CHAPTER I
INTRODUCTION

1.1 Statement and significance of the problem

Mainstream medical science now regards oxidation as a primary cause of degeneration and aging. Oxidation, the slow deterioration of matter as a result of chemical reactions involving oxygen, is a familiar phenomenon found throughout nature. The rusting of metals, the spoilage of foods, the rancidification of oils and the crumbling of rubber are examples of the common process of oxidation.

In our body, oxidative reactions of many kinds occur due to exposure to environmental toxins. In the air we breathe and the water we drink, we are exposed to many chemical toxins of different kinds, including cigarette smoke, car exhaust, vapors cleaning fluids and solvents. In addition, we are subjected to a variety of chemical food additives such as preservatives, artificial colors, flavors emulsifiers, lubricants, bleaching agents, flavor enhancers, synthetic sweeteners and even drugs. Many of these agents contribute to oxidative reactions in the body (1-2).

Oxidative reactions also occur as result of metabolism, the process by which nutrients are broken down used by the body for energy, growth and repair. Like many biological processes, metabolism offers a system of tradeoffs. On the hand metabolism is essential to life. On the other hand, metabolism generates toxic waste products and gives rise to reactive oxygen species (ROS) (2-3).

Reactive oxygen species include the superoxide anion, hydrogen peroxide, hydroxyl radicals and quinones, and come under the title of free radicals. Free radicals cause damage to DNA, lipid and protein, damaging membranes, altering genes and injuring cells. Free radicals cause the generation of more free radicals. Left unchecked, free radicals cause accelerated tissue damage, which is a major contributing factor to the rate and severity of aging. Free radicals are also implicated in the progression of specific degenerative diseases, including, atherosclerosis, cancer, cystic fibrosis,

diabetes, rheumatoid arthritis, and neurodegenerative condition including Alzheimer's and Parkinson's diseases (3-8).

Fortunately, there are nutritional agents that help to prevent against oxidative damage in the body by getting rid of free radicals, and further help to repair damage that has already occurred. Dietary antioxidant factors include nutrients such as tocopherol (vitamin E) and ascorbate (vitamin C), as well as numerous carotenoids and a number of phenols and flavonoids found in common foods (9-12). Dietary fruits and vegetables are the primary sources of dietary antioxidants. Antioxidants are generally available in foods, however, it is difficult to get antioxidant enough to prevent the damage caused by free radicals resulting from air and water pollution and other environmental factors.

While all antioxidants prevent or minimize oxidative damage, not all antioxidants are created equally. Also various antioxidants individually possess additional protective properties that vary one from another. The substantial differences in the structures and biological activities of various antioxidant account for capacity to prevent or mitigate specific diseases.

The screening tests of previous study (13) had searched for novel and high antioxidant activity from several kinds and different parts of plants and mostly found that high amount of antioxidant capacity was obtained from seeds. Particularly, in this study, the great value of antioxidant activity was found in tamarind's seed rather than others, and that is similar to the previous report of Tsuda and colleagues, 1993 (14).

For this reason, the study has been conducted to study the biochemical properties of natural antioxidants isolated from tamarind seed and to find out whether its potent antioxidant activity could be useful for health.

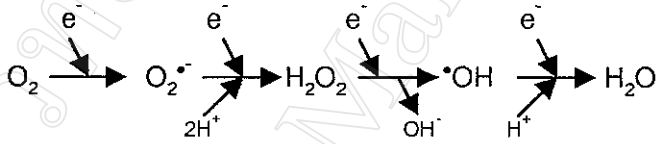
1.2 Literature review

1.2.1 The radical nature of oxygen

Oxidative stress is an inevitable result of life in an oxygen-rich environment. For aerobic organisms oxygen is paradoxically both vital for existence and inherently dangerous. The oxygen paradox derives from the chemical nature of oxygen itself which

in atomic form (O) exists as a free radical, and in molecular form (O_2) is a bi-radical. The outer valence shell of atomic oxygen contains one unpaired electron. When two oxygen atom combine to form molecular oxygen, the outer valence shell electrons do not spin-pair but remain as two unpaired electron. A radical is defined as any atom or molecule with one or more unpaired electron in outer valence shell. Therefore, molecular oxygen is a true bi-radical (1,3).

