

1.1 The "Wear and Tear" theory

Dr. August Weismann, a German biologist, first introduced this theory in 1882. He believed that the body and its cells were damaged by overuse and abuse. The organs-liver, stomach, kidneys, skin, and so on are worn down by toxins in the diet and in the environment; by the excess consumption of fat, sugar, caffeine, alcohol, and nicotine; by the ultraviolet rays of the sun; and by the many other physical and emotional stresses to which we subject our bodies. Wear and tear is not confined to our organs, however; it also takes place on the cellular level. This theory also holds that nutritional supplements and other treatments can help reverse the aging process by stimulating the body's own ability to repair and maintain its organs and cells. The concept of "wear and tear" therefore may be said to differ from person to person, depending upon the lifestyle of each and methods used to reverse the wear and tear (Klatz and Goldman, 2003).

1.2 The neuroendocrine theory

This theory, developed by Dr.Vladimir Dilman, elaborates on the wear and tear theory by focusing on the *neuroendocrine system*, which is a complicated network of biochemicals that governs the release of hormones and other vital bodily elements. When one is young, the hormones work together to regulate many bodily function, including one's responses to heat and cold, one's experiences, and one's

level of sexual activity. Different organs release various hormones, all under the governance of the hypothalamus, a walnut-sized gland located within the brain. Thus, hormone replacement therapy-a frequent component of any anti-aging treatment- helps to reset the body's hormonal clock and so can reverse or delay the effects of aging. If the hormones are being produced at youthful level, in a very real sense the cells of the body are stimulated to be metabolically active, and thus, one can young (Klatz and Goldman, 2003).

1.3 The genetic control theory

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

1.3.1 Cellular aging as programmed phenomenon (the programmed theory of aging)

Hayflick Limit Theory

In 1961 Dr. Hayflick and cell biologist Dr. Moorehead, made a significant contribution to the history of cellular biology, demonstrating the senescence of cultured human cells. Hayflick theorized that the aging process was controlled by a biological clock contained within each living cell. The 1961 studies concluded that human fibroblast cells (lung, skin, muscle, and heart) have a limited life span. They divided approximately fifty times over a period of years and then suddenly stopped. Nutrition seemed to have an effect on the rate of cell division: overfed cells made up to fifty divisions in a year, while underfed cells took up to three times as long as normal cells to make the divisions. Alterations and degenerations occurred within some cells before they reached their growth limit; the most evident changes took place in the cell organelles, membranes, and genetic material. This improper functioning of cells and loss of cells in organs and tissues may be responsible for the effects 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 states 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; Klatz and Gold man., 2003).

1.3.2 The error theory

In 1963, Dr. Leslie Orgel of the Salk Institute suggested that because the "machinery for making protein in cells is so essential to life, an error in that machinery could be catastrophic." The production of proteins and the reproduction of DNA sometimes are not carried out with accuracy (Klatz and Goldman, 2003). 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 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).

1.3.3 Repair failure

The body's DNA is so vital that natural repair processes kick in when an error is made. But the system is incapable of making perfect repairs on these molecules every time, and therefore the accumulation of these flawed molecules can cause diseases and other age-related changes to occur. If DNA repair processes did not exist, scientists estimate that enough damage would accumulate in cells in one year to make them non-functional (Klatz and Goldman, 2003). 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).

1.3.4 Redundancy failure

Like the errors and repairs theory, the redundant DNA theory blames errors accumulating in genes for age changes. But as these errors accumulate, this theory also blames reserve genetic sequences of identical DNA that take over until the system is worn out. Dr. Zhores Medvedev of the National Institute of Medical Research in London proposed that different species' life spans may be a function of the degree of these repeated gene sequences (Klatz and Goldman, 2003). 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 (Kenny, 1982)

1.3.5 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).

Unlike other cells, brain cells or neurons do not replicate. Dr. Donner Denckla, an endocrinologist formerly at Harvard University was convinced that the "death hormone" or DECO (DECreasing Oxygen consumption hormone) released by the pituitary gland may contribute to the loss of neurons. When he removed the pituitary glands of rats, their immune system revitalized, the rate of cross-

linking in cells reduced, and cardiovascular function was restored to the levels of youth. Denckla speculated that as we age, the pituitary begins to release DECO, which inhibits the ability of cells to use thyroxine, a hormone produced by the thyroid gland that governs basal metabolism, the rate at which cells convert food to energy. The resulting changes in metabolic rate bring on and accelerate the process of aging (Klatz and Goldman, 2003).

1.4 The Free-radical theory

This exciting development in anti-aging research was first introduced by R. Gerschman in 1954, but was developed by Dr. Denham Harman of the University of Nebraska College of Medicine. "Free radical" is a term used to describe any molecule that differs from conventional molecules in that it possesses a free electron, a property that makes it react with other molecules in highly volatile and destructive ways (Klatz and Goldman, 2003).

The free radical theory 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, 1992; Jay and Berton, 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).

