CHAPTER II

REVIEW OF LITERATURES

1. Free radicals and antioxidants

Free radicals are chemical species, which have unpaired electrons. Molecules are composed of atoms and electrons. Electrons are present generally in pair. However, under certain condition, molecules have unpaired electrons and as such they are called free radicals. Unpaired electrons usually seek other electrons to become paired. Thus, free radicals are in general reactive and attack other molecules, although some radicals are not reactive and stable enough to have long life. Examples of reactive free radical are the hydroxyl ($HO^{\bullet}$) and alkoxyl ($LO^{\bullet}$) radicals, while the nitric oxide ($NO^{\bullet}$), vitamin E (tocopheroxyl), and vitamin C (dehydroascorbate) radicals are examples of stable radicals.

Active oxygen species (also known as reactive oxygen species) denote oxygen-containing molecules, which are more active than the triplet oxygen molecule present in air. Superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxy radical, and singlet oxygen ($^1O_2$) are accepted as typical active oxygen species, but in broader sense, other species such as alkoxyl radical, peroxyl radical ($LO_2^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), lipid hydroperoxide, and htpochlorite (HOCl) are also consider as active oxygen species. Some of them have unpaired electrons and are free radical, but other are not. Table 2.1 summrizes the active oxygen species, which are relevant to lipid peroxidation and oxidation stress *in vivo*. Nitric oxide and thiyl radical ($RS^{\bullet}$), which do not bear unpaired electrons on oxygen are also included (35).

Currently a number of radical and nonradical species derived from molecular oxygen (reactive oxygen species, ROS) nitrogen (reactive nitrogen species, RNS), and other atom are known to exist in biological systems (Table 2.1). Some are produced as part of normal metabolism (e.g., $O_2^{\bullet -}$ is formed when xanthine is converted to uric

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acid by xanthine oxidase). Others are even considered to be beneficial and play important biological role when their formation are controlled (e.g., $O_2^{\bullet-}$, hydrogen peroxide, hypochlorous, acid, and nitric oxide are the major microbicide of circulating phagocytic leucocytes and stationary cell) (36).

Table 2.1 ROS, RNS and other free radicals sometimes found in biological system

Reactive oxygen species		Reactive nitrogen species		Miscellaneous	
Hydroxyl	$HO^{\bullet}$	Free radicals			
Superoxide	$O_2^{\bullet-}$	Nitric oxide (monoxide)	$NO^{\bullet}$	Thiyl	$RS^{\bullet}$
Alkoxyl	$LO^{\bullet}$	Nitrogen dioxide	$NO_2^{\bullet}$	Hydrogen atom	$H^{\bullet}$
Hydroperoxyl	$HO_2^{\bullet}$			Carbon-centered	e.g., $CCl_3^{\bullet}$
Peroxyl	$LO_2^{\bullet}$	Non radicals		radicals	
Hydrogen peroxide	H_2O_2	Dinitrogen dioxide	N_2O_3	Thiol	RSH
Singlet oxegen	$^1\Delta GO_2$	Dinitrogen tetroxide	N_2O_4		
Lipid peroxides	LO_2H	Dinitrogen pentoxide	N_2O_5		
Ozone	O_3	Peroxynitriles	$ONO_2^{\bullet-}$		
		Alkyl peroxynitriles	$LO_2NO^{\bullet-}$		
		Nitrocarbonate	$O_2NOCO_2^{\bullet-}$		

1.1 Review of chemistry and measurement of reactive oxygen and nitrogen species

1.1.1 Reactive oxygen species (ROS)

Successive addition of electrons to molecular oxygen can give rise to several intermediate ROS culminating in the formation of water (Figure 2.1). Cytochrome oxidase, the terminal enzyme in respiratory chain, adds four electrons to oxygen fairly efficiently to from water during energy generation in the mitochondria. However, during this process ROS are also generated. Some have suggested that 1-3% of oxygen may be converted to $O_2^{\bullet-}$ in the mitochondria. Taking all $O_2^{\bullet-}$ formation together, a 70-kg man would be expected to produce about 2 kg of $O_2^{\bullet-}$ per year.

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This is a considerable amount but, judging from survival of human race, not beyond our antioxidant protective capacity. Not all ROS are equal, however, if 2 kg of the more energetic hydroxyl ($HO^{\bullet}$) free radicals was formed and depending upon their site of production, the resulting damage to macromolecule would probably overpower our protective mechanisms and lead to serious disease (36).

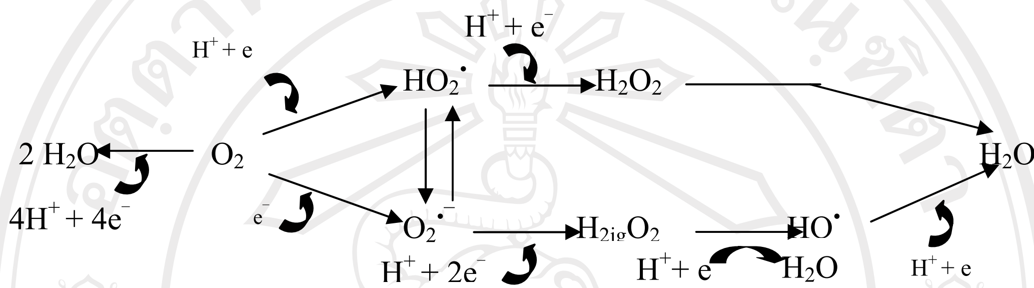

Figure 2.1 The interrelationship between oxygen and the ROS

1.1.1.1 The Hydroxyl Free Radicals

The hydroxyl free radical ($HO^{\bullet}$) 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 carbohydrate (Figure 2.2). $HO^{\bullet}$ are usually generated by two principal mechanism: homolytic fission of water molecule by ionizing radiation (ultraviolet, gamma, microwave, X-rays, etc.) or the breakdown of hydrogen peroxide with metals:

$$Metal^{n} + H_2O_2 \longrightarrow Metal^{(n+1)} + HO^{\bullet} + HO^{-}$$

Several metals can react in this way (iron, copper, chromium, vanadium, etc.), but the most common is Fe^{2+}, this reaction is referred as the Fenton reaction. In the Fenton reaction, Fe^{2+} is likely regenerate from Fe^{3+} by cellular oxidants (reducing agents) such as ascorbate. The resulting ascorbyl free radical is much less reactive and probably dissipates by dismutation into ascorbic acid and dehydroascorbic acid. And second similar reaction, the Haber-Weiss reaction (or $O_2^{\bullet -}$ Fenton reaction), has

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$O_2^{\bullet -}$ reacting with Fe^{3+} to generate Fe^{2+} (and O_2), thereby permitting the Fenton reaction to proceed more effectively. As discuss later, $HO^{\bullet}$ also can be produced from peroxynitrous acid and from the reaction between $O_2^{\bullet}$ and hypochlorous acid.