The radical nature of molecular oxygen permits some very interesting oxidation/reduction chemistry (3). In a non-enzymatic, univalent, reduction pathway oxygen can undergo four successive one-electron reduction pathway as shown in Figure 1. Unlike the concerted four-electron reduction of oxygen catalyzed by the cytochrome oxidase at the end of mitochondrial electron transport chain, the non-enzymatic univalent pathway for oxygen reduction results in the generation of several reactive intermediates.



$O_2^{\bullet -}$ = superoxide

H_2O_2 = hydrogen peroxide

$^{\bullet}OH$ = hydroxyl radical

Figure 1. The univalent pathway for oxygen reduction

The first one-electron reduction of oxygen generates the superoxide anion radical ($O_2^{\bullet -}$) which is generally referred to simply as superoxide. Addition of second electron and two protons (to $O_2^{\bullet -}$) generates the active species hydrogen peroxide (H_2O_2). A third electron addition produces the highly reactive hydroxyl radical($^{\bullet}OH$) and release a hydroxide ion (OH^-). A fourth electron addition generates a water molecule.

1.2.2 Sources of reactive oxygen species (ROS)

The toxic consequences of exposure to ionizing radiation result, almost entirely, from cellular damage caused by reactive oxygen species (ROS), such as hydroxyl radicals and singlet oxygen. Ionizing radiation, or other kinds of high energy radiation, imparts energy to the biological system, resulting in the (i) homeolytic scission of water molecules and the formation of toxic ROS (15). Oxygen radicals also arise from (ii) electron leakage from mitochondrial cytochromes, resulting in reduction of molecular oxygen to superoxide anion (16), (iii) from the futile cycling of cytochromes P450(CYP), whereby the CYP- O_2 complexes do not insert all the activated O_2 into the organic substrate, but release some as superoxide anion into the microsomal lipid environment (17), (iv) activation of cytochrome P4502E1, which release most of the activated O_2 as superoxide anion(17), (v) reduction of tissue oxygen by iron and other transitional metal redox systems(18), (vi) activation of leukocytes and the NADPH oxidase to produce superoxide anion in inflammation and infections (19), (vii) redox cycling of xenobiotic quinones (20), (viii) transoxygenation associated with the conversion of PGG_2 to PGH_2 and HPETE to HETE in prostaglandin synthesis(21).

Besides those sources, ROS are also generated exogenously as components of tobacco smoke or other environmental pollutants (ozone) and, indirectly, through the metabolism of certain solvents, diet (high intake of polyunsaturated fatty acids, diet-derived quinonoid substances), drugs, pesticides and other chemicals that may augment ROS production either directly or via their effects on the cytochrome P450 and other drug-metabolizing enzymes (2,6,7).

1.2.3 Oxidative damage and degenerative diseases

Proteins, lipids, carbohydrates and nucleic acids have all been studied for sensitivity to oxidative modification by a wide variety of free radicals and reactive oxygen species (1,3). Superoxide is not particularly reactive with lipids, carbohydrates, or nucleic acids, but does exhibit limited reaction with reactivity with certain proteins. $O_2^{\bullet -}$ will react with proteins that contain transition-metal prosthetic groups, such as haem

moieties or iron-sulphur clusters. Such transition-metal mediated reactions result in damage to amino acids, usually those directly attached or proximal to the metal catalyst, and loss of protein/enzyme function. Whenever $O_2^{\bullet-}$ is generated, there will always be a concomitant production of H_2O_2.

Hydrogen peroxide is clearly a major common intermediate, generated by multiple oxidation pathways. Hydrogen peroxide is an oxidant for many biological molecules, especially those containing sulphydryl groups, iron-sulphur clusters, reduced haem moieties and copper prosthetic groups. H_2O_2 can also readily react with transition-metal reductants/catalysts to generate the hydroxyl radical ($^{\bullet}OH$) that reduced Fe^{2+} is oxidized by H_2O_2 to generate $^{\bullet}OH$; this is basically the reaction described by Fenton in 1894. However, $O_2^{\bullet-}$ can act as the reductant to re-reduce Fe^{3+} to Fe^{2+}. This is called iron-catalyzed Haber-Weiss reaction. Copper can substitute for iron as the transition metal in the reaction.

The hydroxyl radical is the most reactive one of all the oxygen radicals and will readily oxidize proteins, lipids, carbohydrates, DNA and RNA. For example, haemoglobin can react with H_2O_2 to produce a ferryl-haemoglobin (Fe^{4+}) state. Such high oxidation states can act as $^{\bullet}OH$, or psudo-$^{\bullet}OH$ causing significant oxidative damage. The main difference is that true $^{\bullet}OH$ is a free radical which responds to Brownian motion in solution and reacts at nearly diffusion controlled rates. In contrast, agents like ferryl-haemoglobin have more limited mobility, and reactivity is hindered or determined by the position of the metal on the protein.