This type of free-radical damage begins at birth and continues until we die. At younger age, its effects were relative minor since the body has extensive repair and replacement mechanisms that in healthy young people function to keep cells and organs in working order. With age, however, the accumulated effects of free-radical

damage begin to take their toll. Free radical-induced disruption of cell metabolism is part of what ages our cells. It may also create mutant cells, leading ultimately to cancer and death. Moreover, free radicals attack collagen and elastin, the substances that keep our skin moist, smooth, flexible, and elastic. These vital tissues fray and break under the assaults of free radicals, a process particularly noticeable in the face, where folds of skin and deep-cut wrinkles are testaments to the long-term effect of free-radical attacks (Klatz and Goldman, 2003).

Other theories of aging

While the four theories described above may be the most important to our present-day understanding of the process of aging, there are many other theories that have been put forth to give insight into the process (Klatz and Goldman, 2003).

1.5 Waste accumulation theory

In the course of their life spans, cells produce more waste than they can properly dispose of. This waste can include various toxins, which, when accumulated to a certain level, can interfere with normal cell function, ultimately killing the cell (Klatz and Goldman, 2003).

1.6 Limited number of cell divisions theory

The number of cell divisions is directly affected by the accumulation of the waste products in the cell. The more waste products accumulate over time, the faster cells degenerate. The theory, although eventually overturned, would help explain why cells from older people with more waste divided fewer times than cells from embryos, which divided the most often (Klatz and Goldman, 2003).

1.8 Thymic-stimulating theory

Dr. Alan Goldstein at George Washington University was investigating whether the shrinkage of the thymus contributes to the aging process by weakening the body immune system. Studies have shown that thymic factors are helpful in restoring the immune systems of children born without thymus glands as well as rejuvenating the poorly functioning immune systems of the elderly. Thymic hormones may also play a role in stimulating and controlling the production of neurotransmitters as well as and brain and endocrine system hormones, which means they may be the pacemakers of aging itself as well as key regulators responsible for immunity (Klatz and Goldman, 2003).

1.9 Mitochondrial theory

The free-radical theory is supported by direct experimental observations of mitochondrial aging. Mitochondria are the energy-producing organelles in the cells that are responsible for producing ATP, the primary source of energy. They produce cell energy by a process that leads to the formation of potentially damaging free radicals. Mitochondria are also one of the easiest targets of free-radical injury because they lack most of the defenses found in other parts of the cell. Evidence points to various kinds of accumulated DNA damage over time to be a contributing factor to disease, and new research in mitochondrial repair could play an important part in the fight against aging (Klatz and Goldman, 2003).

1.10 Cross-linkage theory

Developmental aging and cross-linking were first proposed in 1942 by Johan Bjorksten. He applied this theory to aging diseases such as sclerosis, a declining immune system and the most obvious example of cross-linking, loss of elasticity in the skin. One of the most common proteins found in the skin, tendons, ligaments, bone, and cartilage is collagen. The collagen protein can be compared to the legs of a ladder with very few rungs. Each protein is connected to its neighbors by other rungs, forming a cross-link. In young people, there are few cross-links and the ladders are free to move up and down. The collagen stays soft and pliable. With age, however, the number of cross-links increases, causing the skin to shrink and become less soft and pliable. It is thought that these cross-links begin to obstruct the passage of nutrients and waste between cells (Klatz and Goldman, 2003).

Cross-linking also appears to occur when older immune systems are incapable of cleaning out excess glucose, or sugar molecules in the blood. These sugar molecules react with proteins, causing cross-links and the formation of destructive free radicals. Scientists once thought inflexibility of the body with age was due to cross-linking of tendon, bone, and muscle tissues. However, people who lead a more active lifestyle and follow a good diet seem to inhibit or delay the cross-linking process (Klatz and Goldman, 2003).

1.11 Autoimmune theory

The immune system is the most important line of defense against foreign substances that enter the body. With age, the system's ability to produce necessary antibodies that fight disease declines, as does its ability to distinguish antibodies from proteins. In a sense, the immune system becomes self-destructive and reacts against itself. Examples of autoimmune diseases are lupus, scleroderma, and adult-onset diabetes (type II) (Klatz and Goldman, 2003).

1.12 Calorie restriction theory

Calorie restriction, or energy restriction, is a theory proposed by respected gerontologist Dr. Roy Walford of the UCLA Medical School. After years of animal experiments and research on longevity, Walford has developed a high-nutrient, low-calorie diet, which, when followed, demonstrates that "under- nutrition without malnutrition" can dramatically retard the functional if not the chronological aging process. An individual on this program would lose the necessary weight gradually until a point of metabolic efficiency was obtained for maximum health and life span. Walford stresses the importance of not only the high-low diet, but also moderate vitamin and mineral supplements coupled with regular exercise (Klatz and Goldman, 2003).