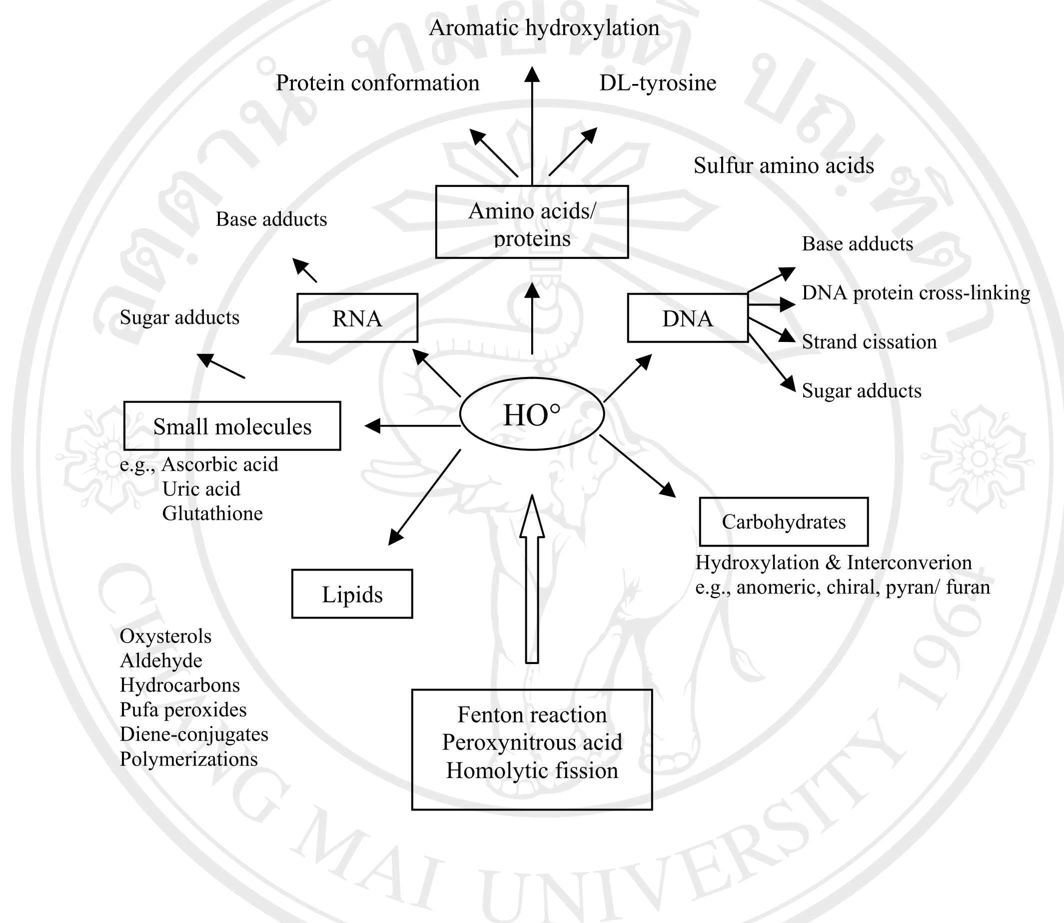

Figure 2.2 The attack of $HO^{\bullet}$ on a variety of biological important molecules produces a great diversity of reaction products

1.1.1.2 The superoxide free radical anion

The addition of one electron to oxygen yields the superoxide free radical anion ($O_2^{\bullet -}$) (Figure 2.1). $O_2^{\bullet -}$ is produced by exposing oxygen to ionizing radiation, as part of normal metabolism (e.g., from the catabolism of xanthine to uric acid catalyzed by xanthine oxidase as well as other flavoprotein oxidase such as aldehyde oxidase) and is deliberately produced during the respiratory burst of phagocytic cell, forming part of the body's defense system for the destruction of the invading organisms. Traces of $O_2^{\bullet -}$ are also formed when oxygen combines with hemoglobin

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and myoglobin and, as discussed earlier, a considerable quantity of $O_2^{\bullet -}$ is accidentally formed in the mitochondria when oxygen is reduced to water.

1.1.1.3 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is another ROS found in biological systems is produced by several enzymatic reactions, including those catalyzed by SOD, d-amino acid oxidase, amine oxidase, glycolate oxidase, and urate oxidase. H_2O_2 appears to play role not only in phagocytic defended by immune system but also in metabolic pathways such as the production of thyroxine in the thyroid gland.

1.1.2 Reactive nitrogen species (RNS)

Due to its electronic structure nitrogen is polyvalent and can from a wide variety of compounds of biological relevance. Of particular interest are the oxides of nitrogen, which show complex chemical interactions and interconversion. A simplified version of this chemistry is presented in Figure 2.3. This section examines the biological importance and analysis of the RNS with particular emphasis on nitric oxide ($NO^{\bullet}$), peroxynitrite anion (ONO_2^-), nitrosoperoxycarbonate anion ($ONO_2CO_2^-$), and nitrocarbonate anion ($O_2NOCO_2^-$).

1.1.2.1 Nitric oxide

Nitric oxide or nitrogen monoxide is one of the nitrogen oxides gases with chemical formula NO. This gas is an important signaling molecule in the body of mammals, including humans, and is an extremely important intermediate in the chemical industry. It is also an air pollutant produced by cigarette smoke, automobile engines and power plants.

NO is an important messenger molecule involved in many physiological and pathological processes within the mammalian body both beneficial and detrimental. Appropriate levels of NO production are important in protecting an organ such as the liver from ischemic damage. However sustained levels of NO production result in direct tissue toxicity and contribute to the vascular collapse associated with septic shock, whereas chronic expression of NO is associated with various carcinomas and

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inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis.

Nitric oxide should not be confused with nitrous oxide (N_2O), a general anaesthetic and greenhouse gas, or with nitrogen dioxide (NO_2) which is another air pollutant. The nitric oxide molecule is a free radical, which is relevant to understanding its high reactivity.