Oxidation of lipid plasma membrane is one of the earliest recognized consequence of free-radical attack leading to the processes of several diseases and aging. Traditionally, most markers of oxidative injury utilized reflect free radical attack on polyunsaturated fatty acids, with the classical route of attack involving lipid peroxidation, generating hydroperoxides, endoperoxides, long-lived aldehydes and the end-products malondialdehyde, ethane, and pentane (33).

Lipid peroxidation is initiated by the attack of an acyl side-chain on a fatty acid by any chemical species that has sufficient reactivity to abstract a hydrogen atom from

methylene carbon in the side-chain. The greater the number of double bonds in a fatty acid side-chain, the easier is the removal of a hydrogen atom, that is why the polyunsaturated fats, arachidonic acid (20:4) and docosahexaenoic acid (22:6), are more susceptible to lipid peroxidation than linoleic acid (18:2). The carbon-centered radical resulting from hydrogen abstraction from a fatty acid can have several fates, but for the most likely one in an aerobic environment is to undergo a molecular rearrangement, followed by reaction with oxygen to give a peroxyl radical (ROO•). Peroxyl radicals are capable of abstracting hydrogen from adjacent fatty acid side-chain in a membrane, and hence propagating a chain reaction of lipid peroxidation resulting in the conversion of hundreds of fatty acid side-chains into lipid hydroperoxides. In biological membranes, this can lead to impairment of membrane function and decreased fluidity and has been associated with a number of diseases.

Iron plays a second important role in lipid peroxidation. Pure lipid peroxides are fairly stable at physiological temperatures, but, in the presence of transition metal complexes, including iron and copper salts, their decomposition is greatly accelerated. Thus a reduced iron complex can react with lipid peroxide in a similar way to its reaction with H_2O_2: it causes fission of an O—O bond to form an alkoxyl radical. R—OOH is lipid hydroperoxide.

$$R—OOH + Fe^{2+}\text{-complex} \longrightarrow Fe^{3+}\text{-complex} + OH^- + R—O^{\bullet}$$

(alkoxyl radical)

An iron complex can form both peroxyl and alkoxyl radicals, according to the overall equation:

$$R—OOH + Fe^{3+}\text{-complex} \longrightarrow RO_2^{\bullet} + H^+ + Fe^{2+}\text{-complex}$$

(peroxyl radical) (further reaction to give alkoxyl radical)

In recent years, oxidative stress has been implicated in a wide variety of degenerative processes, diseases and syndromes, including the following disorders and diseases (22-32): mutagenesis, cell transformation and cancer; fibrogenesis chronic diseases such as cystic fibrosis, atherosclerosis, arteriosclerosis, heart attacks, strokes and ischaemia/reperfusion injury; chronic inflammatory diseases, such as rheumatoid

arthritis, lupus erythematosus and psoriatic arthritis; acute inflammatory problems, such as wound healing; photo-oxidative stress on the eye, such cataract; central nervous system disorder, such as certain form of familial amyotrophic lateral sclerosis(ALS), Parkinson's disease and Alzheimer's dementia; diabetes and a wide variety of age-related disorders, perhaps even including factors underlying the aging process itself.

Some of these oxidation-linked diseases or disorders can be exacerbated, perhaps even initiated by numerous environmental pro-oxidants and/or pro-oxidant drugs and foods. Alternatively, compounds found in certain foods may be able to significantly bolster resistance against oxidants. Currently, great interest centers on the possible protective value of a variety of plant-derived antioxidant compounds, particularly those from fruits and vegetables.

1.2.4 Antioxidant defenses

An antioxidant has been defined as "any substance that, when present at low concentrations compared to dose of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate". Antioxidants can act at different levels in the oxidative sequence. As far as lipid peroxidation is concerned, they could act by (1, 33):

1. Decreasing localized O_2 concentrations (e.g. sealing of food stuffs under nitrogen).

2. Preventing first-chain initiation by scavenging initiating radicals such as $^{\bullet}OH$.

3. Binding metal ions in form that will not generate such initiating species as $^{\bullet}OH$, ferryl, or Fe^{3+} / Fe^{2+} / O_2 and/or will not decompose lipid peroxides to peroxyl or alkokyl radicals.

