CHAPTER 2

LITERATURE REVIEWS

2.1 Polysaccharides and their fundamental analyses

Polysaccharides are biopolymers consisting a long-chain of monosaccharides joined together by glycosidic bonds. These very large, often branched macromolecules, are generally considered to have more than ten monosaccharide residues. Their general formula is $(CH_2O)_n$ with n commonly between 200 and 2500 units [22]. They are water-soluble, generated from renewable natural sources, often biodegradable and non-toxic. Moreover, they can be produced by various living organisms such as plants, microbials, animal-derived and marine algae, and chemically synthesized from biological starting materials.

The polysaccharides from marine algae extracts have a wide range of application in food and pharmaceutical industries. Of the 15,000 varieties of seaweed, only 25 species have commercial value. Seaweed are classified into four major categories based on their pigments, namely, Cyanophyta (blue-green algae), Chlorophyta (green algae), Phaeophyta (brown algae) and Rhodophyta (red algae); only Phaeophyta and Rhodophyta are important sources of polysaccharides [23-24] (Table 2.1). Rhodophyta such as *Gelidium, Gracilaria* and *Porphyra* produce carrageenan and agar [25-27] while Phaeophyta such as *Laminaria, Sargassum* and *Fucus* produce alginates, fucoidan and laminaran [28-31].

Table 2.1 Members of polysaccharides found in Algae

Polysaccharides	Algae
Laminaran Fucoidan Alginic acid	Brown algae: *Laminaria, Sargassum* and *Fucus*
Carrageenan Agarose Agar	Red algae: *Gracilaria, Kappaphycus* and *Porphyra*
Porphyran	Red algae: *Porphyra*
Xylan	Red algae, Green algae
Ulvan	Green algae: *Ulva*

2.1.1 Fundamental analyses of polysaccharides [32]

2.1.1.1 Chemical composition analysis

The first step of characterizing a polysaccharide is the determination of the chemical composition, including total sugar contents (phenol–sulfuric acid assay), levels of uronic acid (carbazole assay), proteins (Bradford assay), ashes and moistures. Colorimetric methods are suitable for estimating the contents of total sugars, uronic acids and proteins.

2.1.1.2 Monosaccharide analysis

The determination of monosaccharide composition is the second step for polysaccharide analysis, which will reveal structural information such as the number of monosaccharides presented in the polysaccharide and each sugar unit. Monosaccharide composition is determined by analyzing monosaccharides using high performance liquid chromatography (HPLC) or gas-liquid chromatography (GC) after a complete acid hydrolysis of sugar residues.

2.1.1.3 Structural analysis of polysaccharides

The ring size and glycosidic linkage positions of sugar units in a polysaccharide is established by methylation analysis and/or cleavage reduction. Elucidation of polysaccharide structures using methylation analysis, anomeric configuration analysis by nuclear magnetic resonance (NMR) and mass spectroscopic techniques.

2.1.1.4 Physical properties of polysaccharides

2.1.1.4.1 Molecular weight determination

Molecular weight is a fundamental characteristic of polysaccharides and its determination is important in relation to many physical properties of these materials. Polysaccharides contain chains of different numbers of monosaccharide units giving a distribution of molecular weight. The statistically described molecular weight averages are in common use; Mn (number average molecular weight), Mw (weight average molecular weight), Mz (z-average molecular weight), and Mv (viscosity average molecular weight). The ratio of Mw/Mn is a polydispersity index presenting the distribution of the molecular weight. The Mw/Mn ratio is determined using fractionation techniques, such as size exclusion chromatography (SEC).

2.1.1.4.2 Thermal analysis

Some polysaccharides form gels upon cooling. Thermoreversible gels will melt if the gels are heated over the melting temperature. Differential scanning calorimetry (DSC) is the most popular technique used for characterization of polysaccharide gels. The information provided by DSC usually includes temperatures for glass transition (Tg), melting (Tm) and gelation (Ts).

2.1.1.4.3 Rheological analysis

This method is more appropriate for elucidating the structural features and gelling mechanisms of polysaccharides which determined by texture analyzer.

2.1.1.4.4 Properties of solutions and dispersions; such as

- Solubility
- Determination of intrinsic viscosity
- Shear rate dependence of viscosity
- Effects of temperature, pH and ionic strength
- Viscoelastic properties

Rodriguez *and* co-woker [33] reported the chemical composition and physical properties of the extracts of *Gracilaria gracilis* (Gracilariales, Red algae), sequential extraction with water at room temperature, 70 and 90 °C and structural analyses by methylation and ^{13}C NMR spectroscopy. The results showed that the extractions were obtained in high yield and in a good quality of agarose after extraction at 70 °C without the requirement of alkaline pretreatment, which usually produces degradation of the polysaccharide and the monosaccharide composition. The extracts showed mainly the presence of galactose and 3,6-anhydrogalactose. The chemical composition of cell wall polysaccharide extracted from the marine green algae *Ulva* (Ulvales, Chlorophyta) was determined in different polysaccharide families of the *Ulva* cell wall. It was found that three main types of polysaccharide consisted of ulvan, glucuronan and glucoxylan [34]. Regarding the sulfate polysaccharide fraction from a green algae, *Caulerpa racemosa*, it was found that the hot water extracts contained the polymer with galactose, glucose, arabinose and xylose as major sugar components. Interestingly, it also showed the antiviral

activity against HSV-1 strain F and HSV-2 strain G and TK^- acyclovir-resistant strains of HSV-1 and HSV-2. The polysaccharides from *C. racemosa* may represent an interesting alternative to be considered against the herpes virus infections [35]. Ciancia and co-workers investigated the polysaccharides from the green seaweeds *Codium fragile* and *C. vermilara*. Both seaweeds contained water-soluble sulfated arabionans and galactans, α-(1→4)-D-glucans and β-(1→4)-D-mannans, which *C. vermilara* showed a higher degree of sulfate and anticoagulant activity [36]. Strugala and co-workers reported that alginates may have a more extensive role in the treatment of reflux diseases by inhibiting pepsin, a damaging component of the refluxate [28]. Pandey and Pandey studied the antioxidant capacity of cyanobacterium *Nostochopsis lobatus* [37]. It showed a potentially high antioxidant capacity of 46.12 μM AEAC (Ascorbic acid Equivalent Antioxidant Capacity) by using Ferric Reducing Ability Power (FRAP) assay. Recently, Boonchum reported that four species of seaweed: *Sargassum binderi* Sonder, *Amphiroa* sp., *Turbinaria conoides* (J. Agardh) Küzting and *Halimeda macroloba* Decaisne from the east coast of the Gulf of Thailand were found to have antimicrobial, antifungal and anti-inflammatory activities. The aqueous extract of *T. conoides* showed no irritation when the tests were conducted on rabbit's skin. Thus, it indicated, that the *T. conoides* aqueous extract could be safe to be used in cosmeceutical or pharmaceutical products for human skin [38].

2.1.2 Classification of polysaccharides

2.1.2.1 **Classification of polysaccharides by structures** [22].

2.1.2.1.1 **Homopolysaccharides** (homoglycans), a polysaccharide with a single sugar constituent or consisting of only one type of monosaccharide unit.

2.1.2.1.2 **Heteropolysaccharides** (heteroglycans), a polysaccharide composed of more than one different type of monosaccharides in the main chain.