1.13 Gene mutation and DNA damage theory

In the 1940s scientists investigated the role of mutations in aging. Mutations are changes that occur in the genes that are fundamental to the creation of life. Evidence supporting this idea came from experiments with radiation. It was observed that radiation not only increased the gene mutation rate in animals but accelerated their aging process as well. However, later studies showed the radiation-induced changes were only mimicking age-related changes. In April 2002, Dr. deBoer of Erasmus University in Rotterdam and colleagues announced findings suggesting that damage to the body's DNA is responsible for aging. Molecules called *reactive oxygen species*, by-products of normal metabolic processes, cause harm to DNA and are suspected of contributing to diseases such as cancer and heart disease. Dr. deBoer's team identified a defect in a gene involved in DNA repair. Errors in this gene caused mice to age more quickly upon reaching adulthood (Klatz and Goldman, 2003).

1.14 Rate of living theory

German physiologist Max Rubner, who discovered the relationship between metabolic rate, body size, and longevity, first introduced this theory in 1908. It simply states that we are each born with a limited amount of energy. If use this energy slowly, then rate of aging is slowed. If energy is consumed quickly, aging is hastened. Other "rate of living" theories focus on limiting factors such as the amount of oxygen breathed or the number of heartbeats spent (Klatz and Gold man., 2003).

1.15 Order to disorder theory

From the time of conception to sexual maturation, body is undergoing a system of orderliness. After sexual maturation, however, these same energies start to diminish in efficiency. Disorder occurs in molecules, in turn causing other molecules to produce errors and so on. These chaotic changes in our cells, tissues, and organs are what cause aging. Disorderliness varies from individual to individual, and this may be the reason why human tissues and organs deteriorate at different rates (Klatz and Goldman, 2003).

1.16 Telomerase theory of aging

A new theory of aging that holds many promising possibilities for the field of anti-aging medicine is the telomerase theory of aging. This theory was born from the surge of technological breakthroughs in genetics and genetic engineering. First discovered by a group of scientists at the Geron Corporation in Menlo Park, California, telomeres are sequences of nucleic acids extending from the ends of chromosomes. Telomeres act to maintain the integrity of chromosomes. Every time your cells divide, telomeres are shortened, leading to cellular damage and cellular death associated with aging (Klatz and Goldman, 2003).

2. Skin and aging

2.1 skin

The skin, which is the major component of the integumentary system, separates the body from the external environment by forming an uninterrupted covering over the entire body surface (Spence, 1989). The skin is divided into three layers called epidermis, dermis and the subcutaneous layer (Figure 3).

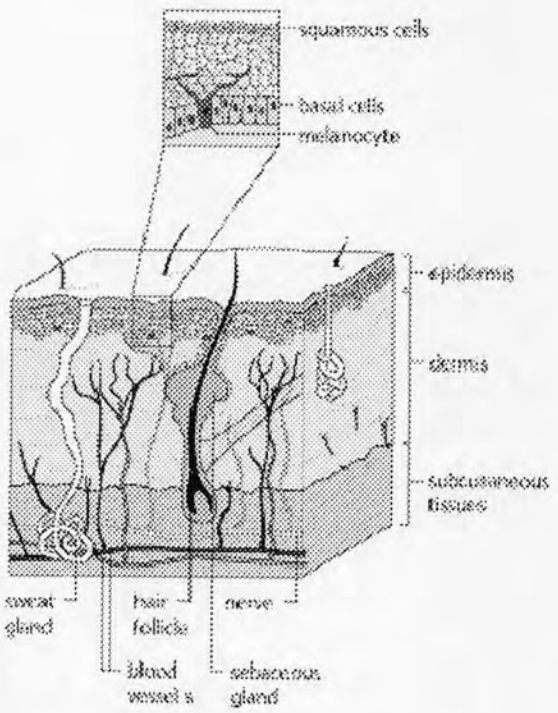


Figure 3. Basic structure of the skin

The superficial portion of the skin is the *epidermis*: the epidermis consists of several layers of thin, flat cells (squamous cells) that form a tissue referred to as stratified squamous epithelium. It is generally quite thin, but becomes thicker in regions that are subjected to constant pressure or friction, such as the soles of the feet and the palms of the hands. In fact, continued pressure at a particular site causes the epidermis to thicken to the extent that calluses or corns may be formed (Spence, 1989). The epidermis is composed of several cell layers about 0.1-0.3 mm thick. From the external surface inwards, these layer are called stratum corneum, stratum

spinosum, stratum lucidum, stratum granulosum, and sratum 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 or corneocytes in the outer layer of stratum corneum 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 horney 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.

Unlike most tissues, there are no blood vessels or nerve fibers within the epidermis. Because of the lack of blood vessels, nutrients reach the cells of the epidermis, and wastes are carried away from them, only by diffusing from, or to, blood vessels located in the dermis. This indirect method of exchange is adequate for those cells located closest to the dermis. But as these basal cells divide, forcing the daughter cells toward the body surface and thus further away from the dermal blood vessel, the rates of exchange of nutrients and wastes are not sufficient to maintain the cells, and they die. The cytoplasm of the dead cells is gradually replaced with a harder substance known as keratin. In this manner the outermost layer of the epidermis (stratum corneum) becomes composed of thin, dead cells containing a horny material (Spence, 1989).