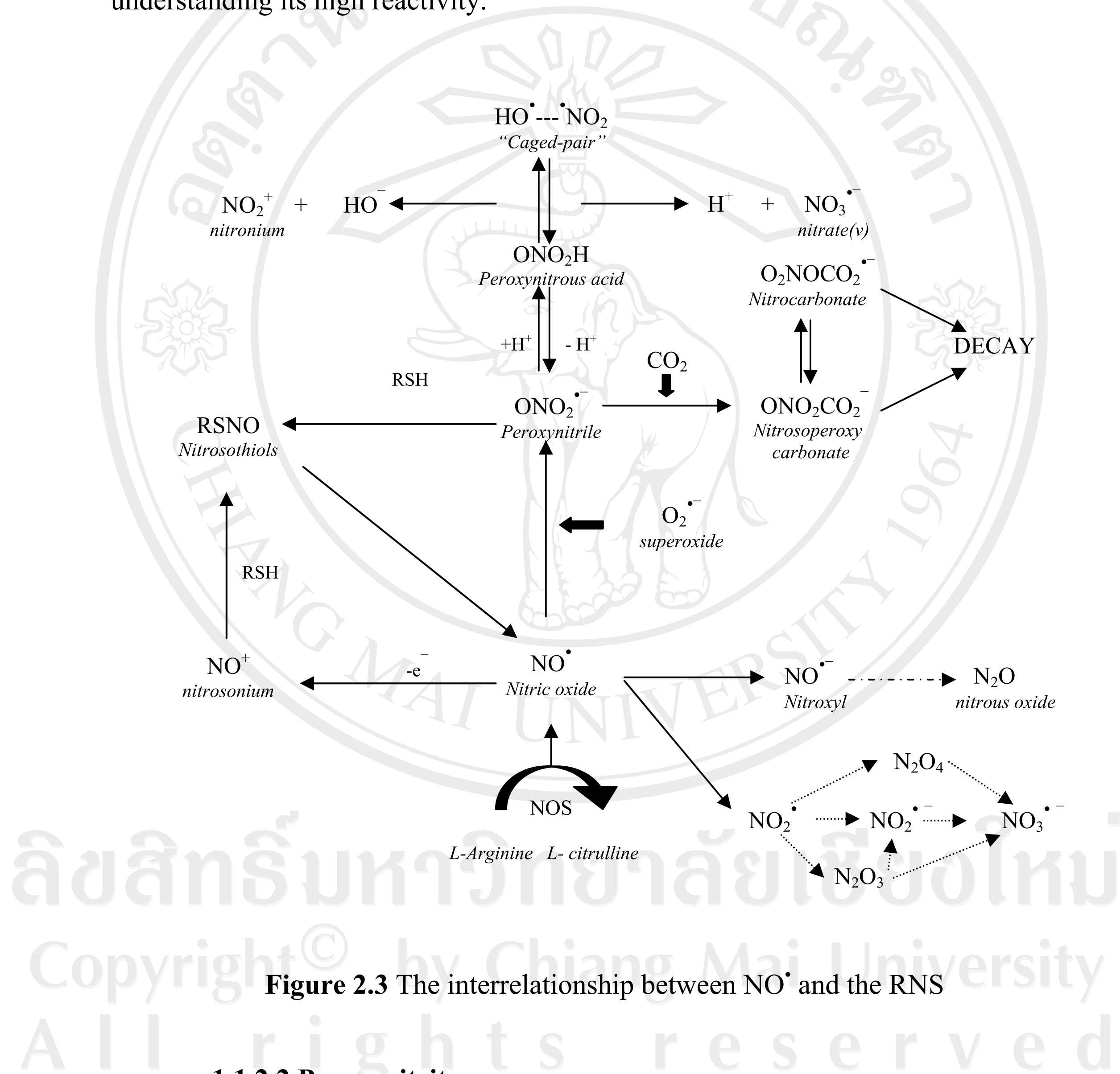

Figure 2.3 The interrelationship between $NO^{\bullet}$ and the RNS

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1.1.2.2 Peroxynitrite

Peroxynitrite (ONO_2^-) is readily formed from the reaction between $NO^{\bullet}$ and $O_2^{\bullet-}$. ONO_2^- and ONO_2H attack many biological important compounds. Examples include the oxidation of nonprotein and protein sulfhydryls, sulfides, deoxyribose,

lipid, ascorbate, the nitration of guanine and the formatiom of carbonyls and other oxidation products in proteins.

1.1.2.3 Nitrocabonate anion

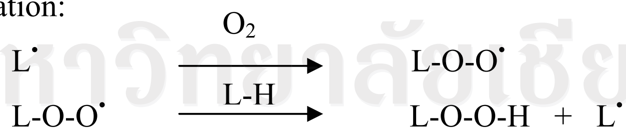
Peroxynitrite (ONO_2^-) rapidly reacts with carbon dioxide to from the unstable nitrosocarboxycarbonate anion ($ONOOCO_2^-$), which quickly rearranges to form the nitrocarbonate anion ($O_2NOCO_2^-$). $ONO_2CO_2^-$ may serve two important biological functions (37). First, it acts as a scavenger of ONO_2^- and, due to its instability, will limit the site of action of ONO_2^-. Second, it may be superior microbicide to H_2O_2, as it will not be deactivated by microbial catalase (38).

1.2 Chain reaction

Many, though certainly not all, radical reactions are chain processes, meaning that one "initiating" event will produce many product molecules. The process normally involves three stages: initiation, propagation, and termination. Any process affording the necessary starting radicals can be an initiating step, while any process destroying the species in the chain can be a terminating step.

Chain Initiation:

Chain propagation:



Chain termination:

Figure 2.4 Sequence describing lipid peroxidation, L represents a lipid and hydrogen being lost generally is allylic, i.e., C=C-C-H.

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Steps other than those shown in Figure 2.4 are possible, in cluding the conversion of the hydroperoxide LO_2H to an alkoxyl radical, $LO^{\bullet}$, and its subsequent abstraction of a hydrogen atom from L-H to form an alcohol (LOH) and a new $L^{\bullet}$ radical. This particular sequence illustrates “chain branching” and ultimately leads to a new radical sesquence and a molecule of conversion to $L\text{-}O\text{-}O^{\bullet}$. While chain termination may involve coupling of various radicals to form several stable species, such as L-L, L-O-O-L, and L-O-L, it is common to show the initiating radical (e.g., $L^{\bullet}$) undergoing dimerization (36).

1.3 Oxidative stress and the principles of protection

Aerobic metabolism entails the production of reactive oxygen species, even under basal conditions, hence there is a continuous requirement for inactivation of these reactive oxygen species. The steady-state of prooxidants and antioxidants may be disrupted. A disbalance in favor of the prooxidants and disfavoring the antioxidants, potentially leading to damage, has been called ‘oxidative stress’. Oxidative stress is a term used to denote the imbalance between the concentrations of reactive oxygen and nitrogen species and the defense mechanisms of the body (39). Such damage may afflict all types of biological molecules, including DNA, lipids, proteins and carbohydrates. Thus, oxidative stress may be involved in processes such as mutagenesis, carcinogenesis, membrane damage, lipid peroxidation, protein oxidation and fragmentation, as well as carbohydrate damage.

In principle, protection against such deleterious effects can be by prevention, interception and repair. All these forms of protection are realized in biology, and examples will be given below. In order to lay out the variety of problems afflicting protective measures, the nature of the prooxidants and antioxidants will frst be presented (40).

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1.4 Antioxidants

Aerobic organisms are protected from oxidative stress insuced by free radicals and active oxygen species by an array of defense systems. As summarized in Table 2.2, various kides of antioxidants with different functions play an important role

in these defense systems. The preventive antioxidants acting in the first defense line suppress the formation of free radicals and active oxygen species. The radical scavenging antioxidants are responsible in the second defense line and inhibit chain initiation and/or break the chain propagation. The antioxidant enzymes such as phospholipases, proteases, DNA repair enzymes, and transferases act as the third line defense. In addition, the appropriate antioxidant is generated and transferred to the right site at the right time and at the right concentration when the oxidative stress takes place. This adaptation mechanism is also important in the total defense system.

With increasing experimental, clinical, and epidemiological evidence which shows the involvement of free radicals and active oxygen species in a variety of diseases, cancer, and aging, the role of antioxidants has received increasing attention. For example, recent epidemiological studies showed that high intake of coronary heart disease and that low levels of vitamin E seem to correlate with an increased incidence of myocardial infarction. Furthermore, probucol, a synthetic radical-scavenging antioxidant, is widely used as a drug in the treatment of hypercholesterolemia and atherosclerosis (35).