4. Decomposing peroxide by converting them to non-radical products, such as alcohol.

5. Chain breaking, i.e. scavenging intermediate radicals such as peroxyl and alkoxyl radicals to prevent continued hydrogen abstraction. Chain-breaking are often phenols and amines.

Antioxidants acting by mechanisms 1, 2 and 3 can be called preventive antioxidants. Those acting by mechanism 3 are not usually consumed of the course of

the reactions. Antioxidants of the fourth type are also preventive antioxidants, but they may or may not be consumed during the reaction, depending on their chemical behavior (e.g. glutathione peroxidase acts by this mechanism and being an enzyme, is a catalyst and is not consumed). Chain-breaking antioxidants, acting by combining with the intermediate radicals, will be consumed, as antioxidants of type 2 as above. It should be stressed that many antioxidants have multiple mechanisms of action.

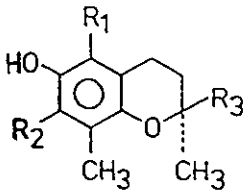
All aerobic organisms, including human beings, utilize a series of primary antioxidant defenses in an attempt to protect against oxidant damage, and numerous damage removal and repair enzymes to remove and/or repair molecules that do get damage. Those antioxidants concentrate on the non-enzymic and enzymic primary antioxidant defenses.

Such antioxidants (33-38) include vitamin C, which acts as a cytosolic antioxidant; vitamin E, which acts as a membrane antioxidant; and glutathione, which acts to protect both cytosol and membranes against free radicals attack. Also present are the glutathione-dependent enzymes, glutathione peroxidase(GPX), glutathione reductase, glutathione transferase, and catalase, which break down H_2O_2 to oxygen and water, and the enzyme superoxide dismutase (SOD), which converts $O_2^{\bullet -}$ into H_2O_2. Some enzymes exist in several different forms. Membrane, cytosolic, and plasma forms of GPX have been reported. Similarly, there are mitochondrial, cytosolic, and extracellular forms of SOD. Other important antioxidants include carotenoid, ubiquinones, bilirubin and uric acid.

1.2.5 Properties of some vitamins as antioxidant

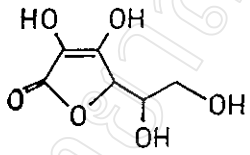
1.2.5.1 Vitamin C

Vitamin C has long been known to be essential for the protection of humans against scurvy. The ascorbic activity of vitamin C lies in the role of ascorbic acid (the reduced form of vitamin C) known as an essential cofactor in hydroxylation reactions involved in the biosynthesis of stable cross-linked collagen. This and other metabolic functions of ascorbate depend on its strong reducing potential, and its structure is

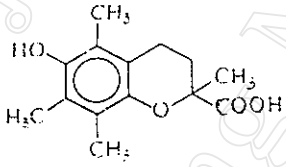


Vitamin E

	R_1	R_2	R_3
α-Tocopherol	CH_3	CH_3	$C_{16}H_{33}$
β-Tocopherol	CH_3	H	$C_{16}H_{33}$
γ-Tocopherol	H	CH_3	$C_{16}H_{33}$
δ-Tocopherol	H	H	$C_{16}H_{33}$



Vitamin C



Trolox

Figure 2: Structures of vitamin E (Tocopherol), vitamin C and Trolox.

(From references 1 and 39)

shown in Figure 2. The same property makes this vitamin an excellent antioxidant, capable of scavenging a wide variety of different oxidants. For example, ascorbate has been shown effectively scavenge superoxide , hydrogen peroxide, hyperchloric acid, aqueous peroxyl radicals, and singlet oxygen and seems to have a protective effect for many kind of cancers and carcinogeneesis (7,9,39). During its antioxidant action, ascorbate undergoes a two-electron oxidation to dehydroascorbic acid (the oxidized form of vitamin C) with intermediate formation of relatively unreactive ascorbyl radical. Although dehydroascorbic acid is relative unstable hydrolyzed readily to L-2,3-diketogulonic acid, it can be reduced back to ascorbate a variety of cells or thiols such as homocysteine. Therefore, both ascorbate and dehydroascorbic acid are biologically active forms of vitamin C. Ascorbate is able to interact synergistically with membrane-bound and lipoprotein confined α-tocopherol i.e. it readily reduces α-tocopherol (39). The mechanism is shown in Figure 3. Ascorbate and α-tocopherol may be classified as phase-transfer active antioxidant.

1.2.5.2 α-Tocopherol and other vitamin E compounds

α-Tocopherol, or in the other name vitamin E, is quantitatively the major lipid-soluble antioxidant in human plasma and LDL. The structure is shown in Figure 2. Vitamin E effectively serves as the major lipid soluble chain-breaking antioxidant (7,9,33,39), preventing lipid peroxidation and modulating the metabolism of the arachidonic acid cascade initiated by lipoxygenase and/or cyclooxygenase, and an increased intake of vitamin E is recommended for heart disease prevention and, on current hypotheses, it could be protective against cancers where *N*-nitroso compounds are implicated. Other isomer of vitamin E, such as β-, γ- and δ-tocopherol, are either present in very low concentrations or not detectable at all. Judged by their rate of reaction with peroxyl radicals, the antioxidant activity decreases in the order α>β>γ>δ, in analogy with the biological potencies of these different forms of vitamin E. α-tocopheroxyl radical, the one electron oxidation product of α-tocopherol, is readily

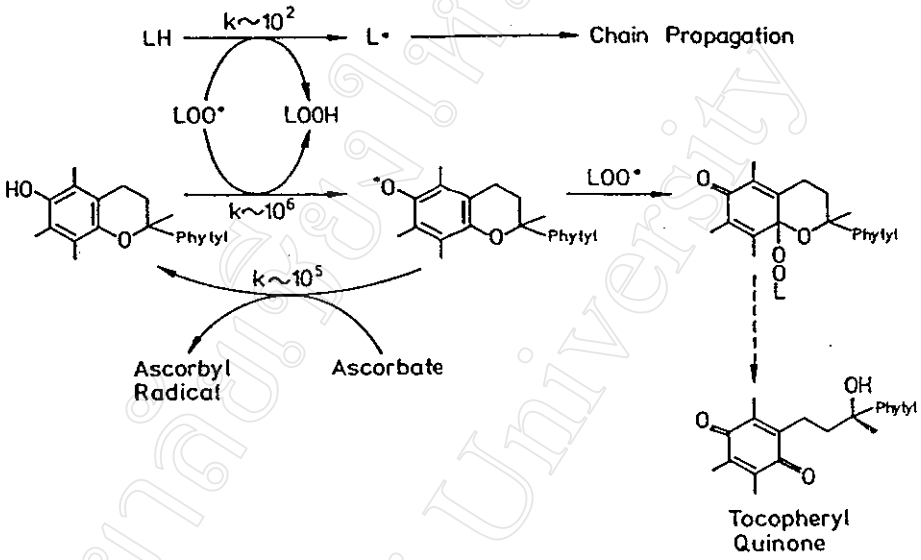


Figure 3: Vitamin E and its reaction with vitamin C. The long, hydrophobic side-chain (phytyl group) makes the molecule lipid-soluble whereas the ring structure allows reaction with radicals. Vitamin C readily reduces tocopheroxyl radical back to tocopherol, and then vitamin C itself undergoes the intermediate formation of unreactive ascorbyl radical. During peroxidation of cell membranes in vitro some tocopherol becomes converted into the quinone form. (From reference 39)

reduced by ascorbate as shown in Figure 3, including by albumin-bound bilirubin and ubiquinol-10 as well. The required radical phase-transfer process may be essential for the integrity of colloidal biological system, such as lipoproteins in plasma. Recent investigations have shown that impairment of radicals export from lipoproteins is the basis for a pro-oxidant rather than antioxidant activity of α-tocopherol alone.

1.2.5.3 Trolox

A water-soluble form of α-tocopherol (1); the hydrophobic side-chain is replaced by a hydrophilic –COOH group, the structure shown in the Figure 2. It is a good scavenger of peroxyl and alkoxyl radicals, giving a Trolox radical that can be repaired by ascorbate. Trolox is commercially available for experimentation.

1.2.6 Other antioxidants

In addition to those natural antioxidants, a huge range of synthetic antioxidants are available, using in the rubber industry to prevent copper-catalyzed oxidative degradation of polypropylene, in the polymer industry to prevent UV-induced free radical degradation of plastics, and for foodstuffs to protect food lipids against oxidative damage (and consequent rancidity) during storage, heat sterilization, or sterilization by ionizing radiation. Table 1 summarizes the structures of several antioxidants used in biology and food technology.

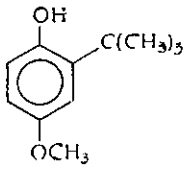
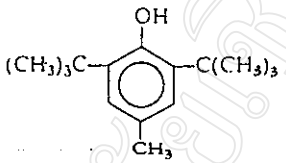
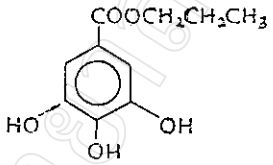
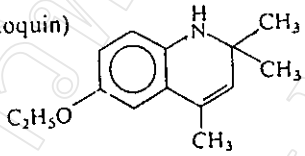
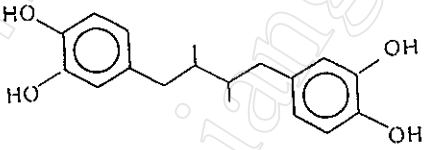
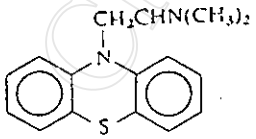
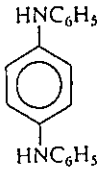
Most antioxidants shown in Table 1 are phenols (A—OH) and aromatic amines (A—NH--) and they usually act by a chain-breaking mechanism similar to that of vitamin E. They efficiently donate a hydrogen atom to a peroxyl and alkoxyl radicals, so interfering with the propagation of lipid peroxidation:

$$A—OH + RO_2^{\bullet}(RO^{\bullet}) \longrightarrow A—O^{\bullet} + R\,O_2H(ROH)$$

$$A—NH\text{--} + RO_2^{\bullet}(RO^{\bullet}) \longrightarrow A—N^{\bullet}\text{--} + R\,O_2H(ROH)$$

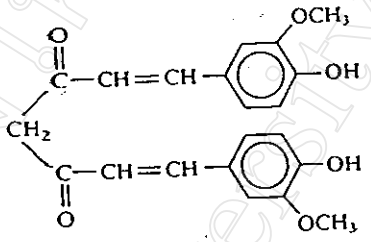
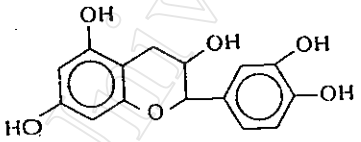
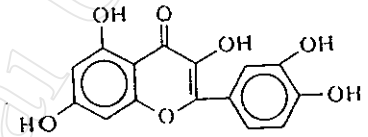
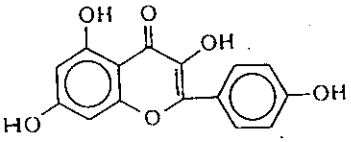
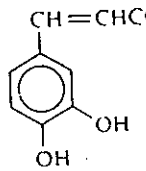
The nitrogen- or oxygen-centered antioxidant radical (A—N•--, A—O•) so produced is insufficiently reactive to abstract hydrogen because of delocalization of the unpaired

Table 1: Structures of some antioxidants used in biology and food technology.

Name	Structure	Comments
Butylated hydroxyanisole (BHA)		Very often added to foodstuffs. Acts as an antioxidant by hydrogen donation, which is common to all the phenolic (and amine) antioxidants.
Butylated hydroxytoluene (BHT)		Very often added to foodstuffs.
Propyl gallate		Fairly water-soluble. Good inhibitor of lipid peroxidation. Often added to foodstuffs. Binds iron ions.
Ethoxyquin (Santoquin)		Frequently used in fruit canning. Powerful enzyme inducer *in vivo*.
Nordihydroguaiaretic acid (NDGA)		Often added to foodstuffs and several polymers (e.g. rubber, lubricants). Binds iron ions.
Promethazine		Inhibits lipid peroxidation *in vitro*.
N,N'-Diphenyl-*p*-phenylene diamine (DPPD)		Popular antioxidant *in vitro*.

Note: from reference 1

Table 2: Structures of some plant compounds with antioxidant activity in certain systems

Name	Structure
Curcumin	
Catecin	
Quercetin	
Kaempferol	
Caffeic acid	

Note: from reference 1

electron into an aromatic ring structure and , unless a mechanism exists for reducing it back to the antioxidant (e.g. ascorbic acid in the case of α-tocopheryl radical), the antioxidant radical can disappear by several mechanisms. These include self-reaction of radicals, e.g. