CHAPTER II

LITERATURE REVIEW

1. Skin and skin aging

Skin is the largest organ of the human body. As our major interface with the environment, the skin is composed of specialized epithelial and connective tissue cells. It has functions in thermoregulation, protection, metabolic functions and sensation. The skin is composed of three primary layers: the epidermis, the dermis, and subcutaneous tissue (Baumann, 2002).

A. Anatomy of the skin

1. Epidermis

The epidermis, the outermost layer, is directly contiguous with the environment. It is formed by an ordered arrangement of cells called keratinocytes, whose basic function is to synthesize keratin, a filamentous protein that serves a protective function (Odom, James and Berger, 2000). The epidermis is the most superficial layer of the skin and provides the first barrier of protection from the invasion of foreign substances into the body. It is very important from a cosmetic standpoint because it is this layer that gives the skin its texture and moisture, and contributes to skin color.

1.1 Layers of the epidermis

The principal cell of the epidermis is called a keratinocyte. The epidermis is subdivided into five layers or strata, the stratum basale (basal layer), the stratum spinosum (spinous or prickle cell layer), the stratum granulosum (granular layer), the stratum lucidum (clear layer) and the stratum corneum (horny layer) in which a keratinocyte gradually migrates to the surface and is sloughed off in a process called desquamation.

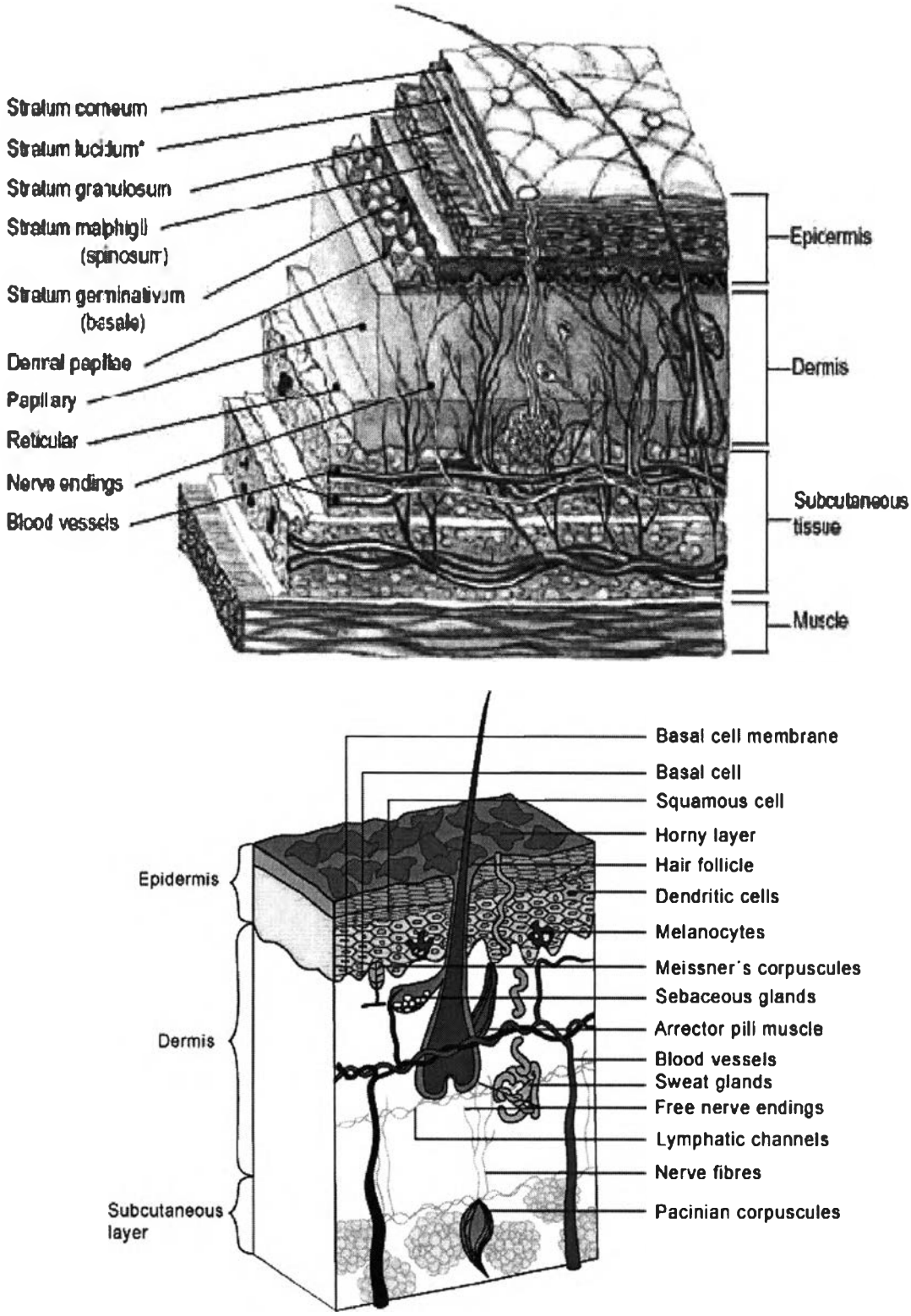


Figure 1. Basic skin structure

1.1.1 The stratum basale (Basal Layer)

The stratum basale (basal layer) provides the germinal cell necessary for the regeneration of the layers of the epidermis. These germinal cells join with other basal cell and the overlying spinous cells via desmosomes to form the basement membrane. After a mitotic division a newly formed cell will undergo a progressive maturation called keratinization as it migrates to the surface. The basal cells are responsible for maintaining the epidermis by continually renewing the cell population.

1.1.2 The stratum spinosum (spinous layer)

The cells that divide in the stratum basale soon begin to accumulate many desmosomes on their outer surface which provide the characteristic "prickles" of the stratum spinosum, which is often called the prickle-cell layer. Keratins 1 and 10 are first seen in this layer of suprabasal keratinocytes. These keratins form a more rigid cytoskeleton that provides a greater mechanical strength to the cell lamellar granules, which are considered the first sign of keratinization, first appear in this layer.

1.1.3 The stratum granulosum (granular layer)

The progressive maturation of a keratinocyte is characterized by the accumulation of keratin, called keratinization. The cells of the stratum granulosum accumulate dense basophilic keratohayalin granules. These granules contain profilaggrin, the precursor to filaggrin. Filaggrin cross-links keratin filaments, providing strength and structure. The proteins of the cornified cell envelope (involucrin, keratolinin, pancornulins, and loricrin) are cross-linked in this layer by the calcium-requiring enzyme transglutaminase to form the cell envelope.

Granular cells exhibit anabolic properties such as synthesis of filaggrin, cornified cell envelope proteins, and high molecular-weight keratins. They also show catabolic events such as dissolution of the nucleus and organelles.

1.1.4 The stratum lucidum

Epidermis varies in thickness throughout the body depending mainly on friction forces and is thickest on the palms of the hands and soles of the feet. The stratum lucidum is normally only well seen in thick epidermis and represents a transition from the stratum granulosum to the stratum corneum.

1.1.5 The stratum corneum

The most superficial layer of the epidermis is the stratum corneum (SC) or horny layer. The keratinocytes that reside in this layer are the most mature and have completed the keratinization process. These keratinocytes have no organelles, and their arrangement resembles a brick wall. The stratum corneum is composed of protein-rich corneocytes embedded in a bilayer lipid matrix arranged in a "brick and mortar" fashion. The "bricks" are composed of keratinocytes, and the "motar" is composed of the contents extruded from the lamellar granules, including lipids and proteins. The stratum corneum is described as the "dead layer" of cells because these cells do not demonstrate protein synthesis and are unresponsive to cellular signaling (Baumann, 2002).

1.2 Cellular components of the epidermis

1.2.1 Keratinocytes

The keratinocytes, or squamous cells, are the cells that make up the majority of the epidermis. They are born at the base of the epidermis at dermal-epidermal junction (DEJ). At the granular layer, keratinocytes are transformed to corneocytes, which are also called squames, to form the stratum corneum. As it migrates upward, there is progressive synthesis of keratin proteins, which constitute part of the protective interface between the body and the environment and not only form the surface coat (stratum corneum) of the epidermis but also is the structural protein of hair and nails (Odom et al., 2000). The migration process normally takes approximately 28 days.

1.2.2 Melanocytes (pigment cell)

The melanocyte is the pigment melanin-producing cell of the epidermis, which is a dendritic (branched) cell that sits in the basal layer of the epidermis. The melanin is produced in melanosomes: membrane-bound organelles (pigment granules) that are transferred to keratinocytes. Melanosomes are synthesized in the Golgi zone of the cell and pass through a series of stages in which the enzyme tyrosinase acts on melanin precursors to produce the densely pigmented granules. Keratinocytes are the reservoir for melanin in the skin. Variations in type and density of melanosomes lead to the wide range of human skin color. Chronic sun exposure can stimulate the melanocyte to produce larger melanosomes, thereby making the distribution of melanosomes within keratinocytes resemble the pattern seen in dark-skinned individuals (Odom et al., 2000; Walters and Roberts, 2002).

1.2.3 Langerhan's cells

Langerhans' cell is the antigen presenting cell of the epidermis which are normally found scattered among keratinocytes of the stratum spinosum, or prickle cell layer of the epidermis. They are dendritic cells intimately involved in immunological response in the skin by providing for the recognition, uptake, processing, and presentation of antigens to sensitized T lymphocytes (Odom et. al., 2000).

1.2.4 Merkel cells

The Merkel cell can be found in the basal layer of the palms and soles, the oral and genital mucosa, the nail bed, and the follicular infundibula. The Merkel cells, located directly above the basement membrane, contain intracytoplasmic neurosecretory-like granules, and, through their association with neuritis, act as slow adapting touch receptors (Odom et. al., 2000).

2. The Dermal-epidermal junction (DEJ)

The junction of epidermis and dermis is formed by the basement membrane zone. This zone is composed of four components: the plasma membranes of the basal cells with the specialized attachment plates (hemidesmosomes); an

electron-lucent zone called the lamina lucida; the basal lamina, including anchoring fibrils, dermal microfibrils, and collagen fibers (Odom et. al., 2000).

3. Dermis

The dermis lies between the epidermis and the subcutaneous fat. The dermis, which is laden with nerves, blood vessels, and sweat glands, consists mostly of collagen. The uppermost portion of this layer, which lies beneath the epidermis, is known as the papillary dermis, and the lower portion is known as the reticular dermis.

3.1 Layers of the dermis

The dermis gives the skin its mechanical strength. There are two main layers of the dermis

3.1.1 Papillary layer

The papillary dermis contains vascular networks that have two important functions. The first, the papillary dermis contains capillary loops being to support the vascular epidermis with vital nutrients and, secondly, to provide a network for thermoregulation. In the papillary dermis, collagen fibers are loosely bundled (Baumann, 2002).

3.1.2 Reticular dermis

The reticular layer of the dermis is tightly packed with collagen and elastin for strength and elasticity. This layer also contains glycosaminoglycans to bind water (Baumann, 2002).

3.2 Components of the dermis

3.2.1 Cellular components

Fibroblasts are the primary cell type in the dermis. They produce collagen, elastin, ground substance, other matrix proteins and enzymes such as collagenase and stromelysin. Immune cells such as mast cells, polymorphonuclear leukocytes (PMNs), lymphocytes, and macrophages are also present in the dermis (Odom et al., 2000).

3.2.2 Extracellular matrix

The extracellular matrix is the material that forms the bulk of the dermis, excluding water and cells (Bernstein and Uitto, 1996). This extracellular matrix (ECM), produced by keratinocytes and fibroblasts, works not only as a physical support, but also as an exchange and communication area that allows nutrients, metabolites and growth factors to diffuse between cells. Collagen, elastin, proteoglycans, fibronectins and other glycosylated proteins are among the main molecules forming the ECM (Thibodeau, 2000). The chief components of the extracellular matrix are collagen and the proteins composing elastic fiber, as well as proteoglycans and glycosaminoglycans, which serve a variety of functions and make up the nonfibrillar ground substance. Most of the dermal extracellular matrix is synthesized by fibroblasts, which respond to variety of stimuli, such as growth factors elaborated by keratinocytes, inflammatory cells, and dermal fibroblast themselves. Physical factors such as sunlight also play a role in altering the content and morphology of dermal extracellular matrix (Bernstein and Uitto, 1996).

3.2.3 Collagen

Collagen, one of the strongest natural proteins, yields the durability and resilience characteristics of skin. Collagen fibers are always seen in the final, mature, state of assembly as opposed to elastin, whose immature fibers are seen in the superficial dermis and whose more mature fibers are found in the deeper layer of the dermis. Each type of collagen is composed of three chains. Collagen is synthesized in the fibroblasts in a precursor form called procollagen. The skin derives its strength from the fibrillar collagens, chiefly collagen type I and III, which form the bulk of the dry weight of the dermis.

- Type I collagen makes up approximately 80% of the dry weight of the dermal matrix and is responsible for the tensile strength of the dermis. Fibrillar collagens contain a very specific amino acid sequence, often referred to as a collagenous sequence, containing glycine as every third amino acid in a repeating fashion. Type I collagen fibers are composed of three polypeptide chains arranged in a triple helical configuration. Procollagen is secreted from the fibroblasts into the

extracellular space and contains nonhelical extensions that are not present on the final mature collagen molecule.

- Type III collagen is the second major fibrillar collagen found in skin, comprising from 10 to 15 percent of the matrix. It is referred to as fetal collagen because it predominates in embryonic life, and is abundant in fetal tissues (Bernstein and Uitto, 1996; Baumann, 2002). Although it is about equal to type I collagen in abundance in fetal skin, type I collagen production quickly outpaces that of type III collagen, making the final ratio in adult skin 6:1 between type I and type III. Type III collagen is composed of three identical α1 (III) chains. It is distributed similarly to type I collagen, although it is particularly abundant in distensible tissues such as the gastrointestinal tract and article blood vessels. It is also the first type of collagen deposited in a healing wound (Bernstein and Uitto, 1996).

- Type IV collagen, which is the other type of collagen that is noteworthy for a cosmetic dermatologist, forms a structure lattice that is a major component of the basement membrane separating the dermis and epidermis, and it forms part of the basement membrane zone of blood vessels in the dermis. Unlike type I and III collagen, type IV collagen has regions interrupted by noncollagenous segments. This is thought to add flexibility to type IV collagen, allowing it to better serve as a scaffold for incorporation of other basement membrane components.

- Type V is diffusely distributed through the dermis and comprises roughly 4 to 5 percent of the matrix.

- Type VI collagen is composed of a short collagenous segment with large amino- and carboxy-terminal domains. It is found in the dermis in the form of microfibrils between collagen fibers in the dermis; it has cell adhesion and collagen-binding properties (Bernstein and Uitto, 1996).

- The anchoring fibrils, which extend from the basement membrane zone at the dermal-epidermal junction into the papillary dermis, are

composed of type VII collagen. These extend from the lamina densa within the basement membrane zone to the papillary dermis; they stabilize the attachment of the epidermis to the dermis. Type VII collagen, like type IV collagen, has several interruptions in its collagenous sequence, which are thought to confer flexibility, highlighting the importance of type VII collagen fibers in maintaining the attachment between the dermis and epidermis. Some investigators have postulated that a weakened bond between the dermis and epidermis caused by loss of the anchoring fibrils (collagen VII) may lead to wrinkle formation (Bernstein and Uitto, 1996; Baumann, 2002).

- Other types of collagen, such as type XII and XIV, known as FACIT collagens (fibril-associated collagens with interrupted triple helixes), are found in association with dermal collagen fibers and are thought to play a role in their formation (Bernstein and Uitto, 1996).

3.2.4 Elastic fiber

Elastic fibers form a fine network throughout the dermis and, although they make up only 1-2% of the dry weight of the skin, are crucial to providing resilience and elasticity. Elastic fibers are composed of at least two distinct proteins, elastin and fibrillin. Elastin fibers are found at the periphery of collagen bundle and endow the skin with recoil properties. These fibers are assembled on bundles of microfibrils composed of fibril. Fibrillin constitutes the fibrillar component of elastic fibers and is analogous to the cloth surrounding a bungee cord, while the stretchy inner component corresponds to elastin. Elastic fibers form a fine network that extends vertically in the dermal papillae and surrounds dermal blood vessels, while in the reticular dermis the fibers are much thicker and run parallel to the epidermis surrounding the larger collagen fibers (Bernstein and Uitto, 1996; Baumann, 2002).

3.2.5 Glycosaminoglycans (GAGs)

Glycosaminoglycans are polysaccharide chains composed of various repeating disaccharide units that are linked to a core protein. Although they

constitute only a small percentage of the dry weight of skin (0.1-0.3%), they can bind up to 1000 times their volume in water. As a result, the hydration of skin is largely dependent on the content and distribution of dermal glycosaminoglycans. The most abundant GAGs in the dermis are hyaluronic acid (HA) and dermatan sulfate. Glycosaminoglycans associated with a protein core are aptly termed proteoglycans. Proteoglycans and glycosaminoglycans maintain dermal hydration, as well as possibly participating in collagen and elastic fiber formation (Bernstein and Uitto, 1996; Baumann, 2002).

3.2.6 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) constitute a protein family that participates in the degradation of ECM macromolecules. There are more than 200 known metalloproteinases, almost all of them dependent on zinc at the active centre for their catalytic function. So far, approximately 20 MMPs have been identified. Table 1 describes a few of these MMPs with examples of the molecular substrates present in the skin's ECM.

- MMP-1 (Collagenase I), or interstitial collagenase, specifically cleaves native triple helical type I collagens, yielding ¾- and ¼-length collagen fragments as result of the hydrolysis of a single Gly-Ile/Leu bond in each α chain of the collagen molecule (Aimes and Quigley, 1995). These matrix metalloproteinases also cleaves type III collagen and pro-MMP-2 (Thibodeau, 2000). The cellular labeling confirmed the high production of MMP-1 (collagenase I) by fibroblast in contact with fibrillar collagen. Furthermore, the localization of MMP-1 in the matrix could be understood by the tissue cellular staining of TIMP-1, specific inhibitor of collagenase 1 (Helary et al. 2005). MMP-1 is involved in UV-induced premature skin aging by degrading various components of the dermal extracellular matrix. The extent of damage caused by MMP-1 presumably correlates with the MMP-1 expression level and its duration, the amount of active MMP-1 in relation to the pro-form and its inactivation through inhibitors (Sudel et al., 2003).

Table 1. Selected MMPs with known substrates (Adapted from Suzuki et al., 1990; Kuroda and Shinkai, 1997; Berton et al., 2000; Thibodeau, 2000; Woessmer and Nagase, 2000).

MMP-No.	Group name	Other names	Examples of substrate
MMP-1	Collagenase I	Interstitial collagenase	Collagen I and III: gelatin; pro-MMP2
MMP-2	Gelatinase A	72kDa gelatinase, Type IV collagenase	Collagen I, IV and VII; elastin; pro-MMP-9
MMP-3	Stromelysin 1	Transin, proteoglycanase	Proteoglycans, fibronectin, pro-MMP1
MMP-9	Gelatinase B	92kDa gelatinase , Type V collagenase	Collagen V; elastin, fibrillin
MMP-12	Macrophage elastase	Metalloelastase	Collagen IV; elastin; basal membrane

- MMP-2 (Gelatinase A, Collagenase IV) is a member of MMP family of zinc-dependent endopeptidases, which cleaves a number of substrates including native type I collagen to N-terminal three-quarter and C-terminal one-quarter fragments identical to those generated by collagenases (Tournier et al., 1994; Aimes and Quigley, 1995). MMP-2 plays an important role in degrading type IV collagen (Thibodeau, 2000; Malina et al., 2004). MMP-2 also cleaves type V collagen (Malina et al., 2004). Besides its action on dermal collagen fibers, it was suggested that MMP-2 could be related to the known hydrolytic action of the proteinase against anchoring fibrils (collagen type VII) (Seltzer et al., 1989.). It has been observed that application of MMP-2 to skin tissue sections led to intense alteration of collagen fibers with nearly total disappearance of type III collagen immunostaining (Berton et al., 2000). Also, a series of experiments suggested that this matrix metalloproteinase played a prominent function in degradation of soft connective tissue collagen (Creemers et al., 1998). Moreover, MMP-2 cleaves type XI collagen (Malina et al.,

2004), gelatin (degraded collagen type I) (Thibodeau, 2000), denatured collagens (Tournier et al.; 1994, Malina et al., 2004), elastin, proteoglycans, laminin and fibronectin (Thibodeau, 2000; Malina et al., 2004). MMP-2 is produced by keratinocyte in the epidermis and located in the basal layer of the epidermis and also in the upper layers of the dermis (papillary dermis) (Thibodeau, 2000). MMP-2 (gelatinase A) is of particular interest, considering its broad substrate specificity and its widespread occurrence in numerous non-inflamed healthy connective tissues. This enzyme appears as the enzyme primarily responsible for the digestion of soft connective tissue collagen (Creemers et al., 1998). Furthermore, the enzymatic activity of MMP-2 in the skin increases with age while the quantity of the selective endogenous inhibitors of MMP-2, called TIMP-2, decrease with age. These observations emphasize the role played by MMP-2 in the atrophy of the organization of the ECM (Thibodeau, 2000).

- MMP-3 (Stromelysin 1, Transin-1) is a member of the MMP family of extracellular proteases which has a broad spectrum of proteolytic activities, including degradation of proteoglycans and fibronectin as well as native type III, IV, V collagens. MMP-3 is required for the conversion of pro-MMP-1 to active form (Suzuki et al., 1990).

- MMP-9 (Gellatinase B) is produced by keratinocytes and stored in tertiary granules of neutrophils. MMP-9 is the largest of MMPs, it includes three fibronectin-like domains and a collagen type V-like domain which is able to digest type V collagen (Woessmer and Nagase, 2000). Nevertheless, inflammatory reaction, as following intense UV exposure, leads to its secretion from neutrophils, its overproduction by keratinocytes and its expression from fibroblasts. MMP-9 displays the greatest elastolytic and fibrillin-degrading activity (Berton et al., 2000).

4. Subcutaneous layer (hypodermis)

The hypodermis, or subcutis, located beneath the dermis, is composed mostly of fat, which is an important energy source for the body. This layer consists of cells containing fatty deposits, called adipose cells. The blood vessels and nerves it contains are larger than those in the dermis. It may also house the hair follicles when they are in the growing phase. (Gray, 2000). One of the functions of this fatty layer may be to act as an insulation to conserve body heat. This layer also contains collagen type I, III, and V. As humans age, some of the subcutaneous fat is lost or redistributed into undesired areas. This phenomenon contributes to the aged appearance.

5. Skin appendages

There are four skin appendages: the hair follicles with their associated sebaceous glands, eccrine sweat glands, apocrine sweat glands, and nails (Walters and Roberts, 2002).

5.1 The hair follicle

Epidermal buds grow down into the dermis. The developing follicle forms at an angle to the skin surface and continues its downward growth. The hair is formed from cells just above the bulb, which also give rise to concentric zones of differentiated epithelial cells destined to form the inner and outer root sheaths. Along one side of the follicle, two buds are formed: an upper, which develops into the sebaceous gland, and a lower, which becomes the attachment for the arrector pili muscle which enables hair to stand up in response to fear (Walters and Roberts, 2002). At skin sites destined to have apocrine units, a third epithelial bud develops from the opposite side of the follicle above the level of the sebaceous gland anlage (Odom et al., 2000).

5.2 The sebaceous glands

The sebaceous glands are found in greatest abundance on the face and scalp, though they are distributed throughout all skin sites except the palms and soles. They are always associated with hair follicles except at the following sites: the eyelids (meibomain glands), the buccal mucosa and vermilion border of the lip, the prepuce,

and the female areolas (Odom et al., 2000). These glands produce grease, or sebum, which is a mixture of waxes and fats. Sebum is secreted through the sebaceous duct into the hair follicle. It forms a mixture with the watery secretion of sweat, which covers the skin and spreads along the hair.

5.3 Sweat glands

Sweat glands are found in almost every part of the skin, forming tiny coiled tubes embedded in the dermis or subcutaneous fat. There are two types of sweat gland: eccrine glands and apocrine glands. The eccrine sweat glands secrete a dilute salt solution with a pH of about 5, this secretion being stimulated by temperature-controlling determinants, such as exercise and high environmental temperature, as well as emotional stress through the automatic (sympathetic) nervous system. The apocrine sweat glands are limited to specific body regions and are also coiled tubes. These glands are about ten times the size of the eccrine ducts, extend as low as the subcutaneous tissues and are paired with hair (Walters and Roberts, 2002).

5.4 The nails

Nails act to assist in grasping small objects and in protecting the fingertip from trauma. The matrix keratinization leads to the formation of the nail plate (Odom et al., 2000). Certainly, nail plate composition, layers of flattened keratinized cells fused into a dense, but somewhat elastic mass, will afford some protection to the highly sensitive terminal phalanx in the keratinization process the cells undergo shape and other changes, similar to those experienced by epidermal cells forming the stratum corneum. The nail plate comprises two major layers (the dorsal and intermediate layer) with, possibly, a third layer adjacent to the nail bed. There are differences in the chemical composition of the two layers, which further suggests that applied drugs may possess differing partitioning tendencies between the layers (Walters and Roberts, 2002).

B. Skin aging

Aging of the skin is commonly associated with increased wrinkling, sagging and increased laxity, but when considering the underlying reasons for these changes, it is important to distinguish between the effects of true biological aging (intrinsic chronologic aging) and environmental factors (extrinsic aging). The first comprises innate or intrinsic aging mechanisms that –similar to internal organs- affect the skin by a slow and partly reversible degeneration of connective tissues. The second process designated as extrinsic or photoaging is mainly due to ultraviolet radiation of sunlight that overwhelmingly contributes to a premature aging phenotype even in young individuals (Scharffetter-Kochanek et. al., 2000). Ultraviolet (UV) irradiation is one of the most ubiquitous environmental hazards that impact every living creature under the sun. Skin is the largest human organ, and is the only organ directly exposed to UV irradiation (Jenkins, 2002; Xu and Fisher, 2005).

Intrinsic chronologic aging is largely genetically determined and clinically associated with increased fragility, loss of elasticity and has a transparent quality. The process of intrinsic skin aging is similar to that occurring in most internal organs, involving slow deterioration in tissue function due to a variety of factors. The stratum corneum remains relatively unchanged, but the epidermis and dermis thins with a flattening of the dermo-epidemal junctions. There is also a reduction in the number and biosynthetic capacity of fibroblasts and progressive disappearance of elastic tissue in the papillary dermis (Jenkins, 2002). An age-associated loss in dermal thickness occurs in elderly persons. Skin collagen content decreases with age and the fine collagen fibers associated with infancy become increasingly dense and tightly packed and far move randomly orientated. The elastic recovery of aged skin is decreased and this function loss is accompanied by a decrease in the number of elastic fibers. The processes associated with intrinsic skin aging are thought to result from a combination of events including (i) decreased proliferation capacity of skin-derived cells; (ii) decreased matrix synthesis in the dermis; and (iii) increased expression of enzymes that degrade the collageneous matrix. It is proposed that this lead to the accumulation of non-dividing senescent cells with altered gene expression and subsequent phenotype, eventually leading to a decline in tissue function and integrity that is

characteristic of aging. These changes are observed over the entire surface of body (Bernstein and Uitto, 1996; Jenkins, 2002).

Extrinsic aging is caused by external factors such as smoking, excessive use of alcohol, poor nutrition, and sun exposure (Baumann, 2002). This process refers to premature skin aging. Enhanced generation of reactive oxygen species and induction of matrix metalloproteinases (MMPs) appear to be the most important components of UVA-modulated signal transduction pathways, ultimate leading to photoaging (Lee et al., 2003) It has been suggested that as much as 80% of facial aging is attributable to sun exposure. Clinically, photodamaged skin is characterized by loss of elasticity, increased roughness and dryness, irregular pigmentation and deep wrinkling (Jenkins, 2002).

2. Biological homeostasis

A. Antioxidant defense mechanism in biological system

Antioxidant status is the balance between the antioxidant system and pro-oxidants in living organisms. A serious imbalance favoring oxidation is defined as oxidative stress. It may result from:

- excessive production of ROS and free radicals and/or
- weakening of the antioxidant system due to lower intake or endogenous production of antioxidants or from increased utilization.

Major factors affecting the antioxidant status are summarized in Table 2.

Table 2. Determinants of antioxidant status in humans (Papas, 1999)

Antioxidant effect	Pro-oxidant effect
Genetic factors	**Genetic factors**
Diet	**Diet**
- Antioxidant vitamins (A, C, E)	- Lipids, especially PUFA
- Phytochemicals	- Divalent minerals (Cu, Fe)
- Minerals, components of antioxidant enzymes (Se, Zn, Cu, Mn, Fe)	- Prooxidant nutrients and phytochemicals
- Food antioxidants and supplements	
	Environment
	- Pollutants
	- Tobacco smoke
	- UV radiation
Alcoholic drinks (wine and other)containing antioxidants	**Alcohol**
	Injury, disease, and medications
	- Trauma, injury/ reperfusion
	- Other diseases
	- Drugs and medical treatment (radiation therapy, etc.)
Exercise program	
	Physiological stage or conditions
	- Prematurity
	- Aging
	- Strenuous exercise
	Stress
	- Physiological
	- Emotional

In healthy individuals, ROS generation and antioxidant defense appear to be approximately in balance, although there may be a slow cumulative oxidative damage that contributes to the aging process and to age-related diseases such as cancer (Halliwell, 1993). The generally accepted hypothesis is that in any biological system an important balance must be maintained between the formation of reactive oxygen and nitrogen species (ROS and RNS, respectively) and their removal (Vaya and Aviram, 2001).

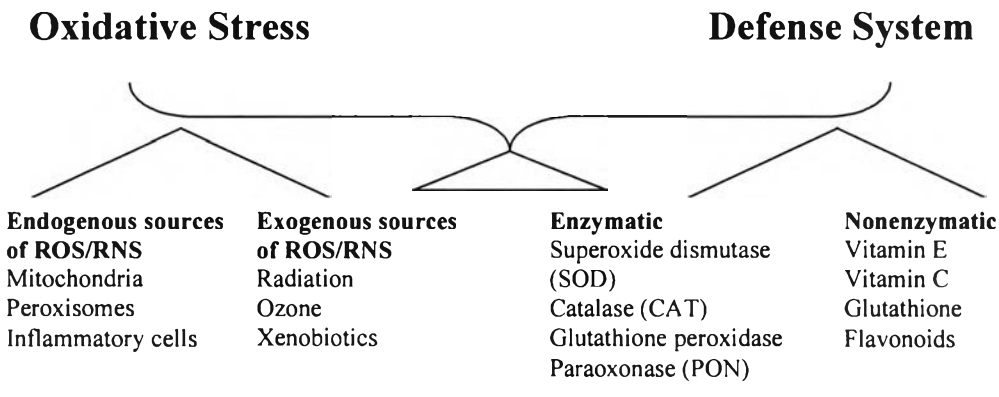


Figure 2. Balance system between oxidative stress and defense system

The skin naturally relies on antioxidants to protect it from oxidant stress generated by sunlight and pollution. A relative symphony of enzymatic and nonenzymatic antioxidants interacts to provide protection in both the intracellular and extracellular space (Pinnell, 2003). Antioxidant enzymes, including superoxide dismutase, catalase, and glutathione peroxidase/reductase, convert reactive oxygen species into nonreactive oxygen molecules.

Superoxide dismutase (SOD) converts superoxide anion into hydrogen peroxide and oxygen. There are 2 types of SOD: a magnesium-containing SOD and a copper-zinc-dependent SOD. Catalase is involved in cellular detoxification and can convert hydrogen peroxide into water and oxygen (Figure 3). Glutathione peroxidase is the most important hydrogen peroxide-removing enzyme existing in the membrane. Glutathione disulfide reductase is a flavoprotein that permits the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) by the oxidation of NADPH to $NADP^+$ (Lee, Koo and Min, 2004).

$2\ O_2^{\cdot-} + 2\ H^+ \longrightarrow H_2O_2 + O_2$ (Superoxide dismutase)

$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$ (Catalase)

$2GSH + H_2O_2 \longrightarrow GSSG + 2\ H_2O$ (Glutathione peroxidase)

$GSSG + NADPH, H^+ \longrightarrow 2\ GSH + NADP^+$ (Glutathione reductase)

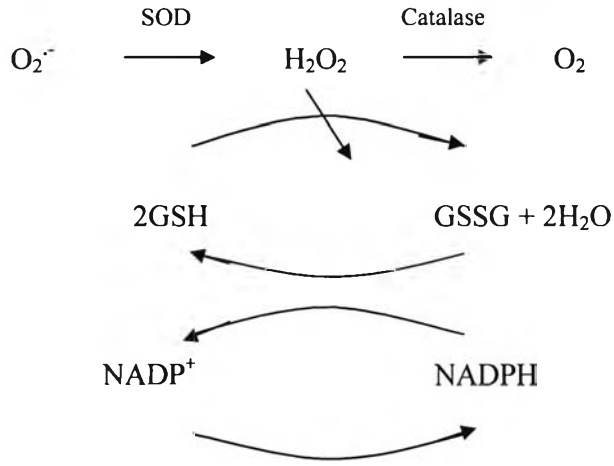


Figure 3. Antioxidant enzymes and their reaction mechanisms.

Low-molecular weight, nonenzymatic antioxidants include L-ascorbic acid in the fluid phase, glutathione in the cellular component, vitamin E in membranes, and ubiquinol in mitochondria. Low-molecular-weight antioxidants work in tissues as a coordinated interactive group of chemicals related to their chemical structure, position in the tissue, and relative redox potential. Thus when a ROS is generated in lipophilic structure and is reduced by α-tocopherol, the oxidized tocopherol can be regenerated by ubiquinol or L-ascorbic acid. In turn, dehydroascorbate can be reduced by glutathione, which, in turn can be reduced by nicotinamide adenine dinucleotide phosphate pool. This balance may be essential for function and the system could potentially fail when any step in the process becomes rate limiting (Pinnell, 2003).

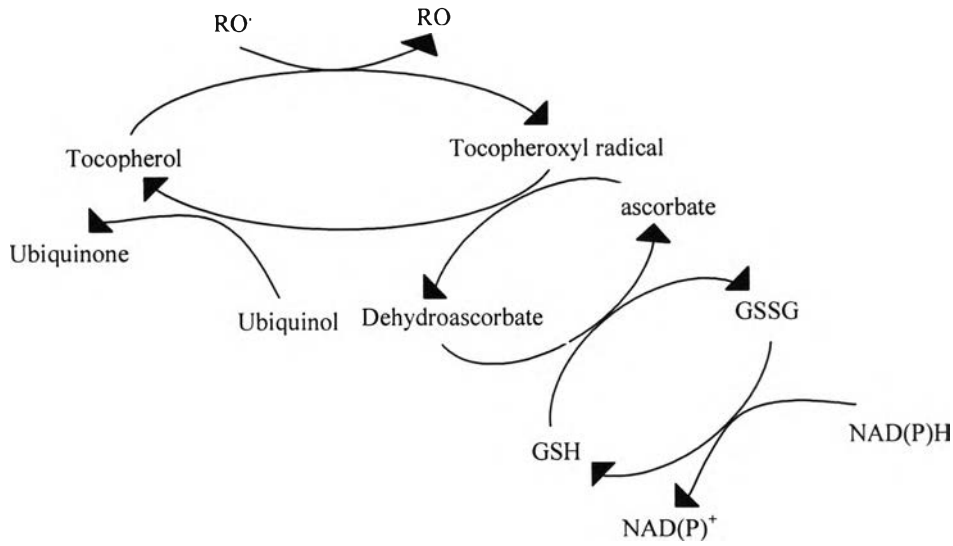


Figure 4. Interaction network of nonenzymatic antioxidants.

B. Iron homeostasis

Organisms take great care in the handling of iron; using both transport (such as transferrin) and storage (such as ferritin and haemosiderin) proteins so as to minimize the amount of 'free' iron within cells and in extracellular fluids (Halliwell, 1993). In plasma, transferrin maintains the concentration of ferric iron sufficiently low to avoid significant hydroxyl radical formation under nonpathological situations. Within the cytosol, iron is incorporated into ferritin, in the form of inorganic ferric iron for which interaction with oxygen is minimal (Galey, 1997). Thus, for example, plasma from healthy human adults contains no 'free' iron capable of promoting Fenton chemistry (Halliwell and Gutteridge, 1992).

Normally, aerobic organisms are protected from oxidative stress induced by free radicals and non-radicals by an array of defense systems. Defense systems *in vivo* against oxidative damage can be summarized in Table 3. The preventive antioxidants act in the first defense line by suppressing 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 (Papas, 1999).

Table 3. 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
Glutahione peroxidase (cellular)	Decomposition of hydrogen peroxide and free fatty acid hydroperoxides
Glutathione peroxidase (plasma)	Decomposition of hydrogen peroxide and Phospholipids hydroperoxides
Phospholipid hydroperoxide	Decomposition of phospholipids hydroper
Glutathione peroxidase	oxides
Peroxidase	Decomposition of hydrogen peroxide and lipid hydroperoxides
Glutathione S-transferase	Decomposition of lipid hydroperoxides
(b) Sequestration of metal by chelation	
Transferrin, lactoferrin	Sequestration of iron
Heptoglobin	Sequestration of hemoglobin
Hemopexin	Stabilization of heme
Ceruloplasmin, albumin	Sequestration of copper
(c) Quenching of active oxygen species	
Superoxide dismutase (SOD)	Disproportionation of superoxide anion
Carotenoids, vitamin E	Quenching singlet oxygen
2. Radical-scavenging antioxidants: scavenge radicals to inhibit chain initiation and break chain propagation	
Hydrophilic: Vitamin C, uric acid, bilirubin, albumin	
Lipophilic: Vitamin E, ubiquinol, carotenoids, flavonoids	
3. Repair enzymes: Repair the damage and reconstitute membranes	
Lipase, protease, DNA repair enzymes, transferase	

3. Photodamage

The skin is always in contact with oxygen and is increasingly exposed to ambient UV-irradiation. Sunlight coupled with living in an oxygen-rich atmosphere causes unwanted and deleterious stresses on skin. The most severe photodamage is skin cancer. Less severe photoaging changes result in wrinkling, scaling, dryness, and mottled pigment abnormalities consisting of hyperpigmentation and hypopigmentation. For a photochemical reaction to occur in the skin, ultraviolet (UV) light from the sun must be absorbed by a chromophore, beginning a series of photochemical reactions that may result in skin cancer or photoaging changes. These photochemical reactions can result in changes to DNA, including oxidation of nucleic acids. Oxidative reactions can also modify proteins and lipids, resulting in changes in function. Their accumulation may result in tissue aging. The body is well equipped to deal with oxidative stress, naturally using antioxidant enzymes and non-enzymatic antioxidants to lessen these changes. However, sunlight and other free-radical generators (e.g., smoking, pollution) can overwhelm the system, making natural protective controls inadequate, resulting in oxidative damage (Pinnell, 2003). In UV-irradiated skin, the level of matrix metalloproteinases (MMPs) that are important enzymes for the proteolysis of extracellular matrix proteins is elevated long before the visible symptoms of photoaging. Among them, MMP-2 (gelatinase A) and MMP-9 (gelatinase B), secreted as proenzymes, play an important role in degradation type IV collagen (Lee et al., 2003).

The depth of penetration of UV irradiation into the skin determines the site of reactive oxygen generation and is clearly dependent on the wavelength of UV and on the dose. Ultraviolet radiation, particularly the band between 280 and 400 nm, is the main cause of oxidative stress (Andreassi and Andreassi, 2004). UVB irradiation (280-320 nm) is mainly absorbed by the epidermis, primarily comprising keratinocytes. Transcription factors such as AP-1 and NF-$_k$B are induced in the epidermis. These factors, in turn, then induce the expression of MMPs in yet uncharacterized fashion. UVB irradiation has recently been shown to generate lipid peroxidation products and hydroxyl radicals ($HO^{\cdot}$) with detrimental long term effects like cancer formation and premature aging of the skin (Brenneisen et al., 1998). UVA

irradiation (320-400 nm) penetrates into the dermis, where it is absorbed by fibroblast, an accessible target even in the deep dermis (Berneburg, Plettenberg and Krutmann, 2000; Scharffetter-Kochanek et. al., 2000). UVA-induced generation of ROS leads to the expression of MMPs and induction of mutation of DNA (Berneburg et al., 2000). Certainly, the transmission also depends on the thickness of the stratum corneum, its state of hydration, and the pigmentation of the epidermis (Scharffetter-Kochanek, 1997). It should be remembered that UV rays can activate photosensitizing substances, both endogenous, e.g. porphyrins and flavins, and exogenous, e.g. some drugs responsible for the formation of highly reactive ROS (Andreassi and Andreassi, 2004). Moreover, UV irradiation of the skin leads to compromise of the antioxidant defences. In particular, under the effect of UV radiation, catalase activity is preferentially destroyed, superoxide dismutase activity is diminished, glutathione peroxidase and glutathione reductase are virtually unchanged (Pinnell, 2003) but their activity decreases and the levels of antioxidant vitamins are greatly reduced. These events favor the formation of additional oxidative stress. It is reasonable to believe that the well-known biological effects manifested after photo-exposure, particularly erythema and hyperpigmentation, are the direct consequence of oxidative stress induced by UV radiation (Andreassi and Andreassi, 2004).

4. Theory of oxidative stress and reactive species

Considerable interest in oxidative stress comes from related pathologies including atherosclerosis, hypertension, ischemia-reperfusion injury, inflammation, cystic fibrosis, cancer, type-2 diabetes, Parkinson's diseases, Alzheimer's disease, and other neurodegenerative diseases. Oxidative stress arises from a significant increase in concentrations of reactive oxygen species (ROS) and reactive nitrogen species (RNS) to levels that are toxic to biomolecules (Jezek and Hlavata, 2005). In a normal healthy human body, the generation of pro-oxidants in the form of ROS and RNS are effectively kept in check by the various levels of antioxidant defense. However, when it gets exposed to adverse physicochemical, environmental or pathological agents such as atmospheric pollutants, cigarette smoking, ultraviolet rays, radiation, toxic chemicals, overnutrition and advanced glycation end products (AGEs) in diabetes, this delicately maintained balance is shifted in favor of pro-oxidants resulting in

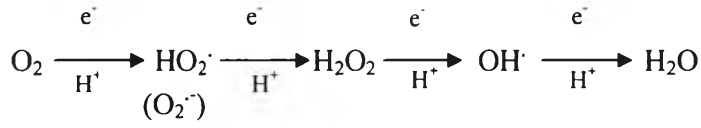
"oxidative stress" (Devasagayam et al., 2004). An **imbalance** between ROS/RNS and antioxidant defense in favor of the former can create the state of **oxidative stress** (Halliwell, 1993; Jezek and Hlavata, 2005). Oxidative stress is mainly caused by ultraviolet radiation (UV) (Andreassi and Andreassi, 2004). Therefore, the risk of photo-oxidative damage of the skin induced by ROS has increased substantially. ROS are important second messengers for the induction of several genes in a variety of physiological and pathological conditions (Brenneisen et al., 1998). The excited photosensitizer subsequently reacts with oxygen, resulting in the generation of ROS including the superoxide anion ($O_2^{\cdot-}$) and singlet oxygen ($^1O_2$). The superoxide anion ($O_2^{\cdot-}$) and singlet oxygen ($^1O_2$) are also produced by neutrophils that are increased in photodamaged skin and contribute to the overall pro-oxidant state. Superoxide dismutase converts $O_2^{\cdot-}$ to hydrogen peroxide (H_2O_2) which is able to cross cell membranes easily and, in conjunction with transitional Fe^{2+}, drives the generation of the highly toxic hydroxyl radical ($OH^{\cdot}$). Both singlet oxygen and $OH^{\cdot}$ can initiate lipid peroxidation of cellular membranes with the generation of carbonyls and still poorly understood consequences (Brenneisen et al., 1998; Scharffetter-Kochanek et al., 2000). As research on the role and involvement of ROS and RNS advances, more and more biological functions are being found to be associated with these species. An excess of oxidative stress can lead to the oxidation of lipids and proteins, which is associated with changes in their structure and functions (Vaya and Aviram, 2001). ROS-mediated oxidative damage involves a vast number of biological molecules since it causes DNA modification, increase lipid peroxidation, which in turn can alter the integrity of membrane structure leading to inactivation of membrane bound enzymes, loss of permeability of the membrane and decrease in membrane fluidity and secretion of inflammatory cytokines (Briganti and Picardo, 2003; Kumar et al., 2004). Aging is also thought to occur as a result of a constant exposure of the organs to ROS/RNS, with a cumulative damage, through the entire life, along with a gradually decreasing repair capacity and increasing degenerative changes in the organs (Vaya and Aviram, 2001). This suggests that cellular redox environment plays a pivotal role in skin homeostasis and that skin diseases could result from an imbalance between pro-oxidant and antioxidant stimuli (Briganti and Picardo, 2003).

A. Reactive species

Free radicals and related species are mainly derived from oxygen (reactive oxygen species/ROS) and nitrogen (reactive nitrogen species/RNS), and are generated in our body by various endogenous systems, exposure to different physicochemical conditions or pathological states (Devasagayam et al., 2004). Free radicals can be defined as molecules or fragments of molecules, capable of independent existence, containing one or more unpaired electrons in their orbital. They tend to react very easily with various types of biomolecules, to acquire another electron and stabilize the orbital (Andreassi and Andreassi, 2004). Generally, free radicals attack the nearest stable molecules, 'stealing' its electrons. The molecule that has been attacked and loses its electron becomes a free radical itself, beginning a chain reaction. Once the process is started, it can cascade, initiating lipid peroxidation which results in destabilization and disintegration of the cell membranes or oxidation of other cellular components such as protein and DNA, finally resulting in the disruption of cells (Kaur and Kapoor, 2001). Free radicals are generally unstable, highly reactive, and energized molecules (Lee et al., 2004). Organisms constantly produce free radicals by different mechanisms. Incomplete reduction of oxygen in the mitochondrial (the major ROS source) electron transport chain releases superoxide anions into the cytoplasm (Andreassi and Andreassi, 2004; Jezek and Hlavata, 2005). Free radicals are formed as a natural consequence of the body's normal metabolic activity and as part of the immune system's strategy for destroying invading microorganisms. For example, $O_2^{\cdot-}$ plays an essential role in the intracellular killing of bacteria by activated phagocytes (for example, neutrophils and macrophages). Exogenous sources of free radicals include ozone, exposure to ultraviolet radiation in sunlight, and cigarette smoke. Free radicals can adversely alter lipids, proteins and DNA and have been implicated in aging and a number of human diseases. In general, free radicals and related species are very short-lived, with half-lives in milli-, micro- or nanoseconds. Details about some of the biologically important reactive species are presented in Table 4 (Devasagayam et al., 2004).

1. Superoxide anion radical ($O_2^{\cdot -}$)

Superoxide anion is a reduced form of molecular oxygen created by receiving one electron. Superoxide anion is an initial free radical formed from mitochondrial electron transport systems (Lee et al., 2004) that is the first reduction of ground state-oxygen, capable of both oxidation and reduction (Cadenas, 1995; Perl-Treves and Perl, 2002). Mitochondria generate energy using 4 electron chain reactions, reducing oxygen to water. Some of the electrons escaping from the chain reaction of mitochondria directly react with oxygen and form superoxide anions (Lee et al., 2004). Superoxide formed *in vivo* is largely –if not completely- converted by SOD-catalysed or non-enzymic dismutation, into H_2O_2 (Halliwell et al., 1995).



The superoxide anion plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical, or singlet oxygen ($2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$) in living organism. The superoxide anion can react with nitric oxide ($NO^{\cdot}$) and form peroxynitrite ($ONOO^-$), which can generate toxic compounds such as hydroxyl radical and nitric dioxide ($ONOO^- + H^+ \rightarrow OH^{\cdot} + NO_2^{\cdot}$) (Lee et al., 2004).

Table 4: Reactive oxygen and nitrogen species of biological interest

Reactive species	Symbol	Half life (in sec)	Reactivity/Remarks
Reactive oxygen species :			
Superoxide	$O_2^{\cdot -}$	10^{-6} s	Generated in mitochondria, in cardiovascular system and others
Hydroxy radical	$OH^{\cdot}$	10^{-9}s	Very highly reactive, generated during iron overload and such conditions in our body
Hydrogen peroxide	H_2O_2	stable	Formed in our body by large number of reactions and yields potent species like $OH^{\cdot}$
Peroxyl radical	$ROO^{\cdot}$	s	Reactive and formed from lipids, proteins, DNA, sugars etc. during oxidative damage
Organic hydroperoxide	ROOH	stable	Reacts with transient metal ions to yield reactive species
Singlet oxygen	$^1O_2$	10^{-6} s	Highly reactive, formed during photosensitization and chemical reactions
Ozone	O_3	s	Present as an atmospheric pollutant, can react with various molecules, yielding $^1O_2$
Reactive nitrogen species:			
Nitric oxide	$NO^{\cdot}$	s	Neurotransmitter and blood pressure regulator, can yield potent oxidants during pathological states
Peroxynitrite	$ONOO^{\cdot}$	10^{-3} s	Formed from $NO^{\cdot}$ and superoxide, highly reactive
Peroxynitrous acid	ONOOH	fairly stable	Protonated form of $ONOO^{-}$
Nitrogen dioxide	NO_2	s	Formed during atmospheric pollution

2. Hydrogen peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) is produced during normal aerobic cell metabolism, and its formation involves a number of enzymatic reaction especially superoxide dismutase enzyme (SOD). H_2O_2 is decomposed to oxygen (O_2) and water (H_2O) by enzymes such as catalase and glutathione peroxidase (Perl-Treves and Perl, 2002). Unlike superoxide radical ($O_2^{\cdot-}$), H_2O_2 is able to cross biological membranes. Both $O_2^{\cdot-}$and H_2O_2 can find some molecular targets to which they can do direct damage, but on the whole their reactivity is limited. Much like superoxide, H_2O_2 is rather stable and therefore less toxic than other reactive oxygen species (Halliwell et al., 1995; Cadenas, 1995; Perl-Treves and Perl, 2002). Hydrogen peroxide is the least reactive molecule among reactive oxygen species and is stable under physiological pH and temperature in the absence of metal ions (Lee et al., 2004). In the presence of transition metals, H_2O_2 can be reduced by Fenton reaction to form hydroxyl radical ($OH^{\cdot}$), which is highly reactive and can result in lipid peroxidation and many biological effects (Keithahn and Lerchl, 2005).

3. Hydroxyl radical ($OH^{\cdot}$)

Hydroxyl radical is the most highly reactive free radical and can be formed from superoxide anion and hydrogen peroxide in the presence of metal ions such as copper or iron. Hydroxyl radicals have the highest 1-electron reduction potential (2310 mV) and can react with everything in living organisms at 2^{nd}-order rate constants of 10^9 to 10^{10} M^{-1} sec^{-1} (Lee et al., 2004; Halliwell et al., 1995). Hydroxyl radicals react with nearly every biomolecule such as nuclear DNA, mitochondrial DNA, proteins and membrane lipids (Keithahn and Lerchl, 2005). The rapid and nonspecific reactivity of $OH^{\cdot}$ renders this free radical particularly dangerous. It may abstract hydrogen from, or hydroxylate, most biomolecules, causing cell injury or death. Hydroxyl radical is believed to be the etiological agent for several diseases and may also be involved in natural aging (Graf et al., 1984). Formation of $OH^{\cdot}$ from $O_2^{\cdot-}$ can be achieved by at least four different mechanisms. One requires traces of catalytic transition metal ions, of which iron and copper seem likely to be the most important *in vivo*. A second mechanism is that background exposure to ionizing radiation causes a steady-state low rate of $OH^{\cdot}$ formation within cells and in food by splitting of water. A

third means of forming some $OH^{\cdot}$ is the reaction of $O_2^{\cdot-}$ with the free radical nitric oxide ($NO^{\cdot}$), a reaction that proceeds at a rate comparable to that of $O_2^{\cdot-}$ with SOD. Reaction of HOCl with $O_2^{\cdot-}$ also makes some $OH^{\cdot}$, the rate constant is close to $10^7\ M^{-1}\ sec^{-1}$ (Halliwell et al., 1995).

$$HOCl + O_2^{\cdot-} \longrightarrow OH^{\cdot} + O_2 + Cl^-$$

The concept of a $O_2^{\cdot-}$ -driven Fenton-type reaction requires both $O_2^{\cdot-}$ and H_2O_2 as precursors of $OH^{\cdot}$, it proceeds via an intermediate catalyst, such as a transition metal chelate (e.g., Fe or Cu), which is reduced by $O_2^{\cdot-}$ and reacts with H_2O_2 in a "Fenton like" reaction to produce $OH^{\cdot}$ as in the following reaction (Cadenas, 1995):

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

$$H_2O_2 + \underset{(Cu^{+})}{Fe^{2+}} \longrightarrow OH^{\cdot} + OH^- + \underset{(Cu^{2+})}{Fe^{3+}} \qquad (2)$$

These well-known reaction sequences give a net balance (Haber-Weiss reaction) as (Cadenas, 1995):

$$O_2^{\cdot-} + H_2O_2 \longrightarrow O_2 + OH^- + OH^{\cdot} \qquad (3)$$

The requirements of transition metal chelate for this reaction are two-fold: on the one hand, the chelators alters the redox potential of the transition metal, thereby facilitating electron transfer reactions with $O_2^{\cdot-}$ and H_2O_2 (e.g., the E^o values for Fe^{3+}/Fe^{2+} and Fe-EDTA$^-$ /Fe-EDTA^{2-} are +770 and +120mV, respectively) and, on the other hand, it maintains the transition metal in solution (Cadenas, 1995). The initial product of reaction may be an oxo-iron complex, possibly ferryl, that then decomposes to form $OH^{\cdot}$. Different ligands to the iron (II) may stabilize this intermediate, so that little $OH^{\cdot}$ is formed. Thus, Fe-EDTA chelates are good sources

of $OH^{\cdot}$ to stabilize the intermediate iron (II) in the presence of H_2O_2. Most ferric (Fe^{3+}) complexes react more slowly with H_2O_2 than ferrous (Fe^{2+}) complexes, so that reducing agents stimulate Fenton reactions. $O_2^{\cdot -}$ in reaction (1) can be replaced by a suitable electron donor (reducing agent), such as ascorbate (reaction 4) (Halliwell et al., 1992; Cadenas, 1995).

$$\underset{(Cu^{2+})}{Fe^{3+}} + \underset{\text{(ascorbate)}}{AH^{-}} \longrightarrow \underset{(Cu^{+})}{Fe^{2+}} + \underset{\text{(semidehydroascorbate)}}{AH^{\cdot}} \qquad (4)$$

For determination of rate constants for reaction of $OH^{\cdot}$, a deoxyribose method was used which is a simple "test-tube" assay. It was found that deoxyribose reacts with $OH^{\cdot}$ with a rate constant of $3.1*10^{9}\ M^{-1}\ sec^{-1}$ (Halliwell et al., 1995; Halliwell et al., 1987).

4. Singlet oxygen ($^1O_2$)

Singlet oxygen is a nonradical and excited status. Singlet oxygen has been shown to be produced in myeloperoxidase and eosinophil peroxidase-catalysed reactions and has been reported to be generated by some cell types including neutrophils, eosinophils, and macrophages. UV light has been shown to generate $^1O_2$ (Wright, Hawkins and Davies, 2000) by photosensitization reactions (Halliwell et al., 1995). Singlet oxygen itself is not only cytotoxic due to the oxidative degradation of cellular membranes, but also it is genotoxic because it can oxidize DNA directly. Singlet oxygen can be produced by living cells and is capable of causing lipid peroxidation, DNA damage and cell death (Maharaj et al., 2005).

5. Nitric oxide and nitric dioxide

Nitric oxide ($NO^{\cdot}$) is a free radical with a single unpaired electron. Nitric oxide is formed from L-arginine by NO synthase. Nitric oxide itself is not a very reactive free radical, but the overproduction of NO is involed in ischemia reperfusion, neurodegenerative and chronic inflammatory disease such as rheumatoid arthritis and inflammatory bowel disease. Nitric oxide, exposed in human blood plasma, can

deplete the concentration of ascorbic acid and uric acid, and initiate lipid peroxidation (Lee et al., 2004).

Nitric dioxide ($NO_2^{\cdot}$) is formed from the reaction of peroxyl radical and NO, polluted air and smoking. Nitric dioxide adds to double bonds and abstract labile hydrogen atoms initiating lipid peroxidation and production of free radicals, it also oxidizes ascorbic acid (Lee et al., 2004).

B. Iron release during oxidative stress

Despite the remarkable efficacy of the iron transport and storage systems, various conditions associated with oxidative stress have been shown to induce a local release of iron from normal sites. The main source of intracellular iron release obviously is ferritin. Release of iron from ferritin requires reduction in the presence of a ferrous iron acceptor. Physiological reductants such as ascorbate and glutathione do not release iron from ferritin at significant rates. Superoxide can release iron from ferritin. Moreover, it is now believed by some authors that one of the main ways by which superoxide induces molecular damage is through its capacity to release iron from various iron proteins. Apart from ferritin, another potentially significant source of free iron may be other iron-containing proteins. Chronic exposure to ultraviolet light has been shown to result in an increased skin level of non-heme iron. This iron accumulation is likely to be the consequence of a UVB radiation-induced capillary damage and leakage of protein-bound iron. Inside red cells, which are rich in antioxidant enzymes, iron is tightly bound to hemoglobin, but injured or lysed red cells can release their iron into the surrounding medium. Indeed, hemoglobin has been shown to release iron ions on exposure to excess hydrogen peroxide. Once released, free iron will catalyze the formation of oxidative damage, mostly through its ability to undergo redox cycling between the two oxidation sites and to generate a hydroxyl radical able to oxidize the surrounding molecules. It is generally believed that because of the presence of physiological reductants inside cytosol, the ferrous form predominates. Once Fe (II) is bound to a target molecule, hydroxyl radicals produced by a reaction with hydrogen peroxide will react very closely to the metal-binding site according to a so-called site-specific Fenton reaction. This type of damage is

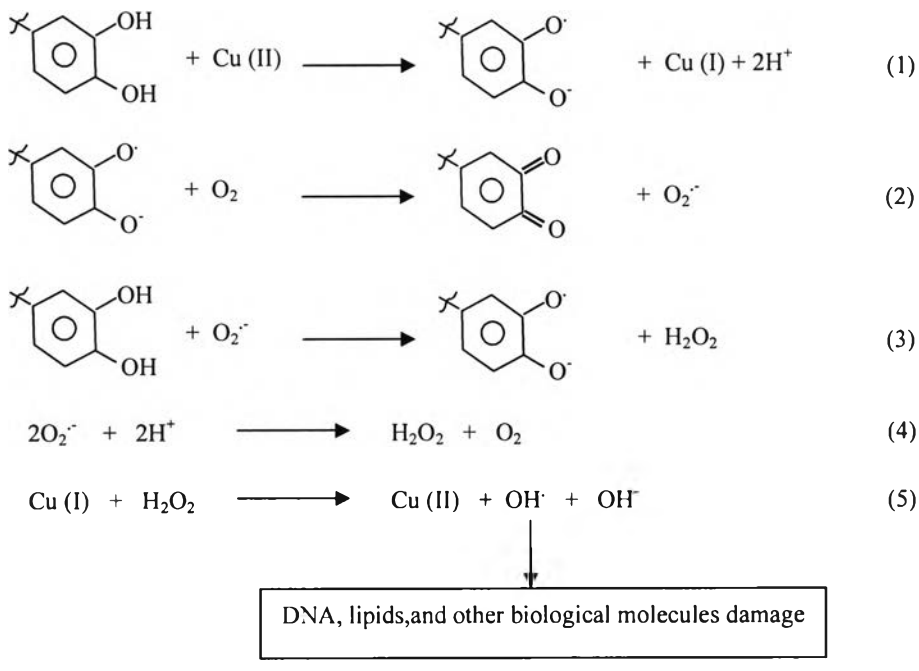
insensitive to inhibition by hydroxyl radical scavengers because the hydroxyl radical reacts immediately at the place where it is formed. In other words, any hydroxyl radical scavenger could not compete for such a reaction except at excessively high concentrations (Galey, 1997).

C. Pro-oxidant effect of antioxidant

Antioxidants also have the potential to act as pro-oxidants under certain conditions. For example, ascorbate, in the presence of high concentration of ferric iron, is a potent potentiator of lipid peroxidation. Vitamin E and beta-carotene also can behave as a pro-oxidant. The paradoxical role (pro-oxidant effect) of antioxidants is also directly related to the recently described "redox signaling" of the antioxidants. The functional role of many antioxidants depends on redox cycling (Devasagayam et al., 2004).

Recent studies have shown that several phenolic food antioxidant additives can accelerate oxidative damage, *in vitro*, to DNA, proteins, and carbohydrates despite antioxidant action toward lipids by acting as pro-oxidants, promoting free radical reactions (Halliwell and Aruoma, 1993; Aruoma et al., 1993).

In the presence of O_2, transition metals such as Cu and Fe catalyze the redox cycling of phenolics, leading to the formation of reactive oxygen species and other organic radicals that can damage DNA, lipids, and other biological molecules. The reactive species responsible for DNA nicking activity of phenolics is the $OH^{\cdot}$ radical or a species with similar oxidative potential. In the presence of metal ion the $OH^{\cdot}$ radical is formed by the following series of reaction (Sakihama et al., 2002):



Iron-catalyzed formation of hydroxyl radical ($OH^{\cdot}$) from superoxide anion radical and H_2O_2 requires the availability of at least one iron coordination site that is openly occupied by a readily dissociable ligand such as water or azide. This principle governing iron reactive may help advance our understanding of the mechanism of oxidative damage in biological systems and may also permit the design of more effective chelators for the control of iron in biological systems (Graf et al., 1984).

The pro-oxidant activity of phenolics depends on their metal reducing properties, chelating behavior, and O_2-reducing capacity. This latter property depends on the redox potential of the oxidized species and the lifetime of the phenoxyl radicals. Two factors that apparently limit this pro-oxidant potential are resonance delocalization of the phenoxyl radical by the side chain double bond and esterification of the terminal carboxyl group (Sakihama et al., 2002).

5. Theory of extrinsic aging

A. UV induced wrinkle (photo-aging)

Photo-aging primarily causes extrinsic aging by exposure to ultraviolet light. Photoaged skin is typically dry, coarse and rough with deep lines and wrinkles and irregular pigmentation. Photoaged skin displays prominent alterations in the cellular component and the extracellular matrix of the connective tissue with an accumulation of disorganized elastin and its microfibrillar component fibrillin in the deep dermis and a severe loss of interstitial collagens, the major structural proteins of the dermal connective tissue. Photoaged skin is characterized by loss of mature dermal collagen, distinct basophilic appearance of collagen. Also, collagen type VII containing anchoring fibrils that contribute to the stabilization of the dermal-epidermal junction are severely reduced in photoaged skin (Scharffetter-Kochanek et al., 2000). There are three principle components involved, namely, collagen fibers, the elastic fiber network and glycosaminoglycans. Collagen is the most abundant extracellular component and provides the strong tensile properties to the dermis. The elastic fiber networks provide elasticity to the skin, whilst the glycosaminoglycan/proteoglycan macromolecules play a role in hydrating the skin and in biological signaling (Jenkins, 2002).

The major histopathological sign of photoaging is the massive accumulation of so called 'elastotic' material in the upper and mid dermis. This material is comprised of extracellular matrix (ECM) components that make up the normal elastic fiber network. This elastotic material involves degradation of existing elastic fibers and dysregulation of elastin and fibrillin production. As the elastic fiber network extends from the dermal-epidermal junction to the deep dermis, the observed changes may contribute to clinical features of photoaging such as loss of elasticity (Scharffetter-Kochanek et al., 2000). The upreguration of elastin and fibrillin gene expression in photodamaged skin and fibroblasts derived from it has been reported (Bernstein et. al., 1994). In contrast with elastic fiber network, components of the collagen fiber network, including collagen I and decorin are downregulated in photodamaged skin. This reduction in collagen fiber production is accompanied by the degeneration of the surrounding collagenous network and, as with the breakdown of the elastic fiber

network, MMPs have been implicated as the key mediators (Jenkins, 2002). Certainly, Photoaging characterized by severe connective tissue damage has been linked to the induced synthesis and activity of different members of the matrix metalloproteinase family.

All matrix macromolecules in the dermis can be digested by matrix metalloproteinase (MMP) acting alone or in combination. The MMP forms a family of structurally related zinc endopeptidases that exhibit different substrate specificities. The activity of MMP is inhibited by a special class of tissue inhibitors of matrix metalloproteinase (TIMP) and by nonspecific inhibitors. An imbalance between the active enzymes and their natural inhibitors leads to the accelerated destruction of connective tissue, which is associated with the pathology of diseases and skin aging (Borkakoti, 2004). The MMPs are a large family of degradative enzymes and four in particular are thought to be important in matrix degradation in skin. The combined actions of collagenase (MMP1), gelatinase A (MMP2), gelatinase (MMP9) and stromelysin1 (MMP3) can fully degrade skin collagen and components of the elastic network (Jenkins, 2002) and are capable of degrading the collagen framework of skin (Pinnell, 2003; Sudel et al., 2003).

From the cosmetic point of view, increased MMP-1 expression after UV exposure has to be regarded as a major reason for premature skin aging, leading to coarse wrinkles and a leathery appearance, which is associated with degradation of connective tissue. Consistent with the role of collagen loss in photoaging, small amount of UV irradiation was reported to result in the induction of a series of MMPs (Jenkins, 2002).

At the same time pro-collagen synthesis is inhibited, perhaps by a mechanism related to degraded collagen. Levels of pro-collagen I protein are decreased, whereas MMP-1 protein and MMP-2 activity are increased in exposed skin compared with unexposed skin (Pinnell, 2003). Several members of the matrix metalloproteinase family were described to display, *in vitro*, elastolytic activity. Among them are MMP-2 (gelatinase A) and MMP-9 (gelatinase B) which contain fibronectin type II like

repeats essential for elastase activity. MMP-2 is expressed constitutively by several cell types in culture including dermal fibroblasts while expression of MMP-9 is more restricted. It is produced by keratinocytes and stored in tertiary granules of neutrophils. In addition, both MMP-2 and MMP-9 (gelatinase A and B, respectively) have the potential to degrade the elastic fiber network. MMP-9 displays the greatest elastotic and fibrillin-degrading activity, whilst MMP-2 shows greater specificity toward collagen III and is capable of degrading constituents of the dermo-epidermal junction (Berton et al. 2000).

B. Reactive oxygen species: the major pathogenic agents for connective tissue alteration in photoaging

An alternative aging hypothesis to that of cellular senescence is the oxidative stress theory of photo-aging. This theory suggests that aging is heavily influenced by external oxidative stresses which influence the genetic program through modulation of redox sensitive genes. Reactive oxygen species cause damages to lipids, proteins and DNA, and also influence cellular senescence. In addition, free radicals also cause damage to connective tissue components of the dermis, particularly collagen, which again is likely to influence cell behavior via cell-matrix interactions (Jenkins, 2002).

Although it is well accepted that low levels of reactive oxygen species are continuously produced *in vivo* and are involved in physiological processes, there is accumulating evidence for the damaging effects of higher concentrations of ROS generated *in vitro* and *in vivo* after UVA and UVB irradiation of the skin (Scharffetter-Kochanek et al., 2000).

1. Reactive oxygen species and activation of cellular MMP in dermal connective tissue

Elastin accumulation and collagen degradation are prominent hallmarks in photodamaged skin. Reactive oxygen species (ROS) also play a substantial role in collagen metabolism. They not only directly destroy interstitial collagen, but also inactivate TIMPs, (tissue inhibitors of matrix-metalloproteases) and induce the synthesis and activation of matrix-degrading metalloproteases (Scharffetter-

Kochanek, 1997; Brenneisen et al., 1998; Scharffetter-Kochanek et al., 2000). Based on this combined approach, indirect evidence was provided that singlet oxygen and H_2O_2 are the major ROS involed in the UVA-dependent induction of MMP-1, MMP-2 and MMP-3 on mRNA and protein levels, whereas the hydroxyl radical and intermediates of lipid peroxidation play a major role in the UVB-induction of MMP-1 and MMP-3 (Brenneisen et al., 1998; Scharffetter-Kochanek et al., 2000).

2. Reactive oxygen species and changes in the cellular component of dermal connective tissue

The notion that ROS are involved in replicative senescence, intrinsic and extrinsic aging comes from several models including models in cell biology, naturally occurring genetic disorders and transgenic organisms. The hypothesis that oxidative metabolism drives the aging process is based on the observation that about two percent of oxygen taken up is chemically reduced in the mitochondria by the addition of single electrons, which are sequentially converted into ROS.

ROS play a major role in photoaging and induce changes in gene expression pathways related to collagen degradation and elastin accumulation. There is evidence that singlet oxygen as generated by UVA-irradiation results in the common deletion mutation of mitochondrial DNA and most likely, via disruption of the oxidative phosphorylation, increases the overall ROS load subsequently activating the transcription of matrix-metalloproteases encoding genes (Scharffetter-Kochanek et al., 2000).

Because ROS may prove particularly relevant to future developments of UV protective agents for the skin, there have been efforts to better define the involvement of distinct oxygen species in the up regulation of matrix metalloproteinases (MMPs). Hence, a decrease of ROS load following UV irradiation by efficient sunscreens and/or antioxidants represents promising strategies to prevent or minimize cutaneous photoaging.

3. The free radical/oxidative stress theory of aging

Bokov, Chaudhuri and Richardson, (2004) has presented a simplified representation of potential biochemical pathways that could lead to increased oxidative damage to lipids, proteins, and DNA in Figure 5. There are two basic pathways that are responsible for the impact of ROS on aging: I. Pathways that affect the net amount of ROS in the whole organism of strategic tissues within the organism. II. Pathways that repair or turnover structures that have been damaged by these ROS.

The first pathway can be divided into two logical subclasses – ROS-producing pathways and ROS-scavenging pathways. ROS are by-products of a variety of pathways in aerobic metabolism. The mitochondrial electron transport chain accounts for the majority of the total oxygen metabolized by the cell, and the by-products produced by the electron transport chain (e.g., superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals) are potential sources of oxidative damage to the mitochondrion itself and to other cellular compartments. The ROS-scavenging pathways involve a complex antioxidant defense system, including both small molecules (tocopherols, vitamin C, glutathione, etc.) and antioxidant enzymes (the superoxide dismutases (SOD), the glutathione peroxidases, catalase, etc.). The balance between these two pathways- the production and the scavenging of ROS- determines the absolute level of oxidative stress, not the activity of any one pathway in isolation. The second class of ROS-related pathways is the repair/turnover pathways for molecules damaged by ROS. This would include pathways for the repair/turnover of DNA, lipids, and proteins (Bokov et al., 2004).

Reactive oxygen species (ROS) are involved in processes of cell damage, apoptosis and aging. The main natural source of ROS in the aerobic cell is located within the mitochondria. Respiratory activity of mitochondria can cause an electron leakage responsible for the generation of superoxide radicals ($O_2^{\cdot-}$) and the subsequent production of hydroxyl radicals ($HO^{\cdot}$). Hydroxyl radicals are highly reactive because of an unpaired electron. They show an average lifetime of 10^{-9} s and can react with nearly every biomolecule such as nuclear DNA, mitochondrial DNA (not histone protected), proteins and membrane lipids (Keithahn and Lerchi, 2005).

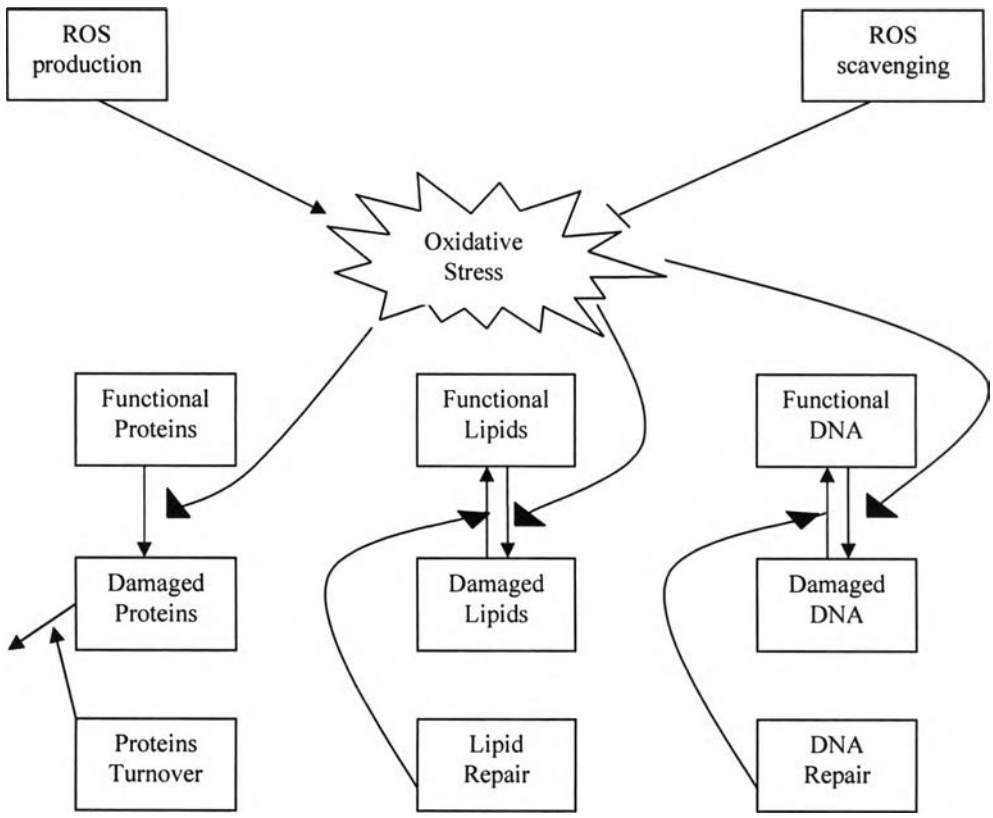


Figure 5. A summary of the biological pathways involved in the free radical/oxidative stress theory of aging.

6. Theory of hyperpigmentation

A. Human skin pigmentation

Melanocytes are specialized cells in the skin that are fond of their embryonic origin at the neutral crest. During embryonic development, melanoblasts migrate to reach the basal layer of the epidermis where they differentiate to mature melanocytes possessing the complete machinery to ensure melanin synthesis and distribution within the skin. Melanin synthesis takes place within specialized intracellular organelles named melanosomes. Melanin-containing melanosomes then move from the perinuclear region to the dendrite tips and are transferred to keratinocytes by a still not well-characterized mechanism. These events, which ensure a uniform distribution of melanin pigments in epidermis, are responsible for skin and hair color in humans and other animals (Busca and Ballotti, 2000).

B. UV-induced pigmentation

Pigmentation of the skin, due to the synthesis and dispersion of melanin in the epidermis, is also the key physiological defense against sun-induced damages (No et al., 1999; Berneburg, Plettenberg and Krutmann, 2000). In physiological conditions human skin pigmentation is increased by UV radiation of the solar light. UV radiation gives both direct and indirect melanogenic effects on melanocytes.

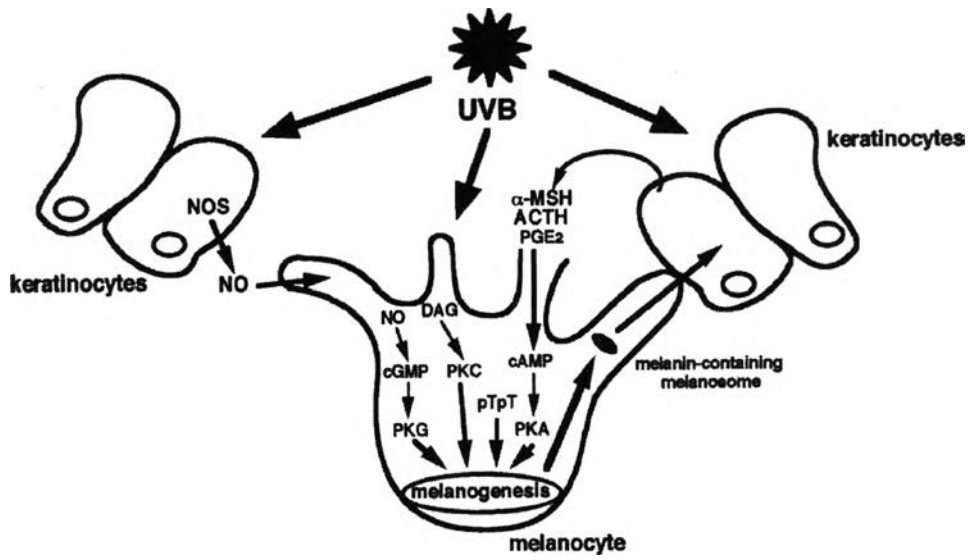


Figure 6. Diagram showing melanogenesis as a result of a complex set of regulatory processes involving direct effects of UV radiation on melanocytes and indirect effects through the release of keratinocyte-derived factors (Busca and Ballotti, 2000).

Direct melanogenic effects of UV on melanocytes might also involve the production of nitric oxide (NO). Nitric oxide (NO) elicits its effects leading to an increase in intracellular cGMP content and the activation of cGMP-dependent protein kinase. In human melanocytes, NO donors and analogs stimulate melanogenesis. On the other hand, indirect melanogenic effects of UV via keratinocyte, strongly suggested that keratinocytes secrete specific factors responsible for melanocyte activation. Among keratinocyte secreted factors, which induce melanocyte activation, are prostaglandins PGE_2, α-MSH and ACTH, endothelin-1 and nitric oxide. They are also capable of stimulating melanogenic enzyme expression (tyrosinase enzyme) (Busca and Ballotti, 2000).

C. Melanogenesis

Melanogenesis is a physiological process resulting in the synthesis of melanin pigments. Melanins are synthesized in melanosomes that contain the specific enzymes required for proper melanin production. Among them, the well-characterized is tyrosinase (Busca and Ballotti, 2000). Figure 7 is a diagram showing pathways of melanogenesis.

Synthesis of melanin starts from the conversion of the amino acid L-tyrosine (monophenols) to 3,4-dihydroxyphenylalanine (o-phenols, L-DOPA) by tyrosinase enzyme with hydroxylation reaction and then the oxidation of L-DOPA yields dopaquinone (o-quinones) by tyrosinase. These quinones are highly reactive and tend to polymerize spontaneously to form brown pigments of high molecular weight (melanins), which determine the color of mammalian skin and hair. Quinones can also react with amino acids and proteins and thus enhance the development of brown color (Nerya et al., 2004). Two types of melanin are eu-melanins (dark-brown) and pheo-melanins (yellow-brown). Therefore, the pathway that DOPA quinone transformed to leucoDOPAchrome, DOPAchrome leading to the formation of eu-melanins is interesting. According to melanogenesis pathway, tyrosinase activity is thought to be a major regulatory factor in the initial steps of this pathway.

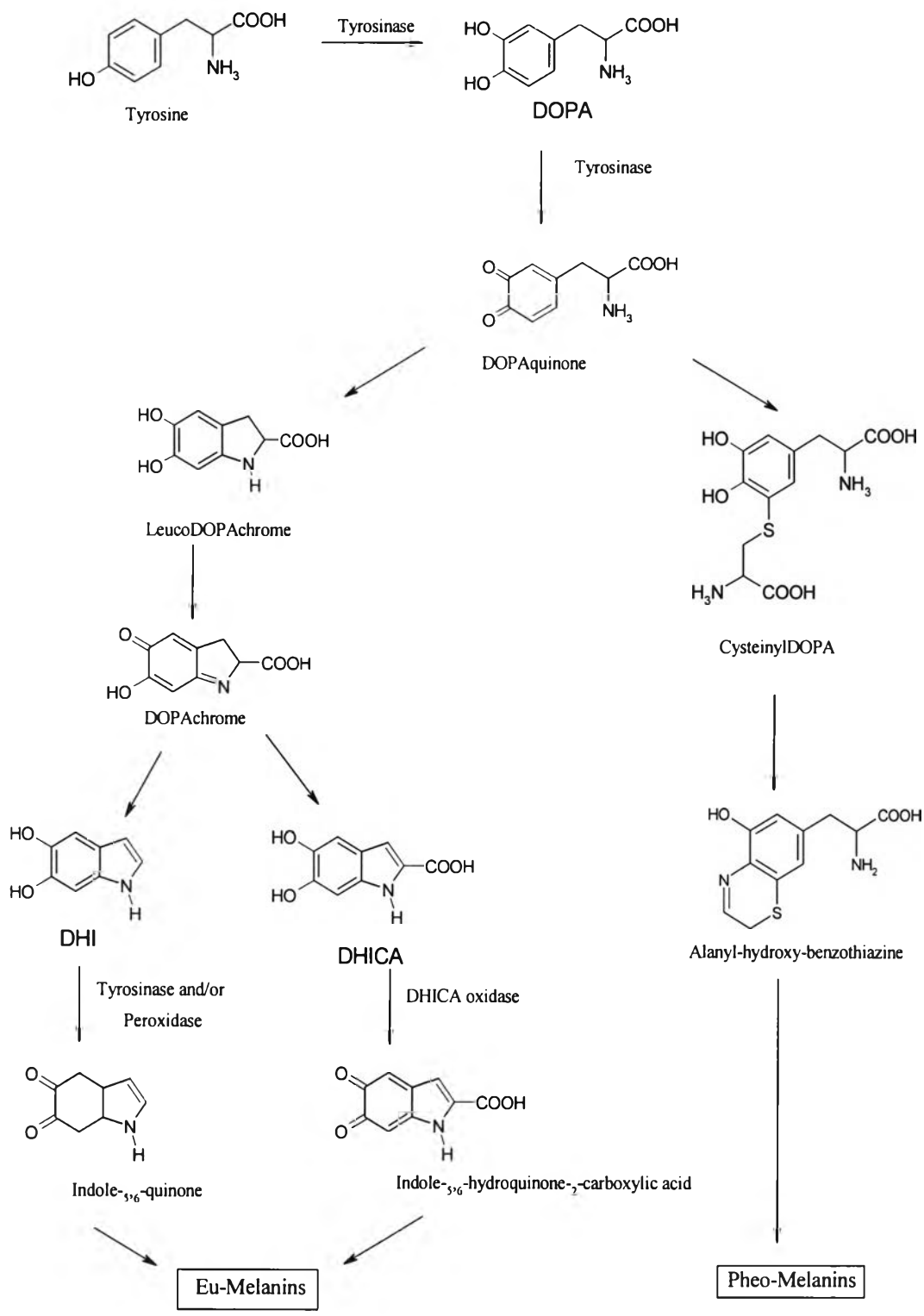


Figure 7. Melanogenesis pathway (Baumann, 2002)

Tyrosinase is a copper-glycoenzyme involved in the biosynthesis of the widespread melanin pigments (Jose, Borron and Solano, 2002; Nerya et al., 2004). Its structure contains two histidine-based regions named Cu A and Cu B which are the peptide segments involved in binding the two coppers. These are the active site of tyrosinase enzyme. Both regions contain three perfectly conserved histidine residues. Concerning oxygen, it binds as a side-on (μ-η2: η2) peroxide bridge rather than in a μ-dioxo disposition. Thus, coppers are penta-coordinated in a distorted square pyramidal geometry (Jose et al., 2002).

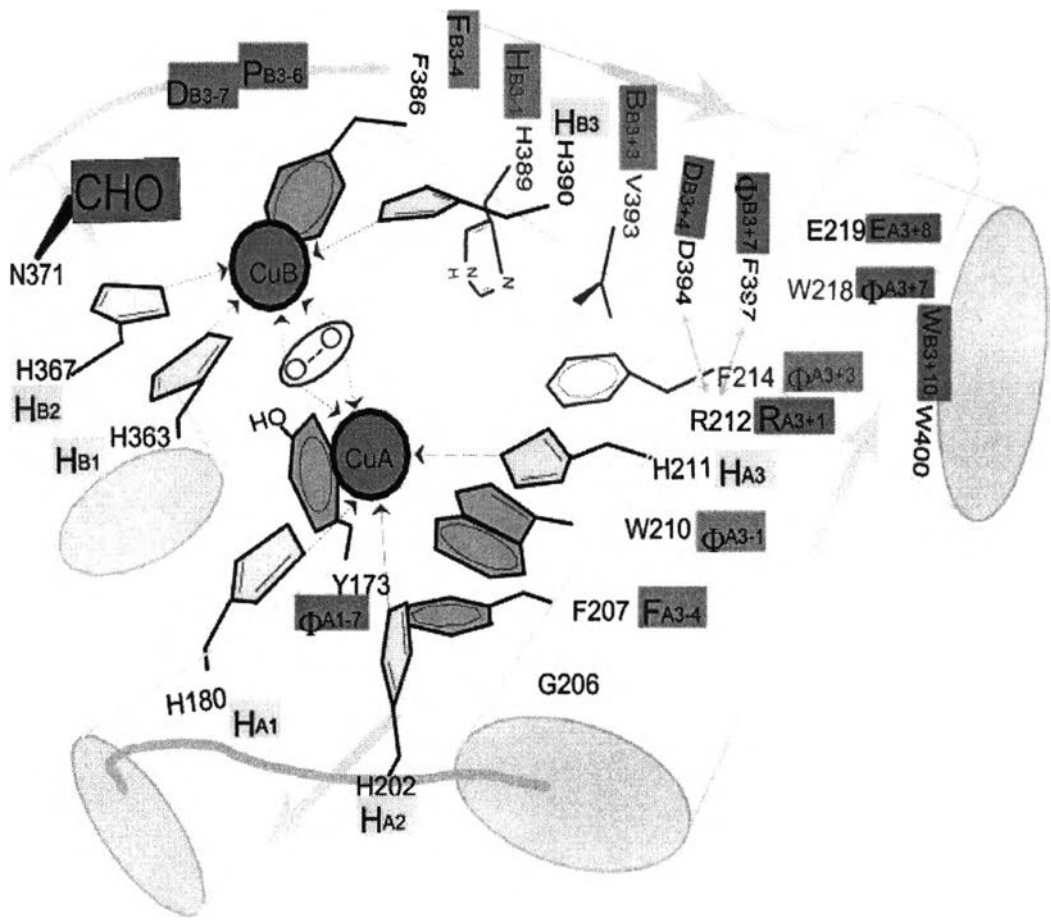


Figure 8. A reasonable model for the active site of mammalian tyrosinase enzyme (Jose et al., 2002).

7. Skin care products

A. Photoprotective product

The most effective approach in protection from environmental damage is avoidance of sun exposure and other potential environmental mutagens such as cigarette smoke (Hadshiew, Eller and Gilchrest, 2000). As shown earlier, ROS play a major role in the UV-dependent upregulation of matrix metalloproteinase, hence, a decrease in ROS load following UV irratdiation by efficient suncreens and/or antioxidants represents promising strategies to prevent or minimize cutaneous photoaging (Scharffetter-Kochanek, 1997).

1. Sunscreens

Sunscreens are the "gold standard" for protecting skin from photodamage. Many chemicals have been developed that absorb UV light efficiently and protect against erythema. However, in actual use, sunscreens provide much less protection than expected. Sunscreens may provide a false sense of security. Finally, no sunscreen provides full spectral protection against UV light. Sunscreen ingredients may become free radicals themselves when activated by UV irradiation, and sunscreen chemicals may be absorbed into skin to cause potentially harmful side effect (Pinnell, 2003).

2. Antioxidants

Antioxidants are a group of substances which, when present at low concentrations, in relation to oxidizable substrates, significantly inhibit or delay oxidative processes, while often being oxidized themselves. In recent years there has been an increased interest in the application of antioxidants to medical treatment as information is constantly gathered linking the development of human diseases to oxidative stress (Vaya and Aviram, 2001).

2.1 Antioxidant mechanisms

2.1.1 Antioxidants as reducing agents

Many compounds with antioxidant activity are, as might be expected, readily oxidizable materials. This property allows them both to intercept primary oxidants such as transition metal ions, molecular oxygen, hydroxyl radical

(OH), or hydrogen peroxide (H_2O_2), and also to compete with chain-carrying free radicals to terminate autooxidation processes. In principle, it should be possible to correlate the antioxidative effectiveness of a compound toward an oxidizing species such as an electrophilic free radical by an electron transfer process *in vitro* (Larson, 1995).

2.1.2 Antioxidants as radical quenchers

A general ability to enter into rapid reactions with free radicals is a great advantage for a potential antioxidant compound. Many synthetic antioxidants are specifically designed to react with oxygen radicals and to form sterically hindered or otherwise inactive radical products that effectively terminate radical chains. This property stop the free radical chain of oxidative reactions by contributing hydrogen from the phenolic hydroxyl groups, themselves forming stable free radicals that do not initiate or propagate further oxidation of lipids (Kaur and Kapoor, 2001). Many naturally occurring compounds could have mechanisms of action that are similar to those that have been established for synthetic materials. Their effectiveness appears to depend on their capacity to form stable radicals, which then react further with polymer free radicals to stop the chain reaction (Larson, 1995).

The properties of hydrogen donation for exhibiting a redox property are critical factors for characterizing antioxidant activity that involves free radical scavenging. The hydrogen donating property of antioxidants can also be confirmed from the result of the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging activity (Hu, Zhang and Kitts, 2000). The principle of this method is that, in the presence of molecule consisting of a stable free radical (DPPH), an antioxidant with the ability to donate a hydrogen atom will quench the stable free radical, a process which is associated with a change in the absorption which can be followed spectroscopically (Vaya and Aviram, 2001).

$$(DPPH)^{\cdot} + A\text{-}H \longrightarrow (DPPH) : H + A^{\cdot}$$

where A-H is the antioxidant and A· is the antioxidant intermediate radical; thus, the DPPH radical scavenging activity can be related to a propensity to donate hydrogen (Hu et al., 2000).

2.1.3 Antioxidants as metal ion complexing agents

Several oxidized transition metal ions such as iron (III) and copper (II) have readily accessible reduced states and, furthermore, are present in high enough concentrations in many tissues to make them plausible reactants for one-electron oxidations or reductions that could generate reactive free radicals. For example, a well-known route to hydroxyl radical (OH·) and other radical generation is Fenton reaction. Agents that could bind reactive transition metal cations by complexation could decrease their biological effects dramatically. A second effect of complexed metal ions could be to change the redox properties of their ligands, and therefore either promote or inhibit their antioxidant capacities (Larson, 1995).

2.1.4 Synergistic effects

It has often been noted that combinations of antioxidants are more effective than would be expected if they were acting independently of one another. For example, ascorbic acid (vitamin C) and tocopherol (vitamin E) have long been known to be highly effective in combination, although vitamin C is significantly less effective when used alone. Ascorbate regenerates tocopherol from the tocopheryl radical, and this recycling action presumably contributes to the synergistic effect of combined ascorbate and tocopherol supplementation (Larson, 1995; Fuchs, 1998).

2.2 Available antioxidant product

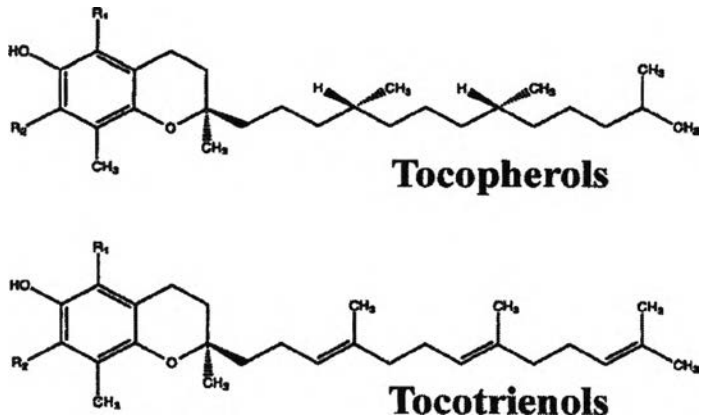
A wide range of antioxidants, both natural and synthetic, has been proposed for use in treatment of human diseases (Halliwell et al., 1992). A compound might exert antioxidant actions *in vivo* in food by inhibiting generation of ROS, or by directly scavenging free radicals. Additionally, *in vivo* an antioxidant might act by raising the levels of endogenous antioxidant defenses (e.g. by up regulating expression of the genes encoding SOD, catalase or glutathione peroxidase). Some quite simple experiments can be performed to examine direct antioxidant ability *in*

vitro and to test for possible pro-oxidant effects on different molecular targets (Halliwell et al., 1995).

2.2.1 Vitamin C (L-ascorbic acid) is one of the important water-soluble vitamins. By a stepwise donation of an electron, the resulting ascorbate free radical that is formed is more stable than other free radicals and can serve as a free-radical scavenger. Topical L-ascorbic acid protected porcine skin from UVB-and UVA-phototoxic injury as measured by erythema and sunburn cell formation (Pinnell, 2003). Vitamin C is significantly decreases the adverse effect of reactive species such as reactive oxygen and nitrogen species that can cause oxidative damage to macromolecules. In addition ascorbic acid can regenerate other antioxidants such as α-tocopheroxyl, urate and β-carotene radical cation from their radical species. Thus, ascorbic acid acts as co-antioxidant for α-tocopherol by converting α-tocopheroxyl radical to α-tocopherol and helps to prevent the α-tocopheroxyl radical mediated peroxidation reactions. However, ascorbic acid has been shown to exhibit both anti-oxidant and pro-oxidant effects in a dose related fashion. The current US recommended daily allowance (RDA) for ascorbic acid ranges between 100-120 mg/day for adults (Naidu, 2003). Ascorbic acid has been reported to exhibit pro-oxidant effect at lower concentration, this activity increase in the range 0.004-0.24mM and reached a maximum when the concentration of ascorbic acid was 1.65 mM and above 1.65 mM, at higher concentration, ascorbic may markedly scavenge $OH^{\cdot}$ and reduce the oxidative damage of deoxyribose (Yen, Duh, and Tsai, 2002). This was supported by Naidu (2003) who postulated that at low concentrations ascorbic acid may act as a pro-oxidant *in vitro*, but as an antioxidant at higher levels. For the pro-oxidant activity, this is related to Fenton reaction that involves the transition metal-catalyzed reduction of H_2O_2 to generate hydroxyl radicals. Ascorbic acid can reduce and then recycle ferric iron (Fe^{3+}) to the more active ferrous iron (Fe^{2+}) facilitating further generation of $OH^{\cdot}$ by subsequent Fenton cycles. Importantly, in the presence of metal ion chelators (e.g. EDTA) other than ascorbic acid the reaction proceeds to give a Fe(II) complex which would react rapidly with H_2O_2 to generate $OH^{\cdot}$ (Halliwell et al., 1992; Minetti, 1992; Fisher and Naughton, 2004). This generation of toxic $OH^{\cdot}$ from a simple system containing metal ions, ascorbic acid and

oxygen has potentially deleterious consequences owing to the ubiquitous nature of these components in diseased tissues. Under these conditions it is imperative to restrict ascorbic acid intake to recommended daily intake levels (Fisher and Naughton, 2004).

2.2.2 Vitamin E (tocopherols) is the body's major lipid phase antioxidant. It consists of 8 molecular forms, 4 tocopherols and 4 tocotrienol (Figure 9). The molecules consist of a hydrophobic prenyl tail that inserts into membranes and a polar chromanol head group exposed to the membrane surface. The chromanol head of each is identical with α-,β-, γ- and δ-isomers, each containing an essential hydroxyl group, necessary for antioxidant activity, and methyl groups varying in number and position. Although all of these isomers are available in dietary sources, human beings use predominantly α-tocopherol because a specific α-tocopherol transfer protein selectively transfers α-tocopherol into lipoproteins (Pinnell, 2003). Since vitamin E is the most effective chain breaking antioxidant present in cell membranes, it is likely that it plays a major role in maintaining cell membrane integrity by limiting lipid peroxidation by ROS (Hughes, 2000). Once oxidized, vitamin E can be regenerated back to its reduced form by l-ascorbic acid and ubiquinol-10 which prevent the peroxidation of LDL (Bowry, Ingold and Stocker, 1992), allowing it to be reactivated without creating a new membrane structure. The relative antioxidant activities of tocopherol in lipid system is $\alpha > \beta > \gamma > \delta$ (Lee et al., 2003).



	R_1	R_2
α	CH_3	CH_3
β	CH_3	H
γ	H	CH_3
δ	H	H

Figure 9. Vitamin E structure. Molecular structures of 4 tocopherols and 4 tocotrienol comprising vitamin E. Substitution of methyl groups (CH_3) at positions R_1 and R_2 determine whether the molecules are α-,β-, γ- and δ-isomers (Lee et al., 2004).

2.2.3 Synthetic antioxidants

Some of the important synthetic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxyl toluene (BHT), *tert*-butylhydroquinone (TBHQ), propyl gallate (PG) and tocopherols. In general, synthetic antioxidants are compounds with phenolic structures and various degrees of alkyl substitutions (Kaur and Kapoor, 2001). **Trolox®** is a cell-permeable, water-soluble derivative of α-tocopherol with the hydrophobic side chain replaced by hydrophilic –COOH group. Its structure is shown in Figure 10. It is a chain-breaking antioxidant that acts as a scavenger of radicals via the H-donating group in its chromanol nucleus. Its protective effects against oxidative damages, particularly against lipid peroxidation, have been demonstrtated *in vitro* and *in vivo*. It is a very efficient antioxidant, good scavenger of peroxyl and alkoxyl radicals, prevents peroxynitrite-mediated oxidative stress and prevents apoptosis in cell culture. Trolox® is commercially available for experimentation especially in an aqueous system (Suarna, Dean and Southwell-Keely, 1997; Feugang et al., 2004). However, Trolox® may be harmful if swallowed and may cause respiratory tract, eye and skin irritation.

2.2.4 Polyphenols

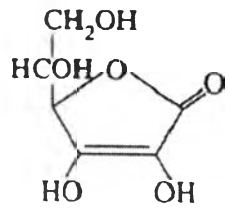
Phenolic compounds or polyphenols are ubiquitous in plants with more than 8000 structures. Natural polyphenols can range from simple molecules (phenolic acids, phenylpropanoids, flavonoids) to highly polymerized compounds (lignins, melanins, tannins), with flavonoids representing the most common and widely distributed sub-group. Polyphenolics exhibit a wide range of biological effects including antibacterial, anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, anticarcinogenic and vasodilatory actions; many of these biological functions have been attributed to their free radical scavenging and antioxidant activity (Soobrattee et al. 2005). Antioxidant mechanisms of polyphenolic compounds are based on reducing properties, hydrogen donating abilities and chelating metal ions. After donating a hydrogen atom, phenolic compounds become resonance-stabilized radicals, which do not easily participate in other radical reactions (Kaur and Kapoor, 2001; Lee et al., 2004). It is known that the degree of glycosylation significantly affects the antioxidant properties of the compound, for example, aglycones of quercetin and myricetin were more active than their glycosides. The molecular scavenging mechanism of these molecules is closely related to their stereo chemical structure (Kaur and Kapoor, 2001). Both the number and configuration of H-donating hydroxyl groups are the main structural features influencing the antioxidant capacity of phenolics (Soobrattee et al., 2005). Catechins and their epimers serve as powerful antioxidants for directly eliminating super oxide anion radicals. These are basically flavonoids and some related compounds (Kaur and Kapoor, 2001).

More recently, attention has been focused on the antioxidant properties of plant polyphenols found in green tea and red wines. But before their relative contribution to preventing oxidative damage can be assessed, considerably more information on the absorption, metabolism and excretion of these compounds in humans is needed (Hughes, 2000).

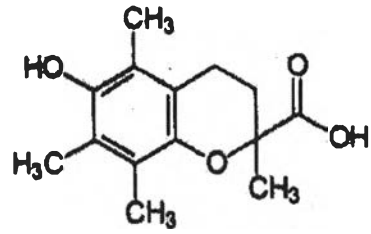
- **EGCG** or (-)-epigallocatechin-3-gallate is the main polyphenolic component of catechins in green tea, which has been consumed as a

popular beverage in Asian countries for many centuries (Baumann, 2002). Its structure is shown in Figure 10. Topical EGCG reduced UVB-induced inflammatory responses and infiltration of leukocytes in human skin. Moreover, topical application of EGCG also inhibited carcinogenesis and selectively increased apoptosis in UVB-induced skin tumors in mice (Pinnell, 2003). EGCG enhance the activity of superoxide dismutase (SOD) and catalase in mouse striatum thus suggesting that flavan-3-ols can also exhibit their neuroprotective effect via regulation of gene expression. The high antioxidant activity of EGCG is explained by the presence of galloyl moiety attached to flavan-3-ol at the 3 position, adding three more hydroxyl groups (Soobrattee et al., 2005). EGCG has been represented as a powerful radical scavenger, as investigated by many *in vivo* and *in vitro* techniques (Geetha et al., 2004; Hsu, 2005; Soobrattee et al., 2005). However, a low concentration of EGCG increased amounts of double base lesions of DNA, especially 8-oxodG in HL-60 cells, further supporting the involvement of H_2O_2 in cellular DNA damage. These results suggested that EGCG can act not only as an antioxidant, but also as a pro-oxidant in the presence of metal ions (Furukawa et al., 2003). Green tea has caused so much excitement in the media that many pharmaceutical and cosmetic companies are supplementing their skin care products with green tea extracts. It will be interesting to see the long-term results of green tea therapy for photoprotection (Baumann, 2002).

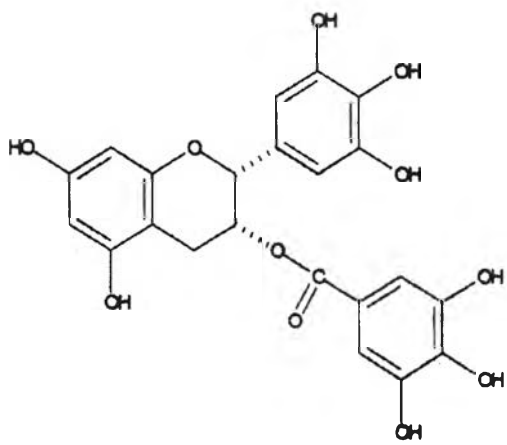
- Red wine has been shown to inhibit *in vitro* oxidation of human LDL. The phenolics substances in wine mainly originate from grapes and include nonflavonoids such as hydroxycinnamates, hydroxybenzoates and stilbene in addition to flavonoids such as flavan-3-ol (catechin), anthocyanins, flavonols and polyphenol tannins. Red wine has great antioxidant potential, because of phenolic compounds (tannins and anthocyanins) which are present in sufficient quantities to ensure optimum free radical scavenging activity of compounds and even combined actions between them leading to synergistic effect of these polyphenols (Kaur and Kapoor, 2001).



Vitamin C



Trolox®



EGCG

Figure 10. Structures of vitamin C, Trolox® and EGCG

However, plant phenolics have sometimes been found to show pro-oxidant properties toward non lipids under certain circumstances. Several flavonoids have been shown to auto-oxidize and generate reactive oxygen species, such as hydrogen peroxide. They are also capable of reducing Fe^{3+} to Fe^{2+}, resulting in the formation of hydroxyl radicals by interaction of Fe^{2+} with H_2O_2 (Laughton et al., 1989; Halliwell et al., 1992; Li and Xie, 2000). Aruoma et al. (1993) reported that several phenolic antioxidants can accelerate oxidative damage of DNA, protein, and carbohydrates *in vitro*. Tea polyphenol (TP) was also reported to have pro-oxidant effects at lower dosages in the aqueous phase. The phenolic compounds act as pro-oxidants under certain conditions, dependent on parameters such as concentrations of phenolic compounds and transition metal ions (Yen, Chen and Peng, 1997). Hagerman et al. (1998) reported that high molecular weight plant polyphenols such as tannins were excellent biological antioxidants and that polygalloyl glucose (polyGG) has no pro-oxidant effect in the deoxyribose assay. Many investigations of the

antioxidant and pro-oxidant effects of simple phenols have been done, and Li and Xie (2000) reported that the scavenging affects of tea catechin oxypolymers (TCOP) to both the hydroxyl radical and superoxide radical was stronger than that of tea catechin (TC), and also they had no pro-oxidant effect.

3. Anti-aging agent

UVB exposure dramatically up-regulates the production of several collagen-degrading enzymes known as matrix metalloproteinases (MMPs). Activation of the MMP genes results in production of collagenase, gelatinase, and stromelysin, which have been shown to fully degrade skin collagen.

3.1 Retinoids, a naturally occurring derivative of vitamin A, is a lipid-soluble molecule known to affect cell growth, differentiation, homeostasis, apoptosis, and embryonic development. Retinoids are beneficial in the treatment of acne, psoriasis, ichthyosis, keratoderma and several other diseases. Multiple studies have examined the use of retinoids for the prevention and treatment of photoaging. Tretinoin, a first-generation retinoid, was the first available topical retinoid, initially marketed as Retin-A (Baumann, 2002). *In vitro* and *in vivo* studies have recently demonstrated that all-trans retinoic acid, which is a known transrepressor of the photoaging-involved transcription factor AP-1, when applied before UVB irradiation substantially abrogated the induction of AP-1 and MMPs (Berneburg et al., 2000; Baumann, 2002). Pre-treatment of the skin with all-trans retinoic acid was shown to inhibit the loss of procollagen synthesis. Therefore, pretreatment of the skin with topical retinoids, when used consistently, is likely to be beneficial in preventing as well as treating photodamage (Baumann, 2002). However, side effects from topically applied retinoids still persist. The most common side effects of topical retinoids are skin irritation, desquamation and redness. Dry skin is also a common compliant of patients with retinoids. The dry skin seen with retinoid use is likely due in part to an increase in transepidermal water loss (TEWL) that accompanies topical retinoid use. These findings seem to be related to the type and dose of the retinoid (Baumann, 2002).

3.2 Other antioxidant products

A new protective strategy has emerged from our understanding that oxidative stress plays a major role in the induction of photoaging. If reactive oxygen species and free radicals are the major causes of aging processes, antioxidant can reduce the level of reactive oxygen species and free radicals, slow the aging process, and these can increase the life span (Lee et al., 2004). A large number of antioxidants have been found to exhibit protective effects against the different ROS involved in photoaging (Berneburg et al., 2000) such as coenzyme Q_{10}, α-Lipoic acid, α-Tocopherol and L-ascorbic acid (Fuchs, 1998; Baumann, 2002).

- Coenzyme Q_{10} (ubiquinone) is a fat-soluble compound found in cells as part of the electron transfer chain responsible for energy production. It has also been found to have antioxidant properties and has been identified as an age-related decline in animals and humans. For this reason, many believe that the antioxidant activities of CoQ_{10} may make it useful as a treatment for aged skin. It had been proved in previous study that after CoQ_{10} application, the reduction in wrinkle depth was reported (Baumann, 2002).

- α-Lipoic acid (ALA) has been proposed as a new antioxidant agent suitable for treatment and prevention of aging skin. It is different than the other antioxidants because it can be used as a superficial chemical peel to resurface the skin in a manner similar to glycolic acid. ALA diminished fine lines and brown spots and improved symptoms of rosacea (stage I and II). ALA may be a useful adjuvant in the treatment of photoaged skin. However, more studies are necessary (Baumann, 2002).

B. Whitening agents

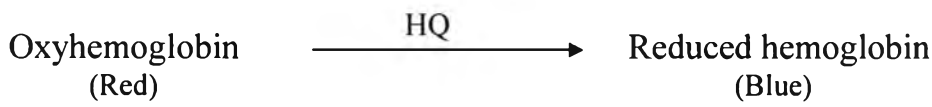
Many modalities of treatment for acquired skin hyperpigmentation are available including chemical agents or physical therapies, but none are completely satisfactory. As a result of the key role played by tyrosinase in melanin biosynthesis, most whitening agents act specifically to reduce the function of this enzyme by means of several mechanisms: interference with its transcription and/or glycosylation, inhibition by different modalities, reduction of by-products and post-transcriptional

control. The classification of tyrosinase inhibitors is difficult because of the capacity of several compounds to work in different ways, interacting with both catalytic and regulatory sites of the enzyme or being metabolized to a product that, in turn, can act as either a non-competitive or a competitive inhibitor. Most of the inhibitors are phenol/catechol derivatives, structurally similar to tyrosine or DOPA, which act as alternative substrates of tyrosinase without producing pigment (Briganti, Camera and Picardo, 2003). A number of naturally occurring tyrosinase inhibitors have been described, the majority of which consisting of a phenol structure or of metal chelating agents. Antioxidants or compounds with redox properties can prevent or delay pigmentation by different mechanisms: by scavenging ROS and RNS, known to induce melanin synthesis, or by reducing *o*-quinones or other intermediates in the melanin biosysnthesis, and thus delaying oxidative polymerization. Chalcones have been reported to have the potency of tyrosinase inhibition and the antioxidant behavior by presenting phenolic hydroxyls and exhibiting the ability to donate electrons (Nerya et al., 2004).

1. Hydroquinone

The most popular depigmenting agent is hydroquinone (dihydroxybenzene; HQ) introduced for clinical use since 1961. For many years, hydroquinone has been the main treatment modality for postinflammatory hyperpigmentation and melasma. Hydroquinone structure has phenolic group. Hydroquinone has been considered as a reference standard in evaluating depigmenting agents. The oxidation products of HQ are quinones and reactive oxygen species (ROS), which lead to an oxidative damage of membrane lipids and proteins, including tyrosinase, and depletion of glutathione contributes to the lightening action. HQ may interfere with pigmentation even through: (i) the covalent binding to histidine or interaction with coppers at the active site of tyrosinase, (ii) the inhibition of DNA and RNA synthesis and (iii) the alteration of melanosome formation and melanization extent (Briganti et al., 2003). The effectiveness of hydroquinone was related directly to the concentration of the preparation but the higher concentrations of hydroquinone were extremely irritating. HQ concentrations higher than 5% are not recommended because they are very irritating without improving the efficacy.

Concentrations of 4-5% are very effective but they are moderate to strong irritants. Results obtained with 3% HQ have been rated as "fair" to "good" in many patients and excellent in a few, although a mild irritation may be expected. Finally, the 2% HQ concentrations are not irritating and their efficacy have been rated from "ineffective" to "very effective" in different studies. Thus, they are recommended for maintenance therapy and not considered for initiating the treatment of melasma (Katsambas and Antoniou, 1995). Prolonged application of hydroquinone can cause exogenous ochronosis which presents as asymptomatic blue-black macules in the area of hydroquinone application. The blue-black macules result from HQ converting oxyhemoglobin (red) to reduced hemoglobin (blue) by taking oxygen out of oxyhemoglobin (Baumann, 2002).



Therefore, because of the hazard of long-term treatments, the use of hydroquinone in cosmetics has been banned by the European Committee (24 th Dir. 2000/6/EC) and formulations are available only by prescription of physicians and dermatologists (Briganti et al., 2003).

2. Arbutin

Arbutin, a naturally occurring HQ β-D-gluconopyranoside, consists of a molecule of hydroquinone bound to glucose. It has depigmenting effect at non-cytotoxic concentrations, and acts similar to HQ. In both normal human melanocytes and melanoma cells, arbutin induces a decrease of tyrosinase activity and exerts an inhibitory effect on melanosome maturation. An interference with the uptake of tyrosine into melanocytes has also been postulated. However, despite encouraging initial results, other researchers have not confirmed the activity of arbutin on intact melanocytes and in clinical trial (Baumann, 2002; Briganti et al., 2003).

3. Kojic acid

Kojic acid (5-hydroxy-2-hydroxymethyl-4H-pyran-4-one) is an antibiotic, fungal metabolite commonly produced by many species of *Aspergillus*, *Acetobacter* and *Penicillium* (Baumann, 2002). The depigmenting action of kojic acid is attributed to the chelating ability (Briganti et al., 2003). Nonetheless, kojic acid has exhibited mild cytotoxicities that were tested using the MTT assay and it is too unstable to be stored for a long time. To overcome these drawbacks, amino acid derivatives of kojic acid were developed, which exhibited higher long term stability and lower toxicity than that of kojic acid but their synthetic yield was very low (Kim et al., 2004). The addition of 2% kojic acid in a gel containing 10% glycolic acid and 2% hydroquinone will improve melasma. Both kojic acid and hydroquinone are tyrosinase inhibitors. The combination of both agents augments this inhibition. The addition of glycolic acid enhances penetration of both agents and hence promotes efficacy. However, the combination can cause redness, stinging and mild exfoliation (Lim, 1999).

4. Hydroxystilbene compounds

Most hydroxystilbene compounds such as resveratrol, naturally contained in herbal medicines, have potent inhibitory effects on mushroom tyrosinase. Resveratrol, one of the ingredients of red wine, possesses several biological properties such as free radical scavenging, anti-inflammatory and anticancer activities. Resveratrol is probably metabolized by tyrosinase leading to the formation of an *o*-hydroxy derivative, which is a competitive inhibitor of the enzyme. Oxyresveratrol, extracted from *Morus alba*, is a stronger inhibitor (IC_{50}= 1 μM) on mushroom DOPA oxidase activity than resveratrol (IC_{50}= 54.6 μM), suggesting that the number and the position of hydroxyl substituents have an important role in determining the activity (Briganti et al., 2003). Ohguchi et al. (2003) reported that the number and position of hydroxyl group on phenyl rings in hydroxystilbene are important for the inhibition of tyrosinase activity, and also that the trans-olefin structure of the parent stilbene skeleton is essential for the inhibition. 3,3',4,4'-Tetrahydroxy-trans-stilbene showed nearly complete inhibition of tyrosinase activity.

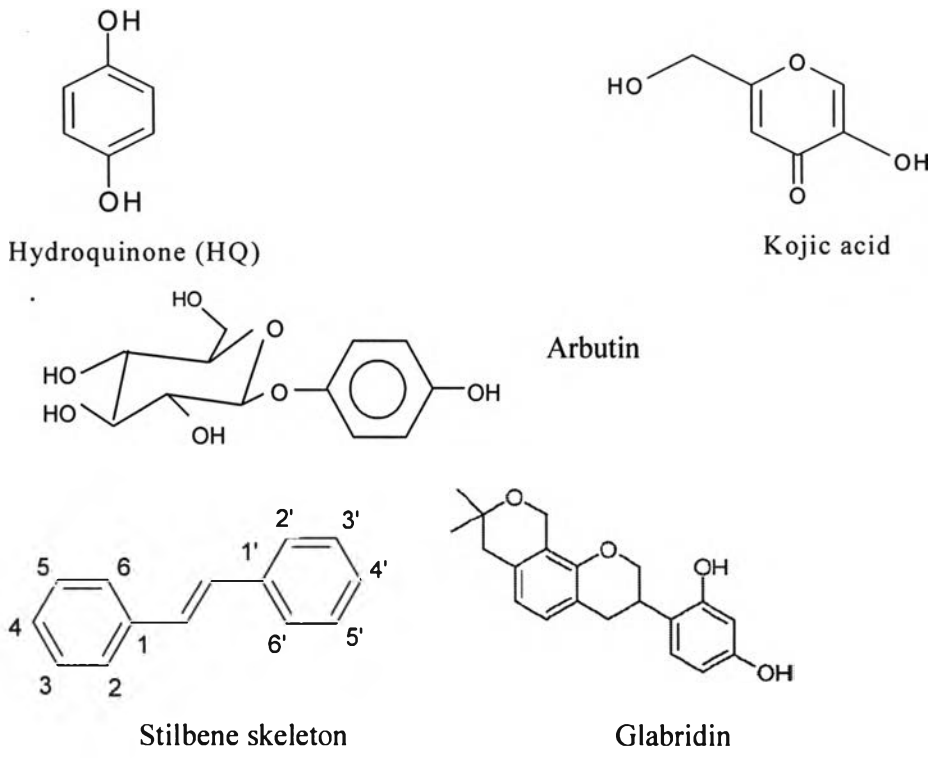


Figure 11. Chemical structures of hydroquinone, arbutin, kojic acid, stilbene skeleton and glabridin.

5. Licorice extract

Licorice is one of the most popular and widely consumed herbs in the world (Cui et al., 2005). The licorice root (*Glycyrrhiza glabra* L.) has long been employed in Western countries as a flavoring and sweetening agent. Glabridin is the main ingredient of licorice extract that affects the skin. Glabridin inhibits tyrosinase activity in cell cultures without affecting DNA synthesis. Topical applications of 0.5% glabridin inhibited ultraviolet B (UVB)-induced pigmentation and erythema in the skin of guinea pigs (Baumann, 2002; Nerya et al., 2003). The main drawbacks of glabridin are its poor skin-penetrating ability and its instability in formulations (Nerya et al., 2003).

6. Redox agents and ROS scavengers

Compounds with redox properties can have depigmenting effects by interacting with *o*-quinones, thus avoiding further oxidative polymerization of

melanin intermediates, or with the copper at the active site of tyrosinase enzyme. Moreover, redox agents, by scavenging ROS generated in skin following UV exposure, can inhibit possible second messengers which are able to stimulate epidermal melanogenesis either directly or indirectly. Ascorbic acid interferes with the different steps of melanization, by interacting with copper ions at the tyrosinase active site and reducing dopaquinone and by blocking DHICA oxidation. α-Tocopherol derivatives inhibit tyrosinase *in vitro* and melanogenesis in epidermal melanocytes. The antioxidant properties of α-tocopherol, which interferes with lipid peroxidation of melanocyte membranes and increases the intracellular glutathione content, could explain its depigmenting effect (Briganti et al., 2003).

8. Review of *Phyllanthus emblica* Linn.

Family : Euphorbiaceae

Synonymn : *Emblica officinalis* Gaertn.

English names: Emblic myrobalan, Malacca tree, Indian gooseberry

Common name: Amalaka, Amla, Avla

In Malaya the emblic is called *melaka*, *Asam melaka*, or *amlaka*. In Thailand, it is called *ma-kham-pom*; in Laos, *mak-kham-pom*; in Cambodia, *kam lam* or *kam lam ko*; in southern Vietnam, *bong ngot*; in North Vietnam, *chu me*. In the Philippines, it is called *nelli* (Morton, 1987).

A. Morphological characteristics

P. emblica is a small to moderate size deciduous tree, normally reaching a height of 8-18 m and, in rare instances, 100 ft (30 m). Its fairly smooth bark is a pale grayish-brown and peels off in conchoidal flakes (Morton, 1987; Scartezzini and Speroni, 2000, สุภาภรณ์ ปิติพร, 2548). Leaves are linear-oblong blunt, small, a hundred or more on each branchlet, arranged in two ranks and thus appearing to form a pinnate leaf, 8-12 mm or more long and 2-5 mm broad, stipulate, entire, obtuse or round at the base, subacute or apiculate apex, hairless, light green outside, pale green or often pubescent beneath, almost stalk-less. Leaves fall in November-December and grow in

February-March (Scartezzini and Speroni, 2000; สุภาภรณ์ ปิติพร, 2548). Small, inconspicuous, greenish-yellow flowers are borne in compact clusters in the axils of the lower leaves, blossom in March-May, unisexual, 0.5-1.5 cm long. Usually, male flowers occur at the lower end of a growing branchlet, with the female flowers above them, but occasional trees are dioecious. The nearly stemless fruit is round or oblate, nearly spherical or globular, indented at the base, and smooth, though 6 to 8 pale lines. Normally, fruit is 12-25 mm wide and 15-20 mm long, ripen from November-February. When ripened the mesocarp is yellow and the endocarp is yellowish brown. The mesocarp is acidulous in fresh fruit and acidulous astringent in dried fruit (Morton, 1987; Scartezzini and Speroni, 2000; สุภาภรณ์ ปิติพร, 2548).

B. Distribution

The *P. emblica* tree is native to tropical southeastern Asia, particularly in central and southern India, Pakistan, Bangladesh, Ceylon, Malaya, southern China, the Mascarene Islands, Thailand and Cambodia (Morton, 1987; สุภาภรณ์ ปิติพร, 2548). It is commonly cultivated in home gardens throughout India and grown commercially in Uttar Pradesh. In India, and to a lesser extent in Malaya, the *P. emblica* is important and esteemed, raw as well as preserved, and it is prominent in folk medicine. Fruits from both wild and dooryard trees and from orchards are gathered for home use and for market. In southern Thailand, fruits from wild trees are gathered for marketing (Morton, 1987).

C. Phytochemistry

The *P. emblica* tree contains different classes of constituents listed in Table 5. The complexity of the mixture of compounds and the presence of several compounds in small concentrations can make the isolation and identification of the substances present in this genus very laborious. Different environment condition of the spectral data of the complex structures has been reported, resulting in considerable confusion (Summanen, 1999).

Chemical constituents present in different parts of the plant:

1. Fruits

Moisture 81.2%, protein 0.5%, fat 0.1%, mineral matter 0.7%, fiber 3.4%, carbohydrates 14.1%, Ca (0.05%), K (0.02%), Fe (1.2 mg/100g), nicotinic acid (0.2 mg/100g), phyllemblin, phyllemblic acid, gallic acid, emblicol, ellagic acid, pectin, putranjivatin A, two new hydrolysable tannins, vitamin C-like, called emblicanin A and B (not ascorbic acid as it was believed by mistake until 1996), punigluconin and pedunculagin (Ghosal, 1996; Scartezzini and Speroni, 2000). The fruit pulp, which constitutes 90.97% of the whole fruit, contains 70.5% moisture. The total soluble solids constitute 23.8% of the juice. The acidity of *P. emblica* is 3.28% on pulp basis. The pulp contains 5.09% total sugars and 5.08% reducing sugars (Parmar and Kaushal, 1982). New organic acid gallates and polyphenols including L-malic acid 2-O-gallate, mucic acid 2-O-gallate together with hydrolysable tannins, 1-O-galloyl-β-D-glucose, corilagin, and chebulagic acid were found to be the major phenolic constituents of fruit juice (Zhang et al., 2004).

Flower

Fruit

Habit

Leaves

Seed

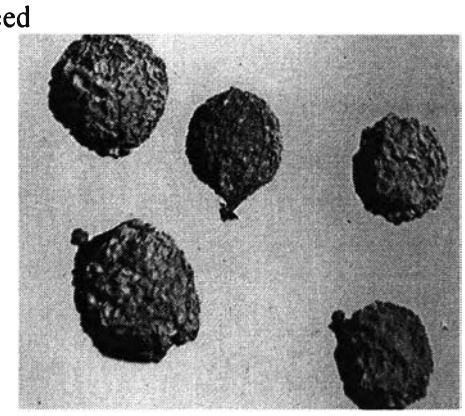


Figure 12 *Phyllanthus emblica* L.

Table 5. Chemical constituents reported from *Phyllanthus emblica* L. (Adapted from Ghosal, 1996; Summanen, 1999; Zhang et al., 2004).

Class	Compound	Occurrence
Alkaloid	Phyllantine Phyllantidine	Leaves, fruit, and tissue cultures
Benzenoid	Chebulic acid Chebulinic acid Chebulagic acid Gallic acid	Leaves
	Ellagic acid	Leaves
	Amlaic acid	Fruit
	Corilagin 3-6-di-O-galloyl-glucose ethyl gallate	Fruit
	β-glucogallin	Leaves, fruit
	1,6-di-O-galloyl-β-D-glucose 1-di-O-galloyl-β-D-glucose putranjivain A digallic acid	Fruit
	Phyllemblic acid Emblicol Mucic (=galactaric) acid	Fruit
Furanolactone	Ascorbic acid	Fruit Leaves
Sesquiterpene	Phyllanemblic acid and its glycosides phyllanemblicuns A-C	Roots
Diterpene	Gibberellin A-1 Gibberellin A-3 Gibberellin A-4	

	Gibberellin A-7 Gibberellin A-9	
Triterpene	Lupeol	Fruit, leaves
Flavonoid	leucodelphinidin	Leaves
	kaemferol	Leaves
	Kaemferol-3-glucoside Rutin	Leaves Leaves
	quercetin	Leaves
	Kaempherol-3-O-β-D-glucoside Quercetin-3-O-β-D-glucoside	Fruit
Hydrolysable tannins	Emblicanin A, B Punigluconin Pedunculagin	Fruit
Sterol	β-sitosterol	Leaves
Carbohydrate	Acidic and neutral polysaccharides glucose	Fruit Leaves

2. Seeds

The seeds contain a fixed oil, phosphatides, and a small quantity of essential oil. The fixed oil (yield 16%) has the following physical and chemical characteristics: acid value 12.7; saponification value 185; iodine value 139.5; acetyl value 2.03; unsaponifiable matter 3.815; sterol 2.70%; saturated fatty acids 7%, linolenic (8.78%), linoleic (44.0%), oleic (28.40%), steric (2.15%), palmitic (2.99%) and miristic acid (0.95%). Proteolytic and lipolytic substances are present (Scartezzini and Speroni, 2000).

3. Leaves

The leaves contain gallic, ellagic, chebulic, chebulagic, chebulinic acids, a gallotannin called amlic acid, alkaloids phyllantidine and phyllantine (Scartezzini and

Speroni, 2000). The branches and leaves of this plant contain ellagitannins and flavonoids, i.e., geraniin, phyllanemblinins C and E, prodelphinidin B_1, prodelphinidin B_2, (-)-epigallocatechin 3-O-gallate, and (S)-eriodictyol 7-[6-o-(E)-p-coumaroyl]-β-D-glucoside (Zhang, et al., 2004).

4. Bark

The bark contains leukodelphinidin, tannin and proanthocyanidin (Scartezzini and Speroni, 2000).

5. Roots

Ellagic acid, lupeol (Scartezzini and Speroni, 2000), several novel sesquiterpenoids including phyllanemblic acid and its glucosides phyllanemblicins A-C were the major sesquiterpenoids from the roots (Zhang et al., 2004).

For many years the therapeutic potential of the fruits was attributed to their high content ascorbic acid. However, in 1996 professor Shibhnath Ghosal of Banaras Hindu University discovered that *P. emblica* fruits do not contain ascorbic acid neither in free nor in conjugated form, but it contains two new hydrolysable tannins with low molecular weight (<1000), called emblicanin A and emblicanin B and other tannins like punigluconin and pedunculagin isolated from the fresh juice or solvent extracts of *P. emblica.* These two new tannins have a very strong antioxidant action.

D. Ethnopharmacology

P. emblica has been used for thousands of years in many of the indigenous medical preparations against a variety of disease condition and is used in traditional medicines of Ayurvedic system.

1. Anti-inflammatory, anti-pyretic and analgesic activity

P. emblica leaves and fruit have been used for fever and inflammatory treatments by rural populations in its growing areas. The ethanol and aqueous extracts of *P. emblica* have shown significant anti-inflammatory activity. The phytochemical screening of the plant extract gave positive test for alkaloids, tannins, phenolic

compounds, carbohydrates and amino acids, which might be in part responsible for anti-pyretic and analgesic activities (Perianayagam et al., 2004). It was found that the water fraction of the methanol leaf extract of *P. emblica* possesses marked anti-migration activity, the IC_{50} being around 10μg/mL for both LTB_4 and FMLP-induced PMN migration (Asmawi et al., 1993). Leaf extracts from different solvents were tested for their inhibitory activity against human PMN functions such as degranulation, migration, and leukotriene B_4 (LTB_4) release and platelet activity and the results showed that the plant leaves contain as yet unidentified polar compound(s) with potent inhibitory activity on PMNs and a chemically different polar molecule (s) which inhibit both prostanoid and leukotriene synthesis (Ihantola-Vormisto et al., 1997).

2. Anti-microbial

Ethanolic extracts of *P. emblica* have been found to show potentially interesting activity against test bacteria using agar well diffusion method at sample concentration of 200 mg/mL, without any indication of cellular toxicity (Ahmad, Mehmood and Mohammad, 1998).

3. Anti-viral

A bioassay-guided fractionation of a methanol extract of the fruit of *P. emblica* led to the isolation of putranjivain A as a potent inhibitory substance on the effects of human immunodeficiency virus-1 reverse transcriptase in the replication of retroviruses such as HIV-1 (El-Mekkawy et al., 1995).

4. Anti-atherogenic

Two compounds from *P. emblica* fruit extract, including corilagin [beta-1-O-galloyl-3,6-(R)-hexahydroxydi-phenoyl-d-glucose] and its analogue Dgg16 [1,6-di-o-galloyl-beta-d-glucose], were effective in inhibiting the progress of atherosclerosis by alleviating oxidation injury and by inhibiting ox-LDL-induced vascular smooth muscle cells (VSMC) proliferation (Daun, Yu and Zhang, 2005).

5. Anti-diabetic

Oral administration of 'Triphala' a traditional medicines used in human diabetes (combination of *Phyllanthus emblica*, *Terminalia chebula* and *Terminalia belerica*) (100 mg/kg body) reduced the blood sugar level in normal and in alloxan-induced (120 mg/kg) diabetic rats significantly within 4 h. It was also found to have a significant antioxidant activity. It may be possible that these extracts may reduce the effect of inflammatory cytokine released during diabetes which may be one of the causative agents for the tissue distraction and insulin resistance (Sabu and Kuttan, 2002). Suryanarayana et al. (2004) showed that tannoid principles of *P. emblica* prevented the sugar-induced polyol stress in cultured rat lenses, implying that *P. emblica* ingredients may be explored as an anticataractogenic agent for diabetic cataract.

6. Anti-ulcerogenic

Methanolic *P. emblica* extract, 10-50 mg/kg administered orally, twice daily for 5 days, showed dose-dependent ulcer protective effects in rat models and significant ulcer healing effect, at the dose of 20 mg/kg, after 5 and 10 healing days treatment. The significant ulcer protective and healing effects of *P. emblica* might be due to its effect both on offensive and defensive mucosal factors (Sairam et al., 2002). Pretreatment with the butanol extract of the water fraction of *P. emblica* fruits was found to enhance secretion of gastric mucus and hexosamine ($P < 0.001$) in the indomethacin induced ulceration of rats (Bandyopadhyay, Pakrashi and Pakrashi, 2000). Bafna and Balaraman (2005) proved that the anti-ulcer effect of *P. emblica* may be due to its antioxidant activity.

7. Anti-tussive

The anti-tussive activity of *P. emblica* in conscious cats was dose-dependent. The research of Nosal et al. (2003) showed that the anti-tussive activity of *P. emblica* is less effective than shown by the classical narcotic antitussive drug codeine, but more effective than the non-narcotic antitussive agent dropropizine. It is supposed that the antitussive activity of the dry extract of *P. emblica* is due to not

only its antiphlogistic, antispasmolytic and antioxidant effects, but also to its effect on mucus secretion in the airways.

8. Hepatoprotective

P. emblica extract significantly inhibited hepatocarcinogenesis induced by *N*- nitrosodiethylamine (NDEA) in a dose dependent manner. Male Wistar rats treated with NDEA alone showed 100 % tumor incidence and significantly elevated tissue levels of drug metabolizing enzymes such as glutathione S-transferase (GST) and aniline hydroxylase (AH). Treatment with *P. emblica* significantly reduced these levels. Serum levels of lipid peroxidase (LPO), alkaline phosphatase (ALP) and glutamate pyruvate transamunase (OPT), which are markers of liver injury, were also significantly reduced in the treated group. Morphology of liver tissue and levels of marker enzymes indicated that *P. emblica* extract offered protection against chemical carcinogenesis. It may be due to the scavenging of the reactive oxygen radicals from the system, as well as inhibition of the enzymes responsible for the activation of NDEA (Jeena et al., 1999).

Another study revealed the aqueous fruit extract of *P. emblica* counteracted the increased lipid peroxide levels induced by acute CCl_4 treatment and offered partial protection against increase in glutamate-pyruvate transaminase (GPT) and alkaline phosphatase (ALP) levels. Moreover, *P. emblica* extract could inhibit the induction of fibrosis due to chronic CCl_4 administration in rats, indicating that *P. emblica* have hepatoprotective activity (Jeena and Kuttan, 2000).

9. Hypolipidemic

Mathur et al. (1996) reported that feeding of *P. emblica* fresh juice at a dose of 5 ml/kg body weight per cholesterol-fed rabbit per day for 60 days leading to reduce serum cholesterol, TG, phospholipids and LDL by 82 %, 66 %, 77 % and 90 %, respectively, and the tissue lipid levels showed a significant reduction following *P. emblica* juice administration. Therefore, *P. emblica* fresh juice is an effective hypolipidemic agent and can be used as a pharmaceutical tool in hyperlipidaemic subjects.

Anila and Vijayalakshmi (2002) showed that flavonoids from *P. emblica* effectively reduce lipid levels in serum and tissues of rats with induced hyperlipidemia. The mechanism of hypolipidemic action is by the concerted action of inhibition of synthesis and enhancement of degradation.

10. Anti-Tumor and Anti-Proliferative

Jeena, Kuttan and Kuttan (2001) reported that aqueous extract of *P. emblica* could inhibit the growth of L929 cells significantly in culture and inhibit cell cycle regulating enzymes cdc 25 phosphatase in a dose dependent manner. Concentration needed for 50 % inhibition was found to be 16.5 and 5 μg/mL, respectively, and that needed for inhibition of cdc2 kinase was > 100 μg/mL. *P. emblica* extract significantly reduced solid tumors induced cell lines, Dalton's lymphoma ascites (DLA) cells while having only a moderate effect on ascites tumor. The results suggest that antitumor activity of *P. emblica* extract may particularly be due to its interaction with cell cycle regulation.

Kaur et al. (2005) studied cytotoxic effect of acetone extract of "Triphala" on cell-lines using Shionogi 115 (S115) and MCF-7 breast cancer cells and PC-3 and DU-145 prostate cancer cells as models. The three fruit, viz. *Terminalia bellerica*, *Terminalia chebula* and *Phyllanthus emblica*, which are components of "triphala" have proven to have cytotoxic effect on these cancer cell-lines. The suppression of the growth of cancer cells in cytotoxic assays may be due to the gallic acid which is the major polyphenol observed in "Triphala".

Khan et al. (2002) showed that pyrogallol, a compound present in dried fruit extract of *P. emblica*, was an active inhibitor of *in vitro* tumor cell growth of K562 human tumor cell lines. *P. emblica* extracts (5-500 ng/mL) were able to fully suppress K562 cell growth. Antiproliferative effects of pyrogallol were therefore determined on human tumor cell lines, thus identifying pyrogallol as the active component.

Lambertni et al. (2004) determined the activity of extracts from Bangladeshi medicinal pants (*P. emblica*, *Aegle marmelos*, *Vernonia anthelmintica*, *Oroxylum indicum*, *Argemone mexicana*) on human breast tumor cell lines and found that only extracts from *P. emblica* induced an increase of ERα mRNA in MCF7 cells. In these assays, *P. emblica* extracts were consistently found to exhibit the highest antiproliferative activity.

Zhang et al. (2004) investigated eighteen main compounds, including four norsesquiterpenoids and 14 phenolic compounds isolated from different parts of *P. emblica*, together with a main constituent, proanthocyanidin polymers identified from the roots, for their antiproliferative activity against MK-a (human gastric adenocarcinoma), HeLa (human uterine carcinoma), and B16F10 (murine melanoma) cells using an MTT method. The results showed that the norsesquiterpenoid glycosides phyllaemblicins B and C showed significant antiproliferative activity against these tumor cells. The antitumor activity of polyphenols might be linked to their anti-inflammatory properties and antioxidant activities.

11. Immunomodulating

Suresh and Vasudevan (1994) found that, administered orally, *P. emblica* enhanced two natural defense mechanisms including natural killer (NK) cell activity and antibody dependent cellular cytotoxicity (ADCC) *in vivo* through the induction of interferon and, thus conferred, protection on Dalton's lymphoma ascites (DLA) tumor (a transplantable murine T cell lymphoma) bearing mice by boosting the host natural immune response. The antitumor activity of *P. emblica* is mediated primarily through the ability of the drug to augment natural cell mediated cytotoxicity.

The immunomodulatory property of *P. emblica* extract was evaluated in adjuvant induced arthritis (AIA) rat model using Complete Freund's Adjuvant (CFA) induced inflammation by Ganju et al. (2003). The result showed that this fruit extract caused immunosuppression in AIA rats, indicating that it may provide an alternative approach to the treatment of arthritis.

12. Antioxidant

The two emblicanins A and B have been found to preserve erythrocytes against oxidative stress induced by asbestos, a generator of superoxide radical. Emblicanin A oxidates when put in contact with asbestos, becoming emblicanin B, and together they have a stronger protective action to erythrocytes than vitamin C (Ghosal, 1996).

In another study, an emblicanin-A and –B enriched fraction of fresh fruit juice of *P. emblica* was investigated for antioxidant activity against oxidative stress in rat heart with vitamin E as the standard antioxidant agent. The results clearly showed that the administration of *P. emblica* extract given orally twice daily for 14 days prior to the sacrifice of the animals provided a significant protection against the stressor agent induced decrease in the activities of cardiac antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, leading to a consequent decrease in lipid peroxidation. The study thus confirmed the antioxidant effect of *P. emblica* and indicates that the fruits of the plant may have a cardioprotective effect (Bhattacharya et al., 2002). This cardioprotective effect was confirmed in a study by Wattanapitayakul et al. (2005), in which the ethanolic extract of *P. emblica* (100 μg/mL) showed the highest cardioprotective effect in patients receiving doxorubicin compared with other medicinal plants tested.

Kumar and Muller (1999) evaluated methanolic extract of *P. emblica* on antioxidant activity against free radical-induced lipid peroxidation using bovine brain phospholipids liposomes as model membranes. The *P. emblica* fruit extract inhibited lipid peroxidation with IC_{50} value of 13 μg/mL.

Kumar et al. (2004) studied the radio protective effect of the fruit pulp of *P. emblica* in adult Swiss albino mice. The radiation can change the antioxidant enzyme levels in the body, leading to induce DNA strand breaks and mutation and induced peroxidative changes to lipids and proteins. *P. emblica* extract has been shown to have significant antioxidant activity, which enhanced the activity of the various antioxidant enzymes and GST as well as glutathione system in the blood.

Treatment with *P. emblica* also lowered the elevated levels of lipid peroxides in the serum. The data clearly indicated that the extract significantly reduced the bioeffects of radiation. *P. emblica* extract may be useful in reducing the side effects produced during therapeutic radiation. These data was supported by the study of Khopde et al., (2001) that examined aqueous *P. emblica* extract for its ability to inhibit γ-radiation-induced lipid peroxidation (LOP) in rat liver microsomes and inhibit the damage to antioxidant enzyme SOD.

Anilakumar, Nagaraj and Santhanam (2004) studied the protective effect of *P. emblica* on oxidative stress and toxicity in rats challenged with dimethyl hydrazine (DMH). Administration of *P. emblica* at 5 % and 10 % levels increased the hepatic GSH and reduced the conjugated dienes. 10 % *P. emblica* enhanced the catalase, glutathione peroxidase (GSH-Px) and superoxide dismutase activities in the liver and increase the hepatic ascorbic acid and glutathione (GSH) in rat treated with DMH. The activity of γ-glutamyl transpeptidase, which was increased significantly ($P < 0.001$) in kidney upon DMH injection, was reduced by 50 % on feeding of *P. emblica*. The results showed that *P. emblica* has the ability to detoxify DMH partly by enhancing the multicompartment antioxidant system in the rat.