Table 2.2 Defense systems *in vitro* against oxidative damage

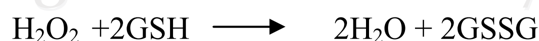
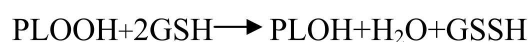
1. Preventive antioxidants: suppress the formation of free radical.	
(a) Non-radical decomposition of hydroperoxides and hydrogen peroxide.	
Catalase	decomposition of hydrogen peroxide. $2H_2O_2 \longrightarrow 2H_2O + O_2$
Glutathione peroxidase (cellular)	decomposition of hydrogen peroxide and fatty acid hydroperoxide.  $H_2O_2 + 2GSH \longrightarrow 2H_2O + 2GSSG$ $LOOH + 2GSH \longrightarrow LOH + H_2O + GSSG$
Glutathione peroxidase (plasma)	decomposition of hydrogen peroxide and phospholipids hydroperoxides.  $PLOOH + 2GSH \longrightarrow PLOH + H_2O + GSSH$

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Table 2.2 Defense systems *in vitro* against oxidative damage (Continue)

Phospholipids hydroperoxide glutathione peroxidase	decomposition of phospholipids hydroperoxide.
Peroxide	decomposition of hydrogen peroxide and lipid hydroperoxide. $OOH + AH_2 \longrightarrow LOH + H_2O + A$ $O_2 + AH_2 \longrightarrow 2H_2O + A$
Glutathione-S-tranferase	decomposition of lipid hydroperoxides.
(b) Sequestration of metal by chelation.	
tranferrin, lactoferrin	sequestration of iron.
haptoglobin	sequestration of hemoglobin.
hemopexin	stabilization of heme.
ceruloplasmin, albumin	sequestration of copper.
(c) Quenching of active oxygens.	
Superoxide dismutase (SOD)	disproportionation of superoxide. $2O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + O_2$
Carotenoids	quenching of singlet oxygen.

2. Radical-scavenging antioxidant: scavenge radical to inhibit chain initiation and break chain propagation.

Hydrophilic: vitamin C, uric acid, bilirubin, albumin.

Lipophilic: vitamin E, ubiquinol, carotenoids.

3. Repair and *de novo* enzymes: repair the damage and reconstitute membranes

lipase, protease, DNA repair enzymes and transferase.

4. Adaptation: generate and transfer them appropriate antioxidant enzymes to the right site at the right concentration.

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1.5 Machaism of Action of Antioxidants

The role and action of antioxidants will be reviewed briefly using lipid peroxidation as an example. Lipid peroxidation can be inhibited by suppressing chain inititio and chain propagation and/or by enhancing chain termination. Metals often play an important role in radical generation. For this reason, proteins such as ferritin and ceruloplasmin, which sequester metal ions are also important antioxidants. Hydroperoxide and hydrogen peroxide are precursors of oxygen radicals and, hence, peroxidases such as glutathione peroxidase (GPX) also act as antioxidants. Superoxide dismutase (SOD) acts as an antioxidant by dismutating superoxide to triplet oxygen and hydrogen peroxide. Various carotenoids may act as quenchers of singlet oxygen.

The chain propagation can be stoped quite efficiently by eliminating oxygen. Thus, foods are often stored *in vitro* or under nitrogen. Aerobic organisms, however, need oxygen. The lipid radical reacts with oxygen quite rapidly to give lipid peroxyl radical, which continues the chain propagation reaction. This chain reaction can be terminated by scavenging the chaincarrying lipid peroxyl radical. Vitamins C and E, uric acid, bilirubin, and ubiquinol, a reduced form of coenzyme Q, are important radical-scavenging antioxidants. These antioxidants must scavenge the radical rapidly before it attacks the lipid. Therefore, the more reactive the compound is toward the radical, the more potent it is as an antioxidant. This reactivity is, however, not the only factor that determines the antioxidant potency. It is also determined by many other factors such as the type and location of the radical, site of antioxidant, concentration and mobility of the antioxidant at the microenvironment, fate of antioxidant-derived radical, and interaction with other antioxidants. These factors will be discussed briefly for vitamin E (35).

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1.6 Type of antioxidants

Antioxidants can be defined as compounds that inhibit or delay, but do not completely prevent, oxidation. There are two basic categories of antioxidant, namely synthetic and natural.

1.6.1 Synthetic antioxidants

Some of the more popular synthetic antioxidants used are phenolic compounds such as butylated hydroxyanisole (BHA), butylated hydroxy-toluene (BHT), tertiary butylhydroquinone (TBHQ), and esters of gallic acid, e.g. propyl gallate (PG). The synthetic antioxidants have been very thoroughly tested for their toxicological behaviours, but some of them are coming, after a long period of use, under heavy pressure as new toxicologocal data impose some caution in their use. In this context, natural products appear as healthier and safer than synthetic antioxidants. Since about 1980 natural antioxidants have appeared as an alternative to synthetic antioxidants (41).

1.6.2 Natural antioxidants

The empirical use of natural compounds as antioxidants is very old. Natural antioxidants can be phenolic compounds, quinines, or lactones, as well as polyphenolics. Natural antioxidants found in many sources such as plants, microorganisms and even animal tissue. Phenolic compounds are also the majority group of natural antioxidants and the three important groups of antioxidant are tocopherol, flavonoids and phenolic acid. Natural antioxidants have been wildly used as alternative antioxidants since the synthetic antioxidants had new toxicology data impose caution in their use. Some natural antioxidants are more potent, efficiency and safer than synthetic antioxidants (41-42).

1.7 Assay methods for antioxidants

Several methods are used to measure the antioxidant activity of a biological material. The most commonly used ones are those involving chromogen compounds of radical nature that stimulate the reductive oxygen species. These methods are popular due to their ease, speed and sensitivity. The presence of antioxidants leads to the disappearance of these radical chromogens; the most widely used ones being the ABTS and DPPH methods. Some other commonly used assays like FRAP assay, ORAC assay are mentioned below.

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1.7.1 Trolox Equivalent Antioxidant Capacity Assay

The Trolox equivalent antioxidant capacity (TEAC) assay was first reported by Miller, Rice-Evans, Davies, Gopinathan, and Milner (1993) and Rice-Evans and Miller (1994) and then modified by Re *et al*. (1999). The TEAC assay is based on the

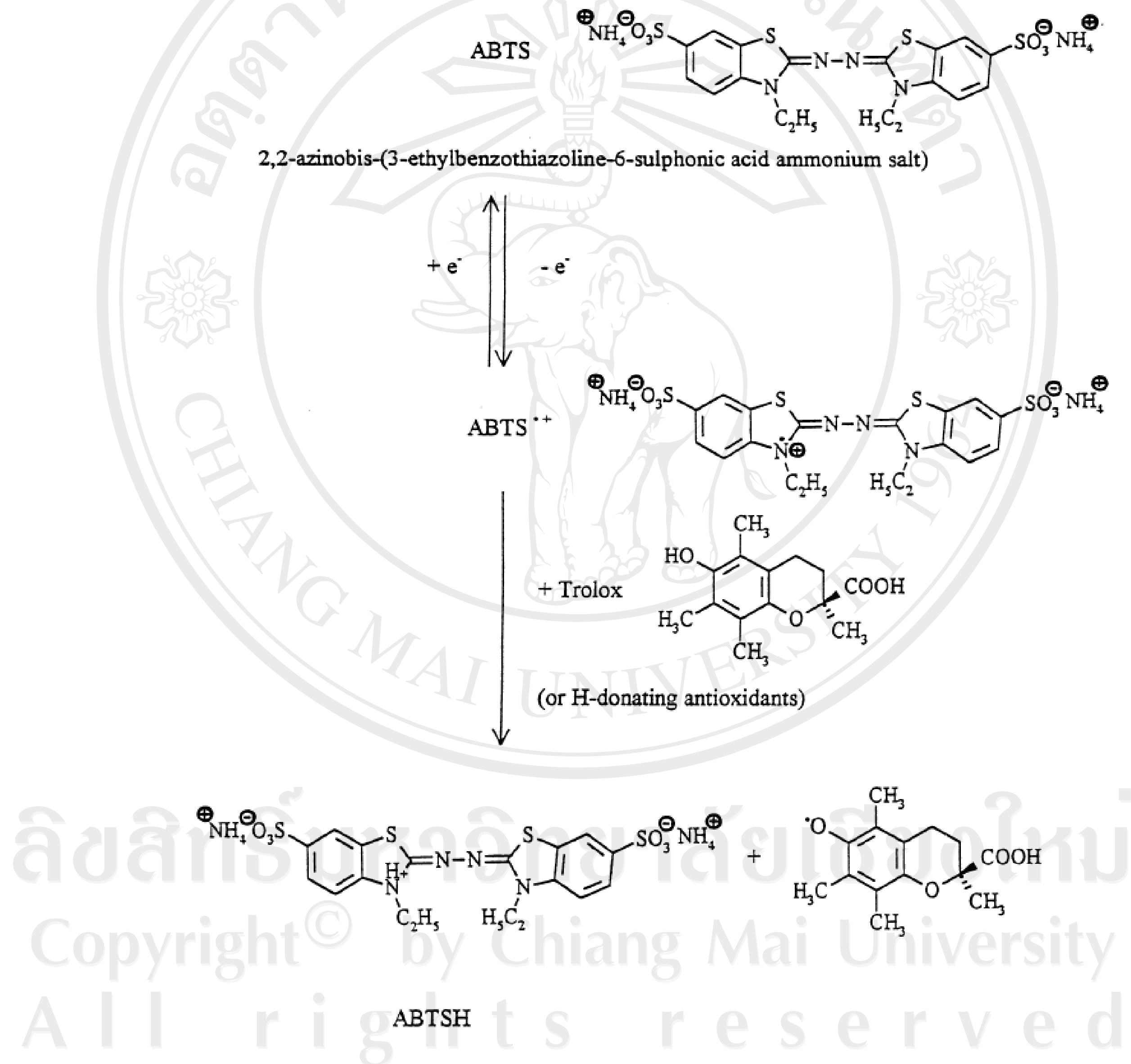

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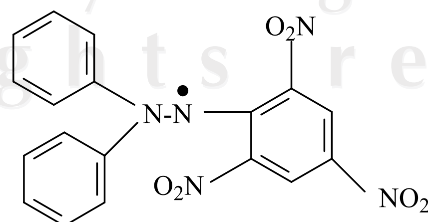
Figure 2.5 The assay for measuring TEAC (Trolox equivalent antioxidant capacity)

Inhibition by antioxidants of the absorbance of the radical cation of 2,2'-azinobis (3-thylbenzothiazoline 6-sulfonate) (ABTS), which has a characteristic long-wavelength absorption spectrum showing maxima at 734 nm. Generation of the ABTS radical cation forms the basis of one of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of various substances. The experiments are carried out using a decolorization assay, which involves the generation of the ABTS chromophore by the oxidation of ABTS with potassium persulphate. It is applicable to both hydrophilic and lipophilic compounds. The assay has been widely used in many recent studies related to detection of antioxidant property of plant (43-44).

1.7.2 2,2-Diphenyl-1-picrylhydrazyl Radical Scavenging Capacity Assay

This method was given by Brand-Williams, Cuvelier, and Berset (1995) and later modified by Sanchez-Moreno, Larrauri, and Saura-Calixto (1998). It is one of the most extensively used antioxidant assay for plant samples. This method is based on scavenging of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) from the antioxidants, which produces a decrease in absorbance at 515 nm. When a solution of DPPH is mixed with a substance that can donate a hydrogen atom, the reduced form of the radical is generated accompanied by loss of colour. This delocalization is also responsible for the deep violet colour, characterized by an absorption band in ethanol solution at about 520 nm (43-45).

Representing the DPPH radical by Z* and the donor molecule by AH, the primary reaction is:



DPPH

Figure 2.6 DPPH structure

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1.7.3 Ferric Ion Reducing Antioxidant Power Assay

Ferric reducing ability of plasma (FRAP) assay is a technique to determine the total antioxidant power interpreted as the reducing capability. The FRAP assay also takes advantage of electron-transfer reactions. Herein a ferric salt, Fe(III)$(TPTZ)_2Cl_3$ (TPTZ) 2,4,6-tripyridyl-triazine), is used as an oxidant. The oxidant in the FRAP assay is prepared by mixing TPTZ (2.5 mL, 10 mM in 40 mM HCl), 25 mL of acetate buffer, and 2.5 mL of $FeCl_3$•H_2O (20 mM). The conglomerate is referred to as "FRAP reagent". The final solution contain Fe(III) of 1.67 mM and TPTZ of 0.83 mM. Therefore, the TPTZ is deficient as the ideal reaction stoichiometry between Fe(III) and TPTZ is 1 to 2. The oxidant is not just Fe(III)$(TPTZ)_2$, it also contains other Fe(III) species which can lead to potential problems as many metal chelators in food extract could bind Fe(III) and form complexes that are also capable of reacting with antioxidants. To measure FRAP value, 300 μL of freshly prepared FRAP reagent is warmed to 37 °C and a reagent blank reading is taken at 593 nm; then 10 μL of sample and 30 μL of water are added. Absorbance readings are taken after 0.5 s and every 15 s until 4 min. The change of absorbance ($\Delta A = A_{4min} - A_{0min}$) is calculated and related to ΔA of an Fe(II) standard solution. ΔA is linearly proportional to the concentration of antioxidant (43-44).