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 material (Fenske, 1986; Thody, 1986; Marieb, 1995).

The *dermis* is located immediately beneath the epidermis. It is thicker than the epidermis and is composed of dense connective tissue. There are many collagenous fibers and some elastic fibers, which are embedded in an interfibrillar gel of glycosaminoglycans, distributed throughout the dermis. The spaces between these fibers are filled with a matrix that varies in consistency from fluid to semisolid (Spence, 1989). Collagen is the major component and is secreted by fibroblasts which are the principle 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, 1986; Marieb, 1995).

In contrast to the epidermis, the dermis is well supplied with blood vessels, lymphatic vessels, and nerves. It also contains *sweat glands* and oil-secreting *sebaceous glands*. Specialized receptors located in the dermis provide information to the nervous system concerning environmental stimuli producing touch, pressure, pain, and temperature changes. These receptors allow us to respond in an appropriate manner to environmental challenges (Spence, 1989).

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

Most of the changes that occur in the skin are associated with age. The changes tend to be so obvious to the person affected as well as to the personal interactions which affect an individual's self-concept (Spence, 1989; Potts, 1984; Yamauchi, 1988; Gilchrest, 1991).

Epidermal changes (Spence, 1989)

The epidermis tends to become thinner with age, due in part to an increased scaling off of its cells and a declining rate of cellular division that typically accompanies aging. As a result, some of the cells lost from the surface of the epidermis are not replaced. There are reports of increased permeability of the surface cells, allowing substances to pass through this barrier more readily than in younger skin.

There is a decrease in the number of cells in the epidermis capable of producing the pigment melanin. However, the pigment cells (melanocytes) present tend to be larger and group together, forming dark pigment plaques called *aging spots* that are typical of older persons.

Dermal changes (Spence, 1989)

The dermis is a connective tissue layer, and most of the cells within this layer are fibroblasts, which produce the fibers of the connective tissue. There is a general reduction in the number of fibroblasts and fibers with aging, and

thus the dermis becomes thin and somewhat translucent. At the same time, the collagenous fibers present become larger and coarser. This reduces the amount of apace available for storage between the fibers, causing the fat, water, and matrix content of the dermis to diminish with age.

During aging the elastic fibers of the dermis become less resilient due to structural changes resulting from the formation of cross-links. In some locations the elastic fibers may undergo slight calcification, which further decreases their resiliency. These changes in the properties of the elastic fibers cause the skin to be less able to smooth out; thus, wrinkles and sags become common with age.

There is a reduction in the numbers of sweat glands and sebaceous glands due to their gradual atrophy. Therefore, older persons tend to sweat less and have drier, scalier skin. Because older persons do not perspire as much as younger persons, their ability to regulate body temperature is diminished, and they are more likely to suffer from heat exhaustion.

At the same time, there is a generalized reduction in blood flow to the skin, causing the skin surface to be cooler in older persons than in younger persons. The lowered skin temperature slows the growth of the fingernails and toenails. Although the nails grow more slowly, they tend to take on a yellowish color, develop ridges, and become thicker due to the deposition of calcium. This is more noticeable in the toenails, which may also become curved.

With age there is a decrease in the number and activity of hair follicles, causing a generalized loss of body hair which is especially noticeable on the head. However, the hair in the eyebrows, nostrils, and ears of old men may become coarse and grow more rapidly. Because of a concomitant decrease in the number of functioning pigment-producing cells, there is a gradual reduction of pigment in the hair with aging. As the amount of pigment in the hair decreases there is a loss of hair color and the hair become gray. In the complete absence of pigments the hair appears white. Heredity plays a role in the loss of hair color, and some people become gray prematurely, while others retain their usual color until quite late in life.

Structural, functional, and numerical changes with age have been reported for some of the dermal sensory receptors. A person's sensitivity to touch diminishes with age in regions of the skin which lack hair but remains at its usual level

in skin with hair. The data on temperature and pain receptors are mixed and make any conclusions concerning their age-related changes tentative at best. There are indications that the sensitivity of temperature receptors declines with age. Data from pain research are particularly confusing in that some support an increased, others a decreased, and still others an unchanged sensitivity to pain.

Hypodermal Changes (Spence, 1989)

The hypodermis, which is also referred to as the *subcutaneous tissue* because it is located just beneath the skin, is technically not part of the skin. The hypodermis is essentially a layer of loose connective tissue in which much fat is stored. With aging there is a generalized loss of fat from the subcutaneous tissue. The loss of subcutaneous fat is a major cause of the wrinkles that are common with age and is largely responsible for the emaciated appearance of many older persons.

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 4 (Jay and Berthon, 1998).

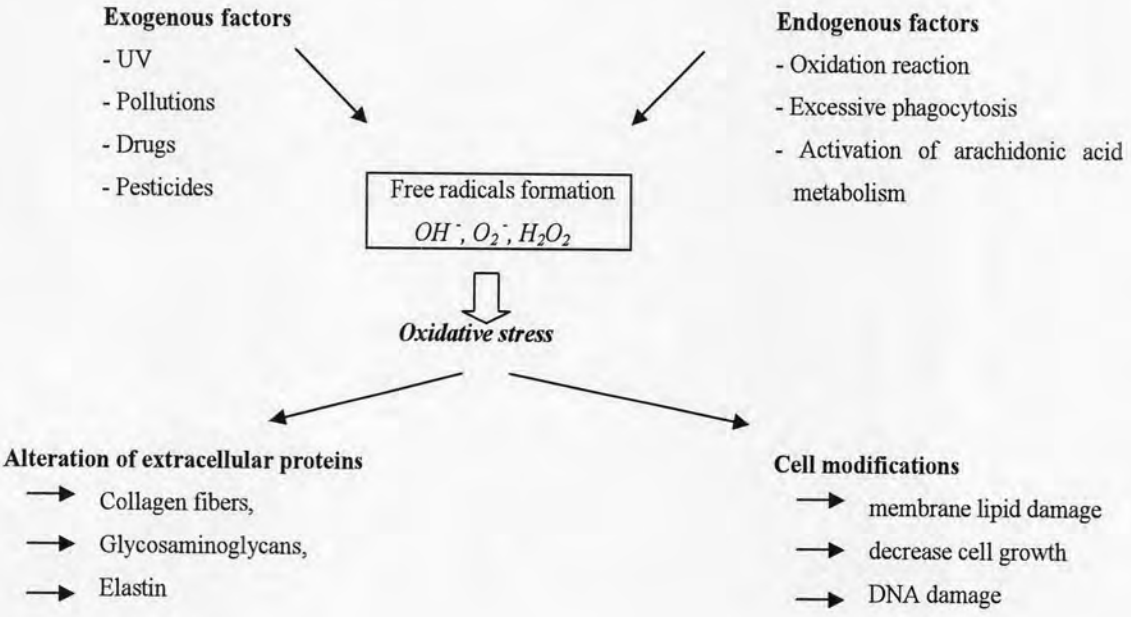


Figure 4. Free radicals formation and its deleterious effects

From Figure 4, 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 and Berthon, 1998).

degrade most of the proteins in the extracellular matrix. As seen in table 1, almost all of them are able to degrade some types of collagen and gelatin, and most of them, especially MT-MMPs, activate other MMPs.

All MMPs are synthesized in the latent form. They are secreted as proenzymes and require extracellular activation. They can be activated *in vitro* by many mechanisms including organomercurials, chaotropic agents and other protease. MMPs need Zn^{2+} to be active. MMPs activity is regulated at many levels. The messenger RNA (mRNA) is transcriptionally regulated by biologically active agents such as hormones, oncogened, growth factor and tumor promoters. The activation processes consist of three different mechanisms: stepwise activation on the cell surface and intracellular activation. In addition, two progelatinases (proMMP-2 and proMMP-9) can bind to endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs).

These enzymes are synthesized and secreted in low amounts by normal cells associated with physiologic tissue remodeling such as wound healing, implantation, trophoblastic invasion and angiogenesis. Increased expression of MMPs is found in various human tumors and cell lines. MMPs are often overexpressed in malignant tumors.

Table 1 The matrix metalloproteinase family.

Subfamily	Name	MMPs	Main substrates
Interstitial collagenase	Fibroblast collagenase	MMP-1	Fibrillar collagen
	Neutrophil collagenase	MMP-8	Fibrillar collagen
	Collagenase-3	MMP-13	Fibrillar collagen
	Collagenase-4	MMP-18	Fibrillar collagen
Gelatinases	Gelatinase A	MMP-2	Gelatin, type IV collagen, fibronectin, elastin, laminin
	Gelatinase B	MMP-9	Gelatin, elastin, fibronectin, vitronectin
Stromelysins	Stromelysin-1	MMP-3	Gelatin, fibronectin, casein, laminin, elastin, MMP-2/TIMP-2
	Stromelysin-2	MMP-10	Same as above
	Stromelysin-3	MMP-11	Fibronectin, laminin, gelatin, aggrecan
	Matrilysin	MMP-7	Fibronectin, vitronectin, laminin, gelatin, aggrecan
Elastases	Metalloelastase	MMP-12	Elastin, gelatin, collagen IV, fibronectin, laminin,vitronectin, proteoglycan
Membrane-type MMPs	MT1-MMP	MMP-14	proMMP-2, procollagenase 3
	MT2-MMP	MMP-15	pro-MMP-2
	MT3-MMP	MMP-16	pro-MMP-2
	MT-4-MMP	MMP-17	Unknown
Other MMPs	Enamelysin	MMP-20	amelogenin

MMP-2 (Gelatinase A, 72 kDa type IV collagenase) is the most widely distributed enzyme of the MMP family and it was originally described and purified as a basement membrane collagen degrading enzyme activity from a metastatic murine tumor. MMP-2 is expressed e.g. by fibroblasts, keratinocytes, epithelial cells, monocytes and osteoblasts (Thibodeau, 2000; Tournier et al., 1994; Creemers et al., 1998).

C. 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, 1997; Halliwell et. al., 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 radical, (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).

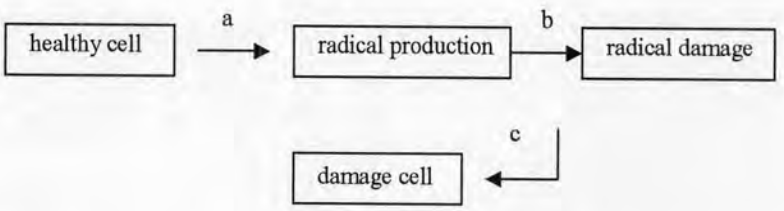


Figure 6. Diminishing radical-induced cell damage; a=radical formation prevention; b=radical scavenging; c=repair of radical-induced damage

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 s formed during superoxide dismutation is also toxic, it can be removed by enzyme catalase. This whole mechanism is necessary for the cell survival.

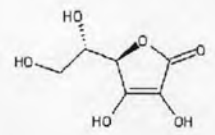
$$O_2 + e^- \xrightarrow[\text{Glutathione peroxidase}]{} O_2^- \xrightarrow[\text{SOD}]{} H_2O_2 \xrightarrow[\text{Catalase}]{} H_2O + O_2$$

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).

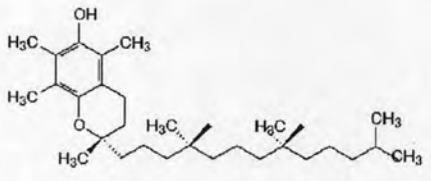
Vitamin C (l-ascorbic acid)

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

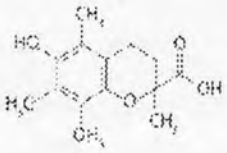
potential, and its structure is shown in Figure 7. The same property makes this vitamin an excellent antioxidant, capable of scavenging a wide variety of different oxidants. For example, ascorbate has been shown to effectively scavenge superoxide, hydrogen peroxide, hyperchloric acid, aqueous peroxyl 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 oxidize form of vitamin C). Although dehydroascorbic acid is relatively unstable and readily hydrolyzed to 1-2, 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 (Sies and Stahl, 1995).



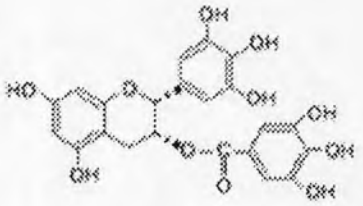
vitamin C



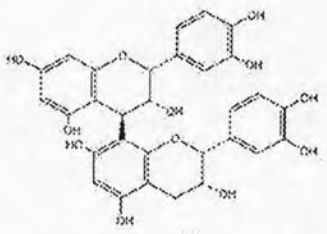
vitamin E



Trolox®



EGCG



French pine bark

Figure 7. Structure of vitamin C, Vitamin E, Trolox®, EGCG and French pine bark

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

Trolox®

It is a water-soluble form of α-tocopherol with the hydrophobic side-chain replaced by a hydrophilic –COOH group. It's 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). 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; Geeta et al., 2004). Moreover, EGCG potent inhibitor of MMP-2 activities, as measured by fluorescence (Demeule et al., 2000).

Pine bark extract

Pine bark extract is a substance obtained from the bark of *Pinus maritime* (French maritime pine) or *Pinus pinaster* (Figure 7). The main constituents of pine bark extract are known to be the mixture of procyanidins, 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 epicatechins) 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 have 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 ingested 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.

D. Cell Culture

(Freshney, 2000)

Cell culture is a method for studying the behavior of animal cells free of systemic variations that might arise in the animal both during normal homeostasis and under the stress of an experiment. Cell cultures may be derived from primary explants, a fragment of tissue is placed at a glass or plastic-liquid interface where, following attachment, migration is promoted in the plane of the solid substrate, or dispersed cell suspensions. Because cell proliferation is often found in such cultures, propagation of cell lines becomes feasible. A monolayer or cell suspension, with a significant growth fraction, may be dispersed by enzymatic treatment or simple dilution and reseeded, or subcultured, into fresh vessels. This constitutes a passage and the daughter cultures so formed are the beginnings of a cell line.

The formation of a cell line from a primary culture implies;

1. An increase in total cell number over several generations
2. That cells or cell lineages with similar high growth capacity will predominate
3. A degree of uniformity in the cell population.

The line may be characterized, and those characteristics will apply for most of its finite life-span. The derivation of continuous cell lines usually implies a phenotypic change or transformation. When cells are selected from a culture, by cloning or by some other method, the subline is known as a cell strain. Detailed characterization is then implied. Cell lines, or cell strains, may be propagated as an adherent monolayer or in suspension.