2.1.2.2 Classification of polysaccharides by charges [39]

2.1.2.2.1 Anionic polysaccharides

Anionic polysaccharides are negative-charged. The anionic polysaccharides which mostly interesting used in cosmetics are comprised of naturally-occurring materials.

a) Naturally-occuring anionic polysaccharides, a major compound in this group is xanthan gum. It occurs on the cell wall of bacteria and is isolated by bacterial fermentation. Xanthan gum is widely used as a thickening agent in the cosmetic industry since it provides the properties to reduce the quantity of primary emulsifiers. Other anionic polysaccharides that have been used in cosmetic are hyaluronic acid and chondroitin sulphate. Both of them possess high water-binding capacity. Due to this property, they are widely used as a moisturizer. In addition, some of the commerically well-established anionic polysaccharides are gum exudates. Arabic, karaya and tragacanthis gums and pectin are exudates isolated from citrus fruit peels, where alginic acid and carrageenan are cellular structural polysaccharide are obtained from seaweed.

b) Semi-natural anionic polysaccharides are derivative of natural polysaccharides such as cellulose gum, which is a majority used in cosmetic industry and carboxymethyl-chitin.

2.1.2.2.2 Cationic polysaccharides

Cationic polysaccharides (positively charged) consist mainly of synthetically-modified polyglycans that bind tightly to anionic surfaces of human skin and hair. Consequently, they have been widely used as film-forming and also damage-

control agents in hair conditioning and skin preparations. Due to they have the distinctive advantage to bind tightly to anionic surfaces like human skin and hair.

a) Naturally-occuring cationic polysaccharides, such as chitosan, which obtained from insect exoskeletons or crustacean. Chitosan is a less widely commercial materials used in cosmetic products.

b) Semi-natural cationic polysaccharides, such as cationic guar gum, cationic hydroxyethylcellulose (HEC), polyquaternium-10, they are a commercially used in cosmetic industry.

2.1.2.2.3 Nonionic polysaccharides

Nonionic polysaccharides are neutral and therefore less affected by negatively or positively charged compounds as surfactants. They can be divided into 2 groups:

a) Naturally-occuring nonionic polysaccharides such as starch, dextrins and guar gum. Starch is one of the most-abundant and mainly used as a thickener. Another nonionic polysaccharide is Guar gum, has found widely used as a natural thickener, stabilizer and emulsifier. Guar gum is sensitive to high acidic condition. It is able to form thick gels if the pH does not become acidic.

b) Semi-natural nonionic polysaccharides are mainly ethers of cellulose, hydroxyethylcellulose (HEC), methylcellulose (MC), hydroxypropylcellulose (HPC), hydroxypropylmethylcellulose (HPMC), nitrocellulose or hydroxypropylguar. They are used as thickeners and film-formers.

2.1.2.2.4 Amphoteric polysaccharides

Amphoteric polysaccharides have both positive and negative charges on the same molecule. There are very few natural amphoteric polysaccharides used in

cosmetics. Mostly found semi-natural amphoteric polysaccharides, as carboxymethylchitosan or hydroxydicarboxyethylchitosan, are rarely used in cosmetics. However, due to their amphoteric behavior similar to surfactants, they are challenging to formulate in cosmetic products.

2.1.2.2.5 Hydrophobic polysaccharides

Hydrophobically modified polysaccharides are of increasing interest in cosmetics. They are a water in-soluble polysaccharide modified by combining the polysaccharides with a hydrophobic substance, therefore, they become less water-loving and often unusual, thickening characteristics. The semi-natural hydrophobic polysaccharides are such as cetyl hydroxyethylcellulose.

2.2 Skin structure and functions

The skin is a key protective barrier from chemicals and environment, the skin is constantly exposed to influences that affect its health and appearance. They are consists of three main layers – the epidermis, the dermis and the hypodermis (or subcutaneous tissue) (Figure 2.1).

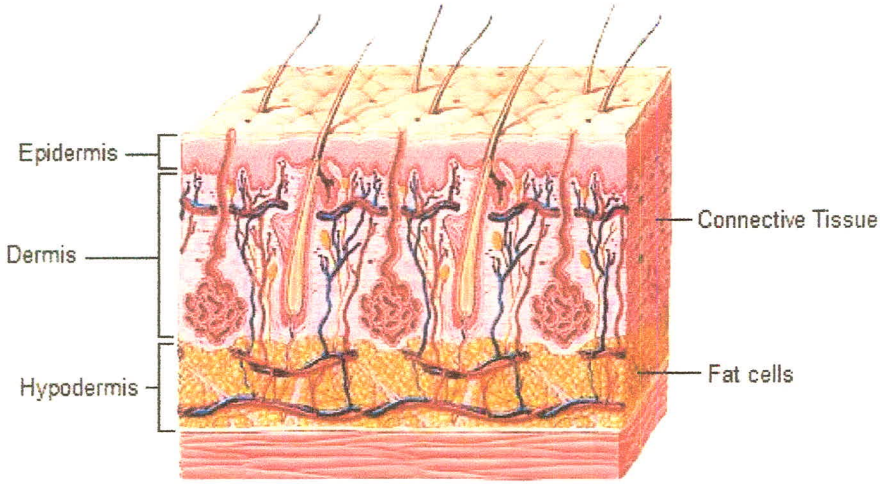


Figure 2.1 Structure and layers of skin [40]

2.2.1 Skin structure [41-45]

- **Epidermis**

The epidermis is the outer layer of the skin which serves as the physical and chemical barrier to the interior body and exterior environment. The thickness of the epidermis varies in different types of skin. It also composes of 5 stratum layers as shown in Figure 2.2.

1. **Stratum corneum** (or horny layer) is the outermost layer exposed to the environment. They are made of dead, flat skin cells that shed about every 2-3 weeks. In this layer, there are many lifeless cells called "corneocytes".

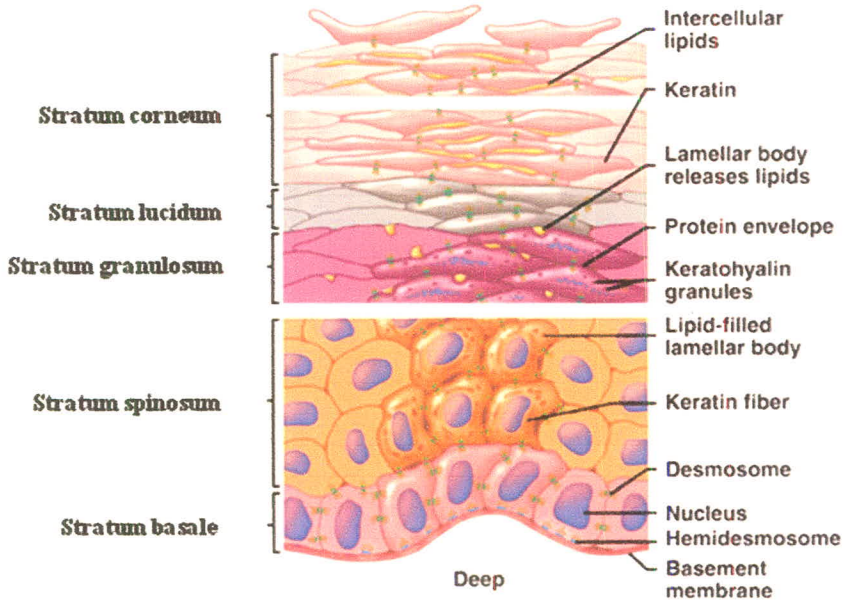


Figure 2.2 Layers of the epidermis

[Copy from http://www.rci.rutgers.edu/~uzwiak/AnatPhys/APFallLect7.html]

2. **Stratum lucidum** (or transparent layer) is a thin layer of keratin sheets (Rein's barrier) as semi-permeable barrier of aqua and minerals.

3. **Stratum granulosum** (or granular layer) is a viable layer containing enzymes (e.g. lipases, glycosidases, phosphatases) that degrade cell organelles.
4. **Stratum spinosum** is the next viable epidermal layer consisting of mature polygonal keratinocytes. The lower level of section also obtains melanin granules produced by melanocytes in basal layer.
5. **Stratum basale** (stratum germinativum or basal layer) is the only layer that is capable of cell division, mainly keratinocytes that differentiate to upper layer.

There are three types of specialized cells in the epidermis.

- Melanocyte: These cells produce a pigment called 'melanin' which gives the skin its dark color. The melanin produced by melanocytes is passed on to keratinocytes.
- Langerhans' cell: These are involved in the body's immune system.
- The Merkel's cell's function is response for cutaneous sensation.

• **Dermis**

The dermis lies beneath the epidermis. Its upper level has projections that extend into corresponding depressions in the epidermis. The dermis is composed of three types of tissue that are present throughout - not in layers. The types of tissue are collagen, elastic tissue and reticular fibers. The dermis is composed of two layers, that are the papillary and reticular layers.

- The upper, papillary layer contains a thin arrangement of collagen fibers.
- The lower, reticular layer is thicker and made of thick collagen fibers that are arranged parallel to the surface of the skin and chondroitin sulfate.

The dermis contains many specialized cells and structures such as hair follicles, sebaceous (oil) glands, sweat glands, blood vessels and nerves.

- **Hypodermis**

Hypodermis (or subcutaneous tissue) is a layer of fat and connective tissue that contains large blood vessels and nerves. This layer acts as a cushioning layer to protect vital inner organs from mechanical trauma and also as regulating of temperature of the body. The size of this layer varies throughout the body. The amount and distribution of the fat depends largely on hereditary factors and on physical activity.

2.2.2 Skin functions

The physiological functions of skin include:

2.2.2.1 Protective layer: The skin serves as a covering that protects the body.

- Protective barrier against mechanical, thermal and external injuries from the environment (sunlight, chemicals) and hazardous substances, protect bacteria from penetrating the skin and prevents loss of moisture from the body.

2.2.2.2 Transmission of sensations

- The dermis is richly supplied with nerves which transmit sensations of touch, pressure, pain and temperature from the skin.

2.2.2.3 Thermoregulation

- The body temperature is regulated by alterations in the amount of blood flowing to the skin, and in the evaporation of water or sweating.

2.2.2.4 Production of vitamin D

- Exposure to sunlight stimulates the production of vitamin D in the skin. Vitamin D is essential for the regulation of calcium levels in the body, and for the structure and growth of the bones.

2.3 Moisture in skin

The water content in the dermis and epidermis is approximately 80%. The outer skin layer, the keratinous layer, is made of dead skin cells with lower water content, approximately 10-30%. When the water content of the skin is normal, the skin appears soft, smooth, supple and glowing. In normal skin, there is a continuous movement of water from the deep layers of the skin to the superficial layers.

2.4 Dry skin

Dry skin is a very common skin condition characterized with water content less than 10% in the most superficial layer of the skin, the epidermis. Skin with normal moisture content will slough off dead cells naturally. In dry skin, the superficial layers do not peel off easily and remain attached. The accumulated keratinous cells are manifested as scales on the dry skin [45-46]. In addition, extremely dry skin, which is tough and less pliable, tends to fissure. These fissures damage the integrity and continuity of the skin and interfere with its function as a protective layer. Subsequently, there is increased water loss and the skin becomes dryer, and more fissures appear [45].

2.4.1 Potential causes of dry skin [45, 47-48]

Dry skin can result from external causes as well as from changes in the skin's ability to retain its moisture.

2.4.1.1 External causes

- Dry environment - winter, central heating and air conditioning

The environment under low humidity conditions and the air condition is cold and dry, the water in skin evaporates more quickly; this makes the skin feel dry and tight.

- Washing - frequent washing repeatedly remove the oily layer that protects the skin. Prolonged exposure to water, especially hot water - can wash away the natural oils that protect the skin.
- Sun exposure - damage from ultraviolet (UV) radiation penetrates far beyond the top layer of skin (epidermis). Sun-damaged skin may have the appearance of dry skin.
- Exposure to certain substances - detergent or substances that remove the natural oily layer from the skin surface.

2.4.1.2 The skin's ability to retain moisture

Usually, dry skin is caused by external factors but sometimes, it can be a sign of something going on internally, whether it is a natural physiological change or an illness. Aging is associated with physiological processes whereby the skin loses its ability to retain moisture. Furthermore, there are diseases in which the skin does not retain body water normally, and significant amounts of water are lost through the skin, for example, atopic dermatitis.

2.4.2 The visible and tactile characteristics of dry skin [49]

- Visible characteristics - redness, lack-luster surface, dry, white patches, flaky appearance, cracks and even fissures
- Tactile characteristics - rough and uneven

- Sensory characteristics - dry, uncomfortable, painful, itchy, stinging and tingling sensation

Dry skin is more prone to skin infections, both bacterial and fungal. The common dermatological term for extremely dry skin is "xerosis" [45].

Products used for treatment or prevention of dry skin are called moisturizers. They are able to break the dry skin cycle and maintain the smoothness of the skin.

2.5 Moisturizers

Moisturizers are used for the treatment of dry, irritated skin and to restore normal barrier function to skin, which is expected to increase skin hydration, improve skin smoothness and decrease symptoms of itching, stinging and burning [17-18, 49-51]. The water content of the skin can be improved by retarding transepidermal water loss (TEWL) while increasing the coursing of water from the dermis to the epidermis [52]. However, moisturizers are also commonly used on normal skin for cosmetic reasons or to relieve subjectively dry skin but not much attention to used on normal skin [17].

2.5.1 Principal ingredients for preserving the moisture of the skin: [53-54]

1. Occlusive ingredients

These substances produce an oily layer on the skin, enriching the skin's natural lipid film which prevents water evaporation from the skin. The keratinous layer becomes more fully saturated with water [45].

An occlusive is one of the best choices to treat dry skin because it provides an emollient effect and coat the stratum corneum to retard TEWL. Two of the best occlusive ingredients currently available are petrolatum and mineral oil. Other

commonly-used occlusive ingredients include lanolin, propylene glycol and beeswax. In addition, natural oils such as sunflower oil have been increasing in popularity. In moisturizers, occlusives are usually combined with humectant ingredients.

2. Humectant ingredients

Humectants are water-soluble materials with high water absorption capabilities. They have the capacity to attract water from the atmosphere (if atmospheric humidity is greater than 80%) and from the underlying epidermis. Humectants are also popular additives to cosmetic moisturizers because they prevent product evaporation and thickening, thereby extending the shelf life of various moisturizers. Substances that function as humectants are glycerin, honey, urea, propylene glycol, hyaluronic acid and some proteins [54-56]. Humectants may also allow the skin to feel smoother by filling holes in the stratum corneum through swelling. However, under low humidity conditions, humectants such as glycerin, will actually attract moisture from the skin and increase TEWL. Therefore, a good moisturizer should combine both occlusive and humectant properties [54-55].

3. Emollient ingredients

Emollient are frequently "oily" substances that include compounds ranging from esters to long-chain alcohols. Although emolliency does not correlate with reduction in TEWL, emollient characteristics do correlate with product preference as a smooth skin texture after application [51]. These products provide increased cohesion causing a flattening of the curled edges of the individual corneocytes. This leads to a smoother surface with less friction and greater light refraction [55]. They function by filling the spaces between desquamating corneocytes to a smooth surface [57]. Many emollients function as humectants and occlusive moisturizers as well [55].

4. Restoration of deficient materials [Natural moisturizing factor (NMF)]

A natural moisturizing factor (NMF) is essential for appropriate stratum corneum hydration, barrier homeostasis and plasticity. NMF is formed from the protein filaggrin, the hygroscopic molecules including amino acids (40%), pyrrolidone carboxylic acids (PCA) (12%), lactate (12%) and sugars, inorganic acids, peptide (8.5%), urea, inorganic salts and other element such as calcium, magnesium etc. were main constituent [54, 58-59].

The role of the NMF is to maintain adequate skin hydration of the stratum corneum, thus, it serves three major functions: (1) it maintains plasticity of the skin, protecting it from damage; (2) it allows hydrolytic enzymes to function in the process of desquamation [59-60] and (3) it contributes to optimum stratum corneum barrier function [59].

2.5.2 Action of moisturizers on the skin

Traditionally, humectants, occlusives and emollients have been, and will continue to be, the mainstay of the medical and cosmetic treatments for xerotic skin [61-62]. The most widely used and effective humectant is glycerol due to its excellent hygroscopicity, other humectants include urea and NMF components [62].

The term "moisturizer" is often used synonymously with emollient, but the term implies the addition of water to the skin. Therefore, moisturizers usually contain humectants to improve the water-binding capacity of the stratum corneum [20, 63].

Moisturizers have multifunctional effects. The desired properties include the reduction of clinical signs of dryness, like scaling and roughness, and decrease in perceived feelings of tightness and itching. Likewise the improvement of skin barrier

function is important [17]. Moisturizers are the obvious treatment for dry skin, and if used properly, are useful treatment adjuncts in inflammatory dermatoses [20].

Application of moisturizers to the skin influence tactile and visual changes in the skin surface. The type of oils used and the ratio between oil and water are both important determinants of product attributes. In addition, other ingredients such as emulsifiers, humectants and preservatives influence the initial feel of the product, its spreading behavior on the skin, whether and how fast it is absorbed and how the skin feels after its use [20].

2.5.3 Hydrating substances /moisturizing agent

1. Glycerin

Glycerin (glycerol) is a strong humectant and has a hygroscopic ability closely resembles that of NMF [55]. The levels of glycerol correlate with SC hydration levels, therefore, it allows the SC to retain high water content in a dry environment and plays an important role in skin hydration [64]. Glycerin causes an expansion of the SC because of increased thickness of the corneocytes and expanded spaces between layers of corneocytes [55].

2. Propylene glycol

Propylene glycol that functions as both humectant and occlusive, is an organic compound with formula $C_3H_8O_2$ or $HO\text{-}CH_2\text{-}CHOH\text{-}CH_3$. It is a colorless, nearly odorless, clear, viscous liquid with a faintly sweet taste, hygroscopic and miscible with water, acetone and chloroform. [55, 65].

3. Hyaluronic acid

Hyaluronic acid is a biopolymer which occurs naturally in the skin and other tissues, consisting of D-glucuronic acid (GlcA) and N-acetyl-D-glucosamin

(GlcNAc) units [9]. It is an important component of the skin matrix and is also a popular skin care ingredient often used topically [55, 66]. Hyaluronic acid is a hygroscopic sugar that can hold thousands of times its weight in water. Characteristically, hyaluronic acid is covalently-bound with various proteins in the animal tissue where the coupled polysaccharide/protein macromolecules are referred to as proteoglycans [7]. These polysaccharides were classified as glucosaminoglycans (GAGs) (amino-containing sugars). The glucuronic acid residues give these polysaccharides their primary anionic charge. Hyaluronic acid is a highly effective humectants used in moisturizing formulas and it provides effective skin surface hydration [55].

4. Mucopolysaccharides (glycosaminoglycans)

Mucopolysaccharides are long unbranched polysaccharides consisting of a repeating disaccharide unit. The repeating unit consists of an amino sugar (*N*-acetylglucose amine or *N*-acetylgalactose amine) which alternates with diverse monosaccharides. They are significant components of the connective tissue and able to retain water content in such a way that the tissue can resist to external pressure.

2.6 Free radicals

A free radical can be defined as any molecular species capable of independent existence that contains one or more unpaired electrons, and unpaired electron being one that is alone in an orbit. The simplest free radical is a hydrogen atom, with one proton and a single electron [67-68]. These unpaired electrons are usually highly reactive and it destabilizes other molecules, so radicals are likely to take part in chemical reactions. A free radical can react with both radical and non-radical

molecules in several manners and may donate its unpaired electron to a non-radical molecule or it might take an electron from another molecule in order to form a paired electron. Free radicals are generally very reactive molecules possessing an unpaired electron [69-70].

2.6.1 Sources of free radical

Free radicals can be generated by several biochemical processes of the human body including cellular metabolism (endogenous sources) and responses to the environment (exogenous sources).

2.6.1.1 Endogenous sources

Free radicals produced continuously in cells either as by-products of metabolism, including mitochondria as main sites of superoxide radical (O_2^-), are generated in an aerobic respiration. Superoxides can be produced by microsomal NADPH-dependent electron transport involving cytochrome P-450 system. Hydrogen peroxide (H_2O_2), the signal transduction molecules, can generate in Microbodies peroxisomes. Phagocytes produce nitric oxide ($NO^{\bullet}$) and hypochlorous acid (HOC1) in inflammatory conditions. Nitric oxide produce in vascular endothelium and other cells. They can be by-products of chemical reactions such as oxidation of catecholamine and activation of the arachidonic acid cascade product electrons and O_2^-, as in the Haber–Weiss reaction and the Fenton reaction, leading to the production of $^{\bullet}OH$ [71-72].

2.6.1.2 Exogenous sources

Environmental factors are known as sources for oxidative stress that can be derived from several sources, for example, pollution, traffic exhaust, cigarette smoke, metal ions, drugs, non-ionising [UV (both UVA and UVB) and infrared] and ionising forms of radiation and nutrition [73].

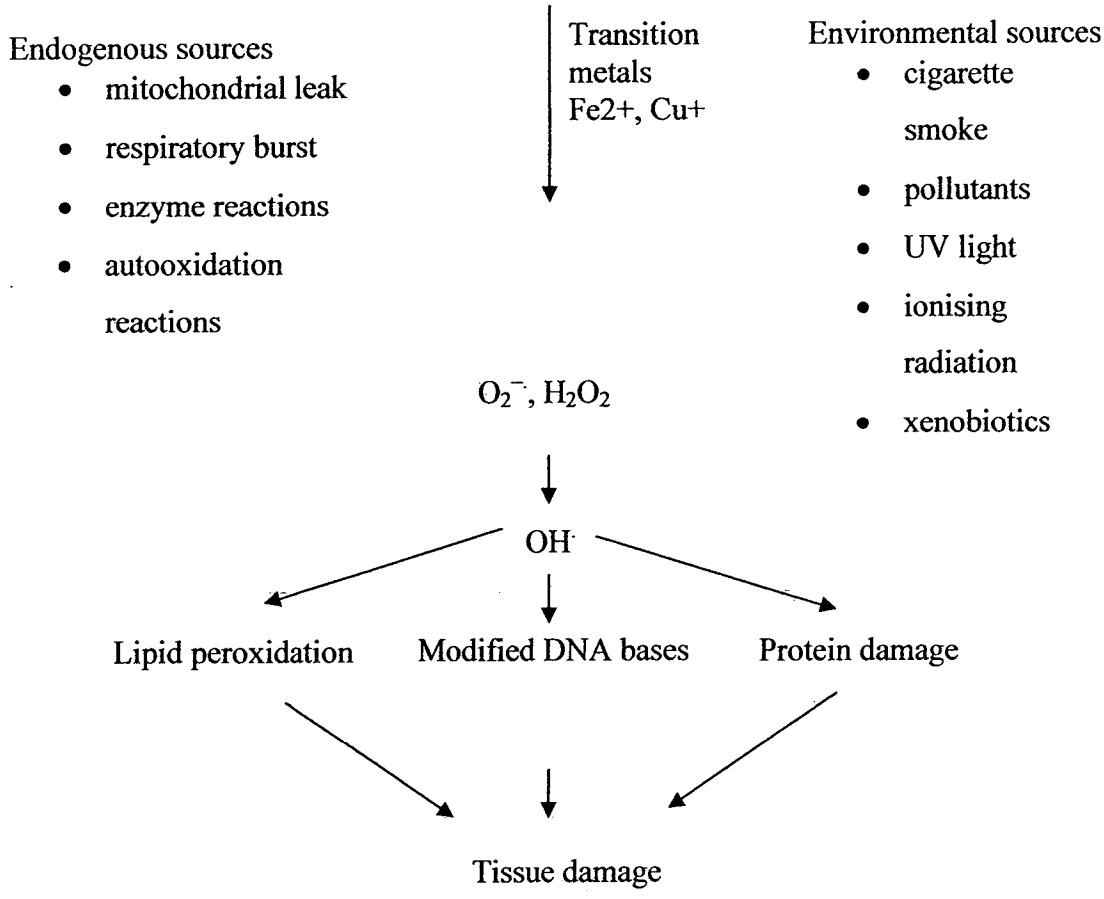


Figure 2.3 Major sources of free radicals in the body and the consequences of free radical damage [70]

The oxidative stress may be caused some human diseases. Free radicals can damage all cellular macromolecules including lipids, DNA and proteins (Figure 2.3) which oxidative stress frequently causes damage to DNA and to specific proteins and such damage may often be of more biological consequence than is damage to lipids (67). Their destructive effects on proteins may play a role in the causation of cataracts. Free radical damage to DNA is also implicated in the causation of cancer and its effect on LDL cholesterol is very likely to be responsible for heart disease. In fact, the theory associating free radicals with the aging process has also gained widespread acceptance [70].

2.6.2 Classification of free radicals

Free radicals and relative substances related in biological systems can be classified into three main groups: reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive chlorine species (RCS) [74].

Table 2.2 Types of free radicals [75-76]

Type of free radicals	Examples
1. Reactive oxygen species (ROS)	superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), hydroperoxyl radical ($HO_2^{\cdot}$), peroxyl radical ($RO_2^{\cdot}$), alkoxyl radical ($RO^{\cdot}$), carbonate anion ($CO_3^{\cdot -}$), carbon dioxide anion ($CO_2^{\cdot -}$), singlet oxygen ($1\Delta gO_2$), hypochlorous acid (HOCl)
2. Reactive nitrogen species (RNS)	nitric oxide radical ($NO^{\cdot}$), nitrogen dioxide ($NO_2^{\cdot}$, $NO_2^{\cdot -}$)
3. Reactive chlorine species (RCS)	Atomic chlorine ($Cl^{\cdot}$)

2.6.2.1 Reactive oxygen species (ROS)

ROS are mostly generated as by-products of the metabolism by several cellular oxygen metabolic pathways in living systems. ROS typically contain not only oxygen centered free radicals such as superoxide radical ($O_2^{\bullet -}$) and hydroxyl radical ($^{\bullet}OH$), but also some non-radical derivatives of oxygen peroxide (H_2O_2), singlet oxygen ($1\Delta gO_2$) and hypochlorous acid (HOCl) [67].

2.6.2.1.1 Superoxide radical (O_2^-)

Superoxide radical ($O_2^{\bullet-}$), the one-electron reduced form of molecular oxygen, is an active oxygen species and its formation is easily increased when exogenous components are present. It is a key substance because the highly toxic hydroxyl radical can be generated by the conversion of superoxide anion through hydrogen peroxide in the presence of metal ions such as copper or iron [77]. Mitochondria and microsomal are the most important reaction sources of free radicals in aerobic cells involving molecular oxygen (O_2). Approximately 1–5% of the total oxygen consumed by mitochondria is converted to ROS by partial reduction of oxygen to $O_2^{\bullet-}$ [78]. The excess of O_2 can lead to a production of $O_2^{\bullet-}$. In addition, $O_2^{\bullet-}$ play important roles in the pathogenesis of many vascular diseases [79].

Superoxide radical can generate other free radicals by enzymatically and non-enzymatically being converted into the hydrogen peroxide (H_2O_2), which is capable of highly reactive hydroxyl radical ($^{\bullet}OH$) formation by the iron-catalyzed Haber-Weiss reaction (the reaction of O_2^- production generates $^{\bullet}OH$ from H_2O_2) [80].

2.6.2.1.2 Hydroxyl radical ($^{\bullet}OH$)

Hydroxyl radical ($^{\bullet}OH$) is the most highly reactive radical. It is one of the most aggressive radicals found in the body reacting at a diffusion-controlled rate with almost every molecule in the living cell including DNA, lipids, proteins and carbohydrates [81]. $^{\bullet}OH$ are usually generated by two principal mechanisms: (1) hemolytic fission of water molecules by ionizing radiation (ultraviolet, gamma, microwave, x-ray, etc.) and (2) the breakdown of H_2O_2 with metals, including iron, copper, chromium, vanadium, etc., but by far the most common is the ferrous iron (Fe^{2+}) which is the Fenton reaction [the reaction to production of $^{\bullet}OH$ by H_2O_2

reduction of the ferrous iron (Fe^{2+}) to the ferric iron (Fe^{3+})]. The $^{\bullet}OH$ is the most powerful oxidant formed in biological systems and can readily attack any biological molecule. $^{\bullet}OH$ can attack polyunsaturated fatty acids to initiate lipid peroxidation.

2.6.2.1.3 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) is an uncharged molecule, a non-radical. H_2O_2 is directly released within DNA and mainly produced by enzymetic reactions. These enzymes are located in mitochondria, peroxisome and other cell organelles. This may lead to cell death and tissue injury in pathway programmed cell death or apoptosis and can oxidize biomolecules [82]. H_2O_2 causes damage because it is not restricted to synthesis only in the cell and it readily diffuses across biological membranes. Moreover, it can enter into various other reactions i.e. the Haber–Weiss reaction and the Fenton reaction [72].

2.6.2.2 Reactive nitrogen species (RNS)

RNS is a nitrogen component free radical. It is generated in the aerobic cellular metabolism.

2.6.2.2.1 Nitric oxide or nitrogen monoxide ($NO^{\bullet}$)

Nitric oxide or nitrogen monoxide ($NO^{\bullet}$) is highly reactive in many biological environments. $NO^{\bullet}$ can freely penetrate the lipid bilayer, can be transported within the cell and easily produced on demand via inducible enzymatic or non-enzymatic reactions [83].

At low concentrations of $NO^{\bullet}$, it is an important oxidative biological signalling on physiologic functions, such as blood pressure regulation, defence mechanisms, smooth muscle relaxation and immune system regulation [84] whereas higher concentrations of $NO^{\bullet}$ can cause cell death and tissue injury leading to impaired

cellular functions such as inflammatory respiratory tract and neurodegenerative diseases [85].

The respiratory epithelium is a major source of $NO^{\bullet}$, in which it regulates normal epithelial cell function and signalling pathways involved in the inflammation. Formation of $NO^{\bullet}$ occurs *via* oxidative reaction with metabolized amino acid arginine to citrulline by nitric oxide synthases (NOSs). NOS has three distinct isoforms, i.e., neuronal nitric oxide synthase (nNOS or NOS-1), inducible nitric oxide synthase (iNOS or NOS-2) and endothelial nitric oxide synthase (eNOS or NOS-3) [84-87]. Each isoform varies in its tissue specificity, several isoforms can be found in the same tissue but may have different functions. For example, nNOS is found in a variety of neurons in both the central and peripheral nervous system, but eNOS is expressed in some neurons and can be stimulated by shear stress in the vascular endothelium while iNOS may occur in normal epithelium such as the lung [87].

2.6.2.2.2 Peroxynitrite ($ONOO^-$)

Nitric oxide ($NO^{\bullet}$) diffuses into mitochondria and oxidant formed by the non-enzymatic reaction with O_2^- in order to produce $ONOO^-$, as a more oxidatively active molecule [71]. $ONOO^-$ is a shot-life, strong oxidant species, it reacts highly with virtually all biological molecules including proteins, lipids and nucleic acids, causing protein damage, lipid peroxidantion and DNA fragmentation [88]. $ONOO^-$ becomes protonated and produces some toxic free radicals $^{\bullet}OH$, NO_2 and nitronium ion (NO_2^+) [67].

2.6.2.3 Reactive chlorine species (RCS)

Hypochlorous acid (HOCl) is the most important RCS in biological systems, generated from H_2O_2 and chlorine (Cl) by enzyme myeloperoxidase (MPO) in

neutrophils under chronic inflammatory conditions [89]. Another reaction is an endogenous defense mechanism to limit HOCl mediation, by reacts HOCl with nitrite anion (NO_2^-) to form nitryl chloride (NO_2Cl), because NO_2Cl would have some cell damage lesser than HOCl [90]. Furthermore, HOCl may give rise to $^{\cdot}OH$ by an iron-independent reaction and/or an iron dependent reaction [91].

2.6.3 Antioxidant defenses

Antioxidants are chemical compounds which is capable of slowing or preventing the oxidation reaction promoted by free radicals. Oxidation is a chemical reaction that transfers electrons from substance to an oxidizing agent. An imbalance between antioxidant and oxidant-generating systems leads to oxidative stress that damages cells or tissues and has also been proposed in the pathogenesis of human disorders [92]. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves. Antioxidant defences systems, both endogenous and exogenous, are present to protect cellular components from free radical-induced damage. These can be divided into three main groups: (1) enzymetic antioxidant defences systems include superoxide dismutases (SOD), Catalase (CAT) and Glutathione peroxidise (GPx); (2) chain breaking antioxidants are represented by ascorbic acid (vitamin C), Glutathione (GSH), α-Tocopherols (vitamin E), carotenoids and Flavonoids, and (3) transition metal binding proteins such as transferring, ferritin and lactoferrin. Antioxidants also counterbalance the production of ROS and thus prevent the harmful effects of these oxygen intermediates on cellular nucleic acids, lipids and proteins (Figure 2.4) [93].

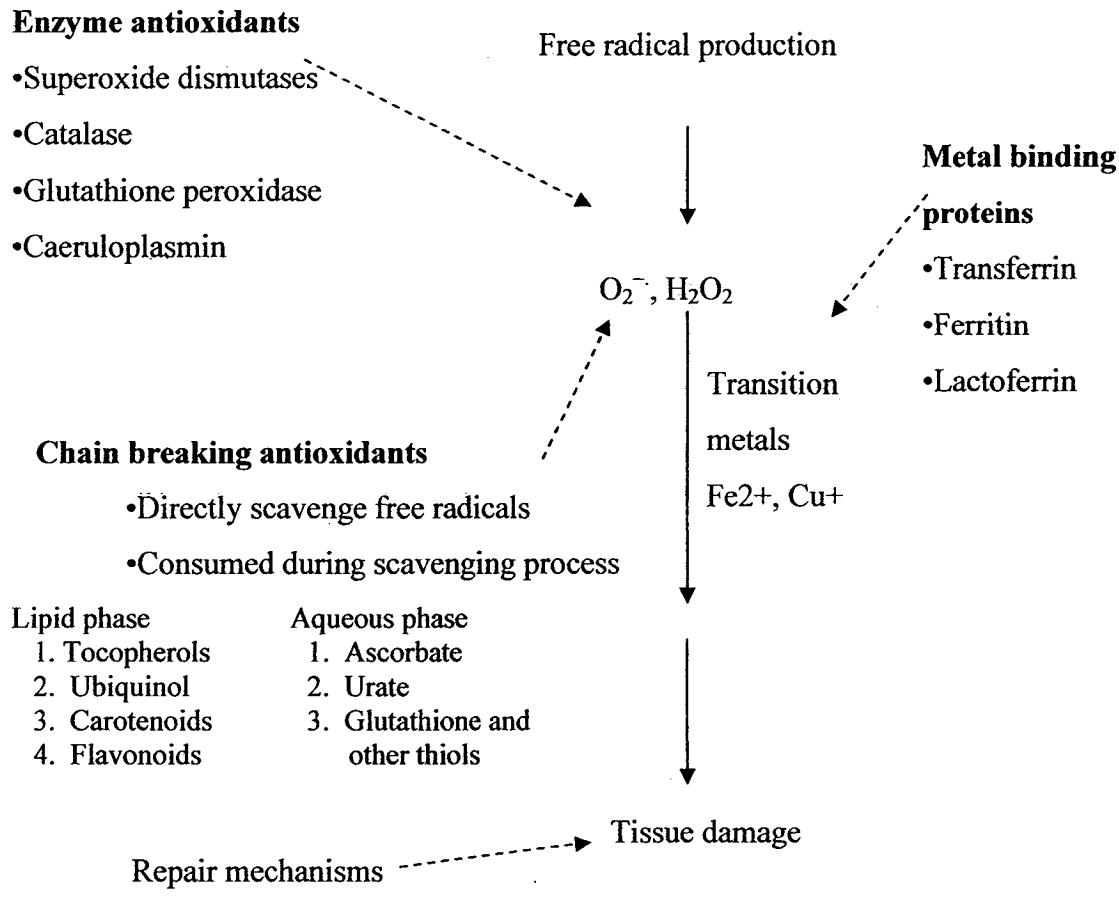
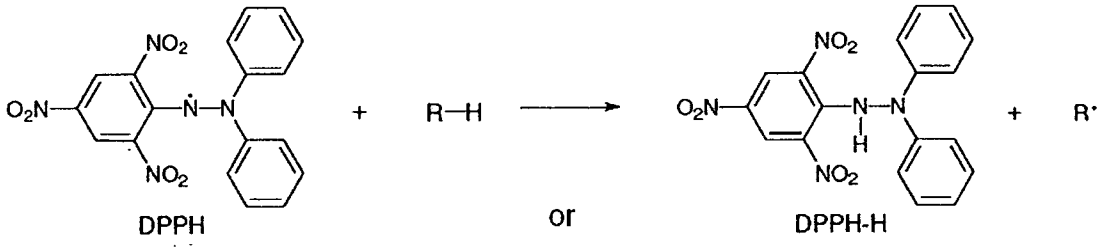


Figure 2.4 Antioxidants defenses against free radicals attack. [70]

2.6.4 *In vitro* methods to determine antioxidant activities

2.6.4.1 DPPH (2, 2-diphenyl-1-picrylhydrazyl radical) radical scavenging assay

The model of scavenging the stable DPPH radical is a widely-used method to evaluate the free radical scavenging ability of various samples [94-96]. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen-donating ability.



Purple Yellow

Figure 2.5 Reaction for DPPH radical scavenging assay [97].

DPPH is a stable free radical with deep purple color in alcoholic solution, and has a UV-vis absorption maximum approximately 515 – 520 nm [98-99]. The color of DPPH solution changes from purple to yellow as the radical is quenched by the electron donating antioxidants, leading to reduction of absorbance; the reaction progress is conveniently monitored by a spectrophotometer [100].

This method is a simple, rapid and convenient method for screening antioxidant activity of many samples, but it has some limitations. The assay can be interfered by carotenoids which also have the nearby maximum absorbance (below 530 nm). Moreover, steric hindrances may occur in large molecules accessing to the radical portion located at the center of DPPH structure [98, 101].

The DPPH assay is valid to quantify samples with hydrophilic or lipophilic antioxidants. DPPH is a stable, long-lived nitrogen radical unlike radicals present in living organisms and has no similarity to the highly-reactive and transient peroxyl radicals that are involved in lipid peroxidation [102]. Thus, antioxidants that react quickly with peroxyl radicals may react slowly or may be inert to the DPPH radical.

The limitation of the DPPH method is that some test compounds, *e.g.*, carotenoids, may have absorbance spectra that overlap with DPPH at 515 nm [103].

2.6.4.2 Trolox equivalent antioxidant capacity (TEAC) assay ($ABTS^{\bullet+}$ decolorization assay)

The TEAC assay is operationally simple and it has been used in many research laboratories for the studying of antioxidant capacity assays [104]. The oxidant $ABTS^{\bullet+}$ radical (2, 2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) is produced through a reaction between the discoloration ABTS radical and potassium persulfate in water. The reaction mixture, which is allowed to stand at room temperature for 12-16 h before use, produces a dark blue solution. Antioxidant capacity assays is measured as the ability of test compounds to decrease the color reacting directly with the $ABTS^{\bullet+}$ radical (Figure 2.5). Results of test compounds are expressed relative to trolox [105].

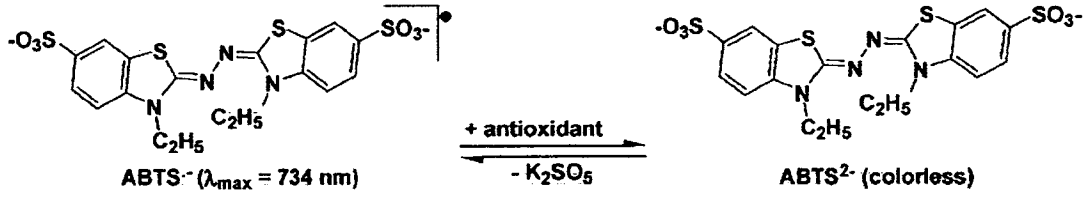


Figure 2.6 Reaction for $ABTS^{\bullet+}$ decolorization assay [102].

$ABTS^{\bullet+}$ reacts rapidly with antioxidants, typically within 30 min. It can be used over a wide pH range and can be used to study effects of pH on antioxidant mechanisms [106]. $ABTS^{\bullet+}$is soluble in aqueous and organic solvents and can be used in both hydrophilic and lipophilic antioxidant capacities of extracts [103]. The radical has a low redox potential (0.68 V) and is suitable for evaluating antioxidant capacity of

phenolics due to their comparatively lower redox potentials. Many phenolic compounds can thus react with the ABTS radical because of this thermodynamic property [106]. A limitation of this method is that the ABTS radical used in TEAC assays is not found in mammalian biology and thus represents a non-physiological radical [102].

2.6.4.3 Thiobarbituric acid-reactive substances (TBARS)

TBARS method is an assay for lipid peroxidation. Lipid peroxidation of polyunsaturated fatty acids (PUFA) generates various reactive products including malondialdehyde (MDA). MDA has been used as a marker for lipid peroxidation because of its reaction with thiobarbituric acid (TBA) to form a strongly pink colored of malondialdehyde-TBA complex:

$$\text{MDA} + 2\text{TBA} \xrightarrow{\text{acid, heat}} \underset{\text{(pink color)}}{\text{MDA-TBA}_2 \text{ complexs}}$$

Absorbance measurements at about 540 nm serve as an indicator of the extent of lipid peroxidation [101, 108]. Inhibiting lipid peroxidation by antioxidant reduces MDA generation which also influences disappear of the absorbance.

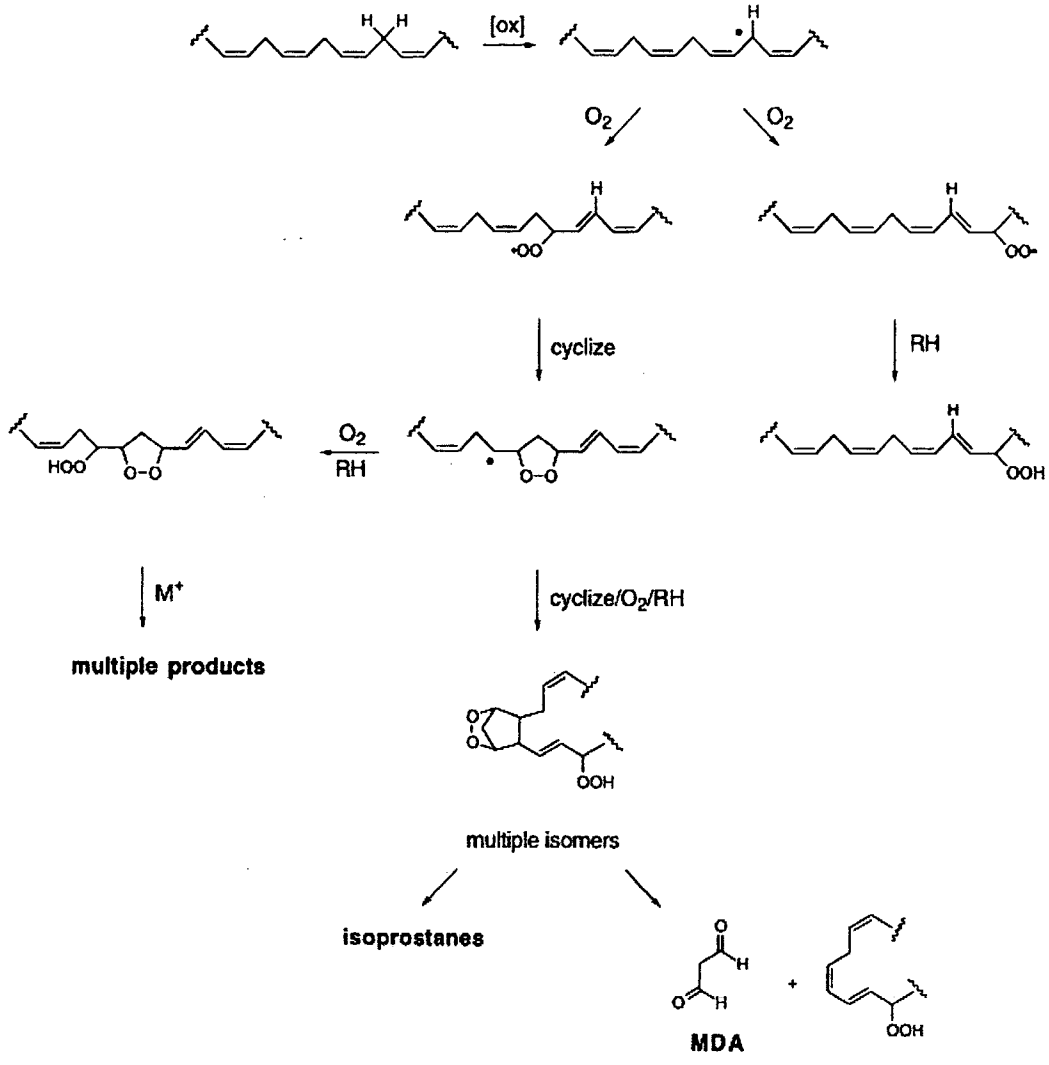


Figure 2.7 Pathways of lipid peroxidation [101]

The TBARS method is a widely acceptable assay for lipid peroxidation because it is a convenient and reliable process to confirm antioxidant activity. Although some limitations occur in this method (e.g., various degrees of saturation (amount of double bonds) in different PUFA providing the distinct TBARS inhibition rate, i.e., the higher degree of saturation shows the greater rate) [109].

2.7 Phenolic compounds

The group of phenolic compounds is the most important class of phytochemicals in plant food sources, and which are derived from the secondary metabolism of plants [110]. It represents a wide range of molecules with a molecular mass from about 100 to 3-4 kDa [111], and also denoted polyphenols, are defined as compounds possessing one or more aromatic rings bearing hydroxyl substituents [110]. Polyphenolic compounds are major group of naturally-occurring antioxidants which are found mostly in higher plants, fungi and algae and have been shown to be more effective antioxidants *in vitro* than vitamins E and C on a molar basis [112-113].

The classification of phenolic compounds is done according to carbon number, which divides the phenolic compounds into five groups [114].

1) the C_6 group comprising simple phenols and benzoquinones;

2) the C_6C_n group, which includes phenolic acid derivatives;

3) hydroxycinnamic acid derivatives, the C_6-C_n-C_6 group, which includes flavonoids (C_6-C_3-C_6);

4) the $(C_6$-$C_3)_n$, group consisting of lignans and lignins; and

5) the tannin group, which are divided into hydrolysable tannins and condensed tannins.

Phenolic hydroxyl groups are good hydrogen or electron-donating antioxidants that can against with ROS and RNS. They are chelating transition-metals involved in free-radical production and inhibiting the enzymes participating in free-radical generation. The radical scavenging of phenolic compounds can be divided into two modes. First, the one-electron reduction potentials of phenolic (phenoxyl) radicals are typically lower than those of oxygen radicals such as superoxide ($O_2^{-}\cdot$), peroxyl

(ROO·), alkoxyl (RO·) and hydroxyl (HO·) radicals, meaning that these species will readily oxidize phenolics to their respective phenoxyl radicals [115-116]. Second, phenoxyl radicals are generally less reactive than oxygen radicals [116]. Consequently, phenolic compounds can directly scavenge harmful reactive oxygen intermediates and inactivate them without promoting further oxidative reactions.

2.8 Algal materials

Algae have been consumed in Asia since ancient times, but to a much lesser extent in the rest of the world. In recent years, many algae resources have attracted attention in the search for bioactive compounds to develop applications in functional foods, supplements, cosmetics, pharmaceutical and other natural health products [117]. Edible algae are a rich source of dietary fibers, minerals and proteins [118].

Rhizoclonium hieroglyphicum (C.Agardh) Kützing is a freshwater macroalgae in the Nan River, located in Nan province. They are unbranched filamentous, cylindrical cells with thick walls and stratified, H-shaped cross wall and chloroplast parietal with a net-like appearance [119-121].

For the freshwater algae, it has been found that the macroalgae such as *Spirogyra* spp. known as "Tao" and *Nostochopsis* spp. or "Lon" are used as a traditional and medicinal food. Additionally, another freshwater macroalgae known as "Kai", consisting of 3 genera; *Cladophora* spp., *Aegagropila* spp. and *Rhizoclonium* spp., is of great interest currently. Peerapornpisal and co-worker in 2006 [1] studied the biodiversity, ecology and nutritional value of "Kai" in Nan River. It was found that "Kai" divided into 2 genera, 6 species, namely *Cladophora glomerata* Kützing, *Cladophora* sp.1, *Microspora floccosa* (Vaucher) Tharet, *Microspora pachyderma*

(Will) Lagerheim, *Microspora* sp.1 and *Microspora* sp.2. Recently, in 2012, diversity of Kai has been reassigned as 3 genera namely *Cladophora* spp., *Aegagropila* spp. and *Rhizoclonium* spp. base on morphology and nuclear ribosomal DNA sequences by Thiamdao and co-worker [2, 122]. The researcher have also found that Kai grow in the clear running water, low water temperature 15-28 °C, pH 6-8 and it is found in the clean to moderate water quality. In addition, it comtained a high protein and carbohydrate content , some vitamins especially vitamin B2, folic acid and pantothenic acid are also found along with major minerals such as calcium, sodium, magnesium and selenium [1]. Moreover, Kai also showed anti-gastric ulcer effect at %inhibition was 27.4 when tested on the restraint water immersion stress-induced ulcers in rats and feeding rat with ethanol extract 100 mg/kg and cimetidine was used as a reference drug which showed %inhibition at 72.4 and it showed an analgesic effect by inhibiting the writhing response in mice induced by acetic acid at %inhibition was 84.5 when feeding with the extract 500 mg/kg using aspirin as a reference drug (150 mg/kg; showed %inhibition=72.6) [6]. In 2009, Utmaung studied the jelly product using Kai algae (*Cladophora* spp. and *Microspora* spp.) as a raw material indicating that Kai may also have a gelling property [123].

Many researchers reported the finding of various antioxidants presented in algae [124-127]. The major groups of antioxidant compounds in marine algae are vitamins, carotenoids, phycobillin pigments sulphated polysaccharindes, phenolic compounds and polyphenols [128]. In addition, the abundant biochemical and pharmacological activities of algae, such as antipyretic activity, analgesic activity, anti-inflammatory activity [33], anti-microbial activity [33, 129], antioxidative activity [130], anti-cancer activity [131] and other activities can be obtained.

Furthermore, the polysaccharides from algae are omnipresent ingredients of cosmetics [9]. Natural bioactive compounds from algae are more acceptable than synthetic compounds as these compounds do not contain chemical contaminants, and display a variety of beneficial functions [38]. Thus, natural compounds are considered to be safe to use as ingredients in medicine, dietary supplements, nutraceuticals and cosmetics with the objective of improving consumer health, reducing the effects of harmful diseases and other broader aspects of immune system function [132].