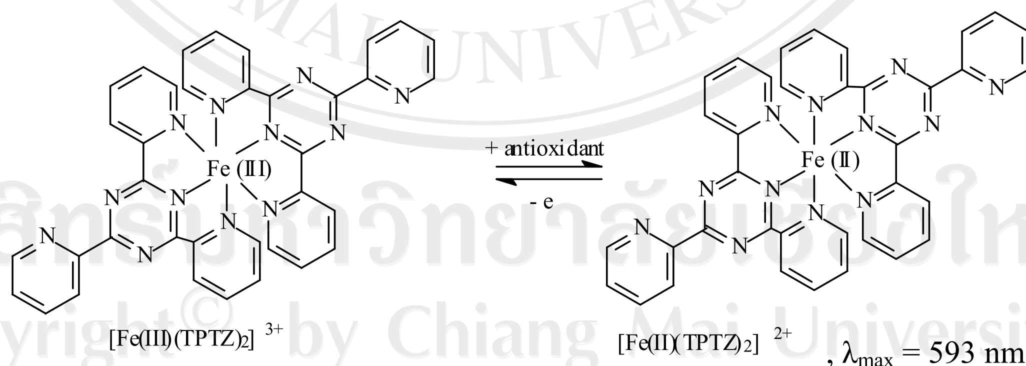

Figure 2.7 Ferric reducing ability of plasma (FRAP) assay

1.7.4 The oxygen radical absorbance capacity assay

Originally developed by Cutler and Cao, the first version of the ORAC assay employed B-phycoerythrin (B-PE, a fluorescent protein) as the probe. The fluorescence decay of B-PE is an indication of damage from its reaction with the

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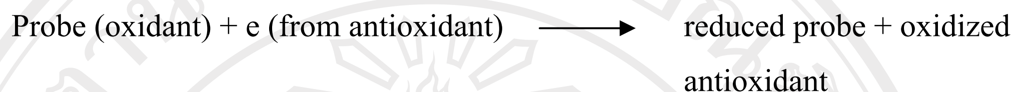
peroxyl radical. Later, Ou and co-workers found that B-PE suffered several disadvantages: (1) μ-PE, a protein product isolated from *Porphyridium cruentum*, has a large lot-to-lot variability; (2) B-PE is photobleached under plate-reader conditions; and (3) B-PE interacts with polyphenols due to the nonspecific protein binding and loses fluorescence even without added radical generator.



Probe (oxidant) + e (from antioxidant) ⟶ reduced probe + oxidized antioxidant

Figure 2.8 The oxygen radical absorbance capacity assay

The detailed procedures of the high-throughput ORAC assay operating on a 96-well plate fluorescence reader are described by Huang *et al*. In general, samples, controls, and standard (Trolox of four or five different concentrations for construction of a standard curve) are mixed with fluorescein solution and incubated at constant temperature (37 °C) before AAPH solution is added to initiate the reaction. The fluorescence emission intensity at 525 nm, exciting at 485 nm (ex) is measured every minute for 35 min at ambient conditions (pH 7.4, 37 °C). A typical ORAC assay kinetic curve is shown in Figure 2.9.

Obtained from the ORAC assay is achieved by (1) calculating of the area under the kinetic curve (AUC) and net AUC (AUCsample - AUCblank), (2) obtaining a standard curve by plotting the concentration of Trolox and the AUC (linear or uadratic fit between 0.78 and 12.6 μM Trolox), and (3) calculating the Trolox equivalents of a sample using the standard curve. These steps can be performed automatically on an Excel or similar data processing program.

It should be noted that in some cases, antioxidant samples exhibited different curves of concentration versus AUC from that of Trolox standard. Forcing the AUC of the sample to the standard equation of Trolox would lead to scattered ORAC values of the sample. This is normally due to sample matrix interference, which is amplified when the antioxidant activity of a sample is low and a large concentration of samples is needed in order to give measurable AUC.

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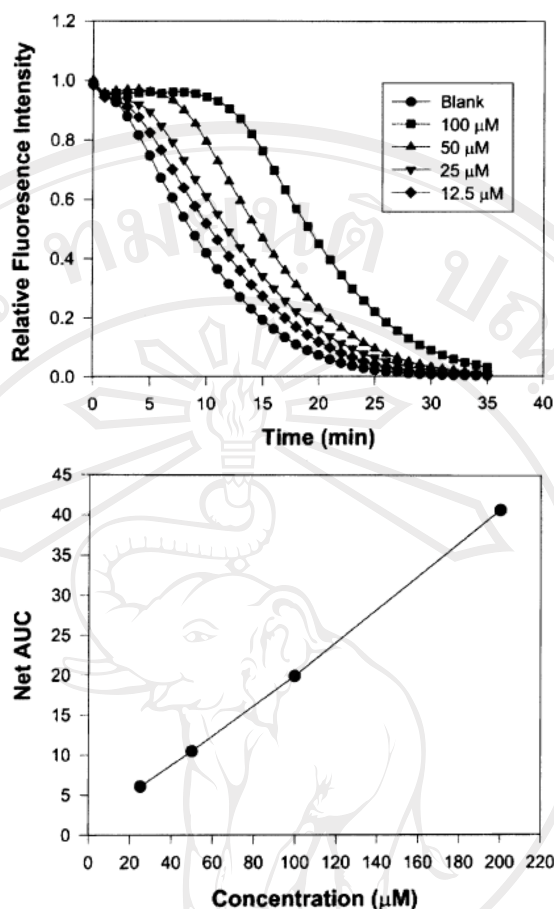

Figure 2.9 (**a**) Fluorescence decay curve of fluorescein in the presence of R-tocopherol and AAPH. (**b**) Linear plot of the net AUC versus R-tocopherol concentration.

The advantages of the AUC approach are that it applies equally well for both antioxidants that exhibit distinct lag phases and those samples that have no lag phases. This approach unifies the lag time method and initial rate method, and it is particularly useful for food samples, which often contain multiple ingredients and have complex reaction kinetics. There is a direct linear correlation of AUC and a broad range of sample types, including raw fruit and vegetable extracts, plasma, and pure phytochemicals. Therefore, the ORAC assay has been broadly applied in academics and the food and supplement industry as a method of choice to quantify antioxidant capacity. In fact, an antioxidant database has been generated applying the ORAC assay in combination with the total phenols assay.

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Many antioxidants are lipophilic, and it is also known that the antioxidant capacity of a compound is dependent upon reaction media. Therefore, an organic solvent based ORAC assay would be particularly useful for lipophilic samples. However, fluorescein is not sufficiently lipid soluble, and its fluorescence intensity in nonpolar organic solvent is rather low. To overcome this problem, Naguib applied 4,4-difluoro-3,5-bis(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene (BO-DIPY 665/676) as a fluorescent probe and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) as a peroxyl radical generator. The reaction could be carried out either in liposome or on an octane and butyronitrile mixture. By applying this assay, the antioxidant capacity of various carotenoids was quantified. However, this assay is 100 times less sensitive than the ORAC assay, probably due to the low efficiency of the radical generator, AMVN. In addition, the fluorescent quenching mechanism of BODIPY by peroxy radical remains to be investigated (43-44).

1.7.5 Thiobarbituric Acid Reactive Substances assay

The thiobarbituric acid (TBA) assay is the most popular and easiest method used as an indicator of lipid peroxidation and free radical activity in biological sample. The assay is based upon the reaction of TBA with malondialdehyde (MDA), one of the aldehyde products of lipid peroxidation. There are numerous variations but basically the sample is heated with TBA under acidic conditions and the amount of pink-coloured MDA-TBA adduct produced is measured at 532 nm. For increased sensitivity the complex can be extracted into an organic sovent such as butanol and measured fluorimetrically. In a few experimental systems the TBA test has been demonstrated actually to be measuring MDA itself. In uncharacterized systems it is usual to refer to the assay of TBA-reactive substances (TBARS) as the test is not specific for MDA (46-47).

The sensitivity of measuring Thiobarbituric Acid Reactive Substances (TBARS) has made this assay the method of choice for screening and monitoring lipid peroxidation, a major indicator of oxidative stress. This rapid, easy-to-use procedure has been modified by researchers for use with many types of samples including drugs, food products and human and animal biological tissues. The assay

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has provided important information regarding free radical activity in disease states and has been used for measurement of anti-oxidant activity of several compounds. Although much controversy has appeared in the literature regarding the specificity of TBARS toward compounds other than MDA, it remains the most widely employed assay used to determine lipid peroxidation. If lipoprotein fractions are first acid precipitated from the sample, interfering soluble TBARS are minimized.

Malondialdehyde (MDA) form a 1:2 adduct with thiobarbituric acid and produces the following:

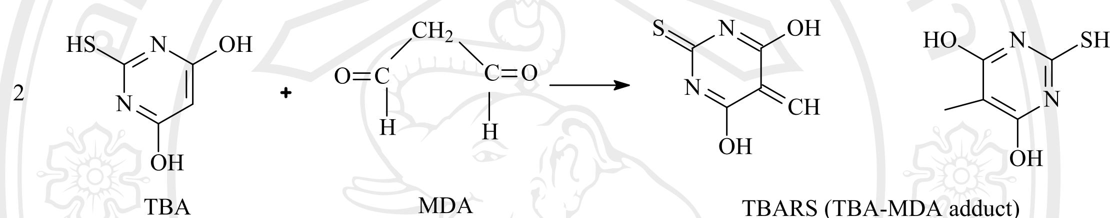

Figure 2.10 Thiobarbituric Acid Reactive Substances assay

This can be measured by fluorometry or spectrophotometry. Although this reaction has a much higher sensitivity when measured via fluorometry, protocols for both methods are provided in the Test Procedure section of this insert. Biological specimens contain a mixture of thiobarbituric acid reactive substances (TBARS), including lipid hydroperoxides and aldehydes, which increase as a result of oxidative stress. TBARS return to normal levels over time, depending upon the presence of anti-oxidants. In practice, TBARS are expressed in terms of malondialdehyde (MDA) equivalents. In this assay, an MDA standard is used to construct a standard curve against which unknown samples can be plotted. The TBARS Assay Kit provides all the necessary reagents to perform 160 tests and is designed for researchers studying oxidative stress and anti-oxidant activity. It is recommended that in-house controls be run with each sample test. Depending on geographic location, normal plasma and serum TBARS should be <1.5 and <2.0 MDA units respectively. Mean and ± SD or SE must be established by each laboratory. It is recommended that samples be run in duplicate.

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1. Hemolyzed, icteric or grossly lipemic plasma samples are not suitable for use in TBARS analysis.

2. Non-lipid TBARS may be present in the sample. It is recommended that a sample with elevated TBARS levels be tested by a more specific test for lipid peroxidation such as HPLC.

3. Normal tissues contain very low levels of free malondialdehyde.

2. Cancer

Cancer (medical term: malignant neoplasm) is a class of diseases in which a group of cells display *uncontrolled growth* (division beyond the normal limits), *invasion* (intrusion on and destruction of adjacent tissues), and sometimes *metastasis* (spread to other locations in the body via lymph or blood). These three malignant properties of cancers differentiate them from benign tumors, which are self-limited, and do not invade or metastasize. Most cancers form a tumor but some, like leukemia, do not. The branch of medicine concerned with the study, diagnosis, treatment, and prevention of cancer is oncology.

Nearly all cancers are caused by abnormalities in the genetic material of the transformed cells. These abnormalities may be due to the effects of carcinogens, such as tobacco smoke, radiation, chemicals, or infections agents. Other cancer-promoting genetic abnormalities may be randomly acquired through errors in DNA replication, or are inherited, and thus present in all cells from birth. The heritability of cancers is usually affected by complex interactions between carcinogens and the host's genome. New aspects of the genetics of cancer pathogenesis, such as DNA methylation, and microRNAs are increasingly recognized as important.

Genetic abnormalities found in cancer typically affect two general classes of genes. Cancer-promoting *oncogenes* are typically activated in cancer cells, giving those cells new properties, such as hyperactive growth and division, protection against programmed cell death, loss of respect for normal tissue boundaries, and the ability to become established in diverse tissue environments. *Tumor suppressor genes* are then inactivated in cancer cells, resulting in the loss of normal functions in those cells, such

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as accurate DNA replication, control over the cell cycle, orientation and adhesion within tissues, and interaction with protective cells of the immune system.

Diagnosis usually requires the histologic examination of a tissue biopsy specimen by a pathologist, although the initial indication of malignancy can be symptoms or radiographic imaging abnormalities. Most cancers can be treated and some cured, depending on the specific type, location, and stage. Once diagnosed, cancer is usually treated with a combination of surgery, chemotherapy and radiotherapy. As research develops, treatments are becoming more specific for different varieties of cancer. There has been significant progress in the development of targeted therapy drugs that act specifically on detectable molecular abnormalities in certain tumors, and which minimize damage to normal cells. The prognosis of cancer patients is most influenced by the type of cancer, as well as the stage, or extent of the disease. In addition, histologic grading and the presence of specific molecular markers can also be useful in establishing prognosis, as well as in determining individual treatments (48-50).

3. Bacteria

The bacteria are a large group of unicellular microorganisms. Typically a few micrometres in length, bacteria have a wide range of shapes, ranging from spheres to rods and spirals. Bacteria are ubiquitous in every habitat on Earth, growing in soil, acidic hot springs, radioactive waste, water, and deep in the Earth's crust, as well as in organic matter and the live bodies of plants and animals. There are typically 40 million bacterial cells in a gram of soil and a million bacterial cells in a millilitre of fresh water; in all, there are approximately five nonillion (5×10^{30}) bacteria on Earth, forming much of the world's biomass. Bacteria are vital in recycling nutrients, with many steps in nutrient cycles depending on these organisms, such as the fixation of nitrogen from the atmosphere and putrefaction. However, most bacteria have not been characterized, and only about half of the phyla of bacteria have species that can be grown in the laboratory. The study of bacteria is known as bacteriology, a branch of microbiology.

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There are approximately ten times as many bacterial cells in the human flora of bacteria as there are human cells in the body, with large numbers of bacteria on the skin and as gus flora. The vast majority of the bacteria in the body are rendered harmless by the protective effects of the immune system, and a few are beneficial. However, a few species of bacteria are pathogenic and cause infections diseases, including cholera, syphilis, anthrax, leprosy and bubonic plague. The most common fatal bacterial diseases are respiratory infections, with tuberculosis alone killing about 2 million people a year, mostly in sub-Saharan Africa. In developed countries, antibiotics are used to treat bacterial infections and in agriculture, so antibiotic resistance is becoming common. In industry, bacteria are important in sewage treatment, the production of cheese and yoghurt through fermentation, as well as in biotechnology, and the manufacture of antibiotics and other chemicals.

Once regarded as plants constituting the class Schizomycetes, bacteria are now classified as prokaryotes. Unlike cells of animals and other eukaryotes, bacterial cells do not contain a nucleus and rarely harbour membrane-bound organelles. Although the term *bacteria* traditionally included all prokaryotes, the scientific classification changed after the discovery in the 1990s that prokaryotes consist of two very different groups of organisms that evolved independently from an ancient common ancestor. These evolutionary domains are called Bacteria and Archaea (51-53).