Advantages of cell culture

The three major advantages of cell culture are the control of the environment, the characterization and homogeneity of sample, and the economy.

In control of the environment, both physiochemical environment (pH, temperature, osmotic pressure, O_2, and CO_2 tension) and physiological conditions are controlled. However, most cell lines still require supplementation of the medium with serum or other poorly define constituents. These supplements are prone to batch

variation and contain undefined elements such as hormones and other regulatory substances.

Tissue samples are invariably heterogeneous. Replicates even from one tissue vary in their constituent cell types. After one or two passages, cultured cell lines assume a homogeneous (or at least uniform) constitution, as the cells are randomly mixed at each transfer and the selective pressure of the culture conditions tends to produce a homogeneous culture of the most vigorous cell type. Hence, at each subculture each replicate sample will be identical, and the characteristics of the line may be perpetuated over several generations or indefinitely if the cell line is stored in liquid N_2. Since experimental replicates are virtually identical, the need for statistical analysis of variance.

Cultures may be exposed directly to a reagent at a lower and defined concentration, and with direct access to the cell. Consequently, less amount is required than for injection *in vivo* where 90% is lost by excretion and distribution to tissues other than those under study. Screening tests with many variables and replicates are cheaper, and the legal, moral, and ethical questions of animal experimentation are avoided.

Disadvantages of cell culture

Culture techniques must be carried out under strict aseptic conditions. This requires a level of skill and understanding to appreciate the requirements of the system and to diagnose problems as they arise. In addition, the expenditure of effort and materials that goes into the production of relatively little tissue is a limitation of cell culture.

In cell line propagation, many workers observed the loss of the phenotypic characteristics typical of the tissue from which the cells had been isolated. Under the correct culture conditions, however, many of the differentiated properties of these cells may be restored. If differentiated properties are lost, it is difficult to relate the cultured cells to functional cells in the tissue. Stable markers are required for characterization, in addition, the culture conditions may need to be modified so that these markers are expressed.

Instability is a major problem with many continuous cell lines, resulting from their unstable aneuploid chromosomal constitution. Even with short-term cultures of untransformed cells, heterogeneity in growth rate and capacity to differentiate within the population can produce variability from one passage to the next.

Although the existence of differences between the environment conditions of a cell *in vitro* and *in vivo* can not be denied, it must be emphasized that many specialized functions are expressed in culture, and as long as the limits of the model are appreciated, it can become a very valuable tool.

E. Flow Cytometry as a Tool to Detect DNA Damage and Death

Patterns of cell death

Cells die in response to a variety of stimuli and during apoptosis they do so in a controlled, regulated fashion. This process of cell death requires time to take place after the initial insult (Kiechle et al., 2002). Apoptosis is a process in which cells play an active role in their own death which is why apoptosis is often referred to as cell suicide. Cells die through either of two distinct processes either necrosis or apoptosis. Apoptosis, or programmed cell death, is a normal component of the development and health of multicellular organisms. The apoptotic pattern is generally triggered by various stimuli including UV, light, and cellular stress (Shao et al., 1997; Saeki et al., 2002). Apoptotic pattern is defined by a series of cellular changes. Firstly the cell shrinks, looses contact with neighbouring cells or surrounding matrix and starts to display intracellular proteins on its surface. The chromatin in the nucleus condenses and the DNA is cleaved into small fragments of 180 base pairs, which lead to a characteristic of DNA laddering when subjected to gel electrophoresis. The plasma membrane then begins to show a bubbled appearance and small membrane bound bodies break off containing intracellular material which can include nuclear matter and cellular. The fragments are known as apoptotic bodies and they are quickly removed by phagocytes or by neighbouring cells. If this does not occur quickly enough, the plasma membrane and intracellular organelles can breakdown resulting in lysis of the fragments. This process is called secondary necrosis. This makes apoptosis distinct from another form of cell death called necrosis. Necrosis is an uncontrolled cell death

that results from acute tissue injury and provokes an inflammatory response, characterised by cell swelling and mitochondrial damage leading to rapid depletion of energy levels, a breakdown of homeostatic control, cell membrane lysis and release of the intracellular contents, leading to an inflammatory response, with oedema and damage to the surrounding cells (Adams, 2003).

Apoptotic mechanism

Apoptotic cell death is an active process mediated by various signaling pathways, which include the caspase cascade and the stress-activated protein kinase pathways (Adams, 2003). There are at least two broad pathways that lead to apoptosis, an "extrinsic" and an "intrinsic" pathway. The extrinsic pathway begins outside a cell, when conditions in the extracellular environment determine that a cell must die. The extracellular signals such as anti-tumor agents, viral infections, and irradiation (Westendorp et al., 1995; Friesen et al., 1996; Muller et al., 1997; Rehemtulla et al., 1997) triggered death receptors that belong to the tumor necrotic factor receptor (TNFR) family. In particular, Fas (CD95/Apo-1), one of the most important receptor of TNFR family, plays a crucial role in maintaining the immune system by inducing apoptosis of immune cells as well as in killing harmful cells such as cancerous cells and bacterial or viral infected cells (Nagata, 1997). Binding of Fas ligand (FasL or CD95L) to the Fas receptor results in clustering of receptors and initiates the extrinsic pathway. Fas clustering recruits Fas-associated death domain (FADD) and pro-caspase-8 to the complex. Concentration of pro-caspase-8 results in its autocatalysis and its activation. The activated-caspase-8 cleaves pro-caspase-3, which then undergoes autocatalysis to form active caspase-3, a principle effector caspase of apoptosis.

The intrinsic apoptotic pathway begins when an injury or the stress occur within the cell. In the intrinsic pathway, mitochondria play a central role in cell death by controlling cellular energy metabolism, production of ROS, and release of apoptotic factors into the cytosol. The most prominent pro-apoptotic factor released from mitochondria is cytochrome *c* (cyt *c*). Once released into the cytosol during apoptosis, cyt *c* binds to Apaf-1 thus forming a complex called the apoptosome, which recruits and activates pro-caspase-9 (Li et al., 1997; Liu et al., 1996). Thereafter,

activated caspase-9 initiates the activation of downstream caspases, which cleave cellular substrates at specific tetra-peptide sequences on the carboxyl termini of aspartate residues. Disruption of an appropriate apoptotic response is implicated in the development of many disease states, including cancer, atherosclerosis and several degenerative diseases.

Reactive oxygen species leading to apoptosis

Cellular stress is caused by multiple factors including injury, inflammation, hypoxia, hormones, high concentrations of metabolites or xenobiotics, and excessive muscle work. "Metabolic stress" is created when the activity of a cell is stimulated, and could result in "oxidative stress" when ROS are produced intracellularly, particularly in mitochondria which participate in apoptosis. Furthermore, exogenous oxidants can generate "cytotoxic stress" that threatens survival of the cell (Kadenbach et al., 2004). At high concentrations, free radicals and radical-derived, nonradical reactive species are hazardous for living organisms and damage all major cellular constituents. At moderate concentrations, however, ROS play an important role as regulatory mediators in signaling processes (Droge et al., 2002). ROS are generated during normal processes of mitochondrial oxidative phosphorylation. Under physiological conditions, electrons carried by the electron transport chain can leak out of the pathway and pass directly to oxygen, generating $O_2^{-\bullet}$. Other sources of $O_2^{-\bullet}$ include enzymes such as cytochrome P450 in the endoplasmic reticulum, lipoxygenases, cyclooxgenases, xanthine oxidase and NADPH oxidase in the cytosol (Chen et al., 2003). The dismutation of $O_2^{\bullet-}$ by SOD results in the generation of H_2O_2. The predominant forms are the copper-containing enzyme and the zinc-containing enzyme, located in the cytosol. The second type is the manganese containing SOD found in the mitochondrial matrix (Bandyopadhyay et al., 1999).

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

Under normal physiological conditions, hydrogen peroxide (H_2O_2) is converted into H_2O by glutathione peroxidase and catalase. GSH is not only present in mitochondria but also present in cytosol (Chen et al., 2003; Bandyopadhyay et al., 1999). In case of catalase, this enzyme is localized in the peroxisomes or the microperoxisomes (Bandyopadhyay et. al., 1999).

$$2\,H_2O_2 \xrightarrow{\text{Catalase}} H_2O + O_2$$

However, this reaction is dependent upon the ratio of GSH : GSSG (oxidized form). If there is not enough reduced substrate (2 GSH→ GSSG) available, H_2O_2 can react with Fe^{2+} to form hydroxyl radicals ($^{\bullet}OH$), the highly toxic free radical, via the Fenton reaction. (Cadenas and Davies, 2000; Carmody and Cotter, 2001; Slater et al., 1995).

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + {}^{\bullet}OH + OH^{-}$$

Additionally, the hydroxyl radical could also be generated through the metal catalyzed Haber-Weiss reaction as follows (Evens et al, 2004).

$$O_2^{-\bullet} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^{-\bullet}$$

High concentration of ROS could induce apoptotic cell death in various cell types (Lee et. al., 2005; Kling et al., 2005), suggesting that ROS contribute to cell death whenever they are generated in the cortex of the apoptotic process (Droge, 2002). ROS as an oxidant could passively attack cellular compound. Increases in ROS are often associated with apoptosis (Kluza et al., 2005; Kling et al., 2005; Takahashi et al., 2003). The generation of ROS disrupted mitochondrial membrane potential, decrease of Bcl-2 and increase of Bax leading to mitochondrial dysfunction. Release of apoptogenic factors from mitochondria, the best known of which is cyt *c*, leads to assembly of a large apoptosisinducing complex called the apoptosome. Cysteine

proteases (called caspases) are recruited to this complex and, following their activation by proteolytic cleavage, activate other caspases, which in turn target for specific cleavage a large number of cellular proteins.