4. Fungi

A fungus is any member of a large group of eukaryotic organisms that includes microorganisms such as yeasts and molds, as well as the more familiar mushrooms. The Fungi are classified as a kingdom that is separate from plants and animals. One major difference is that fungal cells have cell walls that contain chitin, unlike the cell walls of plants, which contain cellulose. These and other differences show that the fungi form a single group of related organisms, named the *Eumycota* (*true fungi* or *Eumycetes*), that share a common ancestor (a *monophyletic group*). This fungal group is distinct from the structurally similar slime molds (myxomycetes) and water molds (oomycetes). The discipline of biology devoted to the study of fungi is

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known as mycology, which is often regarded as a branch of botany, even though genetic studies have shown that fungi are more closely related to animals than to plants.

Abundant worldwide, most fungi are inconspicuous because of the small size of their structures, and their cryptic lifestyles in soil, on dead matter, and as symbionts of plants, animals, or other fungi. They may become noticeable when fruiting, either as mushrooms or molds. Fungi perform an essential role in the decomposition of organic matter and have fundamental roles in nutrient cycling and exchange. They have long been used as a direct source of food, such as mushrooms and truffles, as a leavening agent for bread, and in fermentation of various food products, such as wine, beer, and soy sauce. Since the 1940s, fungi have been used for the production of antibiotics, and more recently, various enzymes produced by fungi are used industrially and in detergents. Fungi are also used as biological agents to control weeds and pests. Many species produce bioactive compounds called mycotoxins, such as alkaloids and polyketides that are toxic to animals including humans. The fruiting structures of a few species contain psychotropic compounds and are consumed recreationally or in traditional ceremonies. Fungi can break down manufactured materials and buildings, and become significant pathogens of humans and other animals. Losses of crops due to fungal diseases (e.g. rice blast disease) or food spoilage can have a large impact on human food supplies and local economies.

The fungus kingdom encompasses an enormous diversity of taxa with varied ecologies, life cycle strategies, and morphologies ranging from single-celled aquatic chytrids to large mushrooms. However, little is known of the true biodiversity of Kingdom Fungi, which has been estimated at around 1.5 million species, with about 5% of these having been formally classified. Ever since the pioneering 18th and 19th century taxonomical works of Carl Linnaeus, Christian Hendrik Persoon, and Elias Magnus Fries, fungi have been classified according to their morphology (e.g., characteristics such as spore color or microscopic features) or physiology. Advances in molecular genetics have opened the way for DNA analysis to be incorporated into taxonomy, which has sometimes challenged the historical groupings based on morphology and other traits. Phylogenetic studies published in the last decade have

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helped reshape the classification of Kingdom Fungi, which is divided into one subkingdom, seven phyla, and ten subphyla (54-56).

5. Review of Chemical Constituents of *C. hystrix* Leaves

Previously reported chemical constituents isolated from *C. hystrix* leaves were 1,2-di-O-α-linolenoyl-3-O-β-galactopyranosyl-sn-glycerol (DLGG) (inhibitors of tumor promoter-induced Epstein-Barr virus (EBV) activation), 1-O-α-Palmitoyl-2-O-linolenoyl-3-O-β-galactopyranosyl-sn-glycerol (LPGG) (inhibitors of tumor promoter-induced Epstein-Barr virus (EBV) activation), β-Sitosterol and 3-[O-α- ga-lactopyranosyl-(1" → 6')-O-β-galactopyranosyl]-1-O-linolenyl-2-O-palmitylglyceride (57-59).

The constituents found in the volatile oil of *C. hystrix* leaves were citronellal**,** linalool, citronellol as major compounds and camphene, β-pinene, sabinene, citronellyl acetate, geranyl acetate, δ-cadiene as minor compounds (60-65).

6. Review of Chemical Constituents of *F. limonia* Leaves

The reported chemical constituents isolated from *F. limonia* leaves in the literature were β-amyrin, lupeol, psoralen, bergapten, xanthotoxin, umbelliferone, marmesin, imperatorin, isopimpinellin, stigmasterol, β-sitosterol, orientin, vitexin and saponarin (66-70).

The major compounds found in the volatile oil of *F. limonia* leaves were estragole, methylchavicol, thymol and *trans*-anethol. The minor compounds were *p*-cymen-7-ol, fenchone, isoeugenol, anisaldehyde and myristicine (71-73).

7. Review of Chemical Constituents of *A. marmelos* Leaves

The reported chemical constituents isolated from *A. marmelos* leaves in the literature were cardenolide (protecting the doxorubicin induced cardiotoxicity and lipid peroxidn. (LPO) in rats), periplogenin (protecting the doxorubicin induced cardiotoxicity and lipid peroxidn. (LPO) in rats), anhydromarmeline (β-glucosidase inhibitors), anhydroaegeline (β-glucosidase inhibitors), aegelinosides A (β-glucosidase inhibitors), aegelinosides B (β-glucosidase inhibitors), Aegeline 2

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(antihyperglycemic activity), *N*-2-[4-(3′, 3′-dimethylallyloxy)phenyl] ethyl cinnamide, *N*-2-hydroxy-2-[4-(3′,3′-dimethylallyloxy) phenyl] ethyl cinnamide, *N*-2-hydroxy-2-[4-(3′,3′-dimethylallyloxy) phenyl] ethyl cinnamide, *N*-4-methoxystyryl cinnamide and *N*-2-hydroxy-2-(4-hydroxyphenyl)ethyl cinnamide, β-Sitosterol, Aegelin, Shahidine (Gram-positive antibacteria), 24-epibrassinolide (antigenotoxic) and marmenol (74-77).

The major compounds found in the volatile oil of *A. marmelos* leaves were sylvestrene, limonene, α-phellandrene, β-phellandrene, *trans*-ocimene and α-cadinene. The minor constituents were β-caryophyllene, α-copaene, α-humulene, α-pinene, sabinene, menthol, 3-carene, germacrene D and epi-cubebol (78-83).

8. Review of Chemical Constituents of *C. aurantifolia* Leaves

The reported chemical constituents isolated from *C. aurantifolia* in the literature were apigenin-6,8-di-C-β-D-glucopyranoside (vicenin II), diosmetin-6,8-di-C-β-D-glucopyranoside, apigenin-8-C-β-D-glucopyranoside (vitexin), apigenin-8-C-[α-L-arabinopyranosyl-(1→6)]-O-β-D-glucopyranoside, apigenin-6-C-[α-L-arabino-pyranosyl-(1→6)]-O-β-D-glucopyranoside and apigenin-6-C-β-D-glucopyranoside (isovitexin) (84).

The major compounds found in the volatile oil were d-dihydrocarvone, d-limonene, α-terpineol, *m*-mentha-6,8-diene R(+) and the minor compounds were α-pinene, 2,3-dehydro-1,8-cineole, camphene, *p*-cymene, *m*-cymene, β-cis-ocimene, trans-*p*-mentha-2,8dienol, β-linalool, fenchol, *p*-menth-8-en-1-ol, d-verbenone, myrtenol, γ-terpineol, verbenone, δ-elemene, neryl acetate, caryophyllene and trans-α-bergamotene (85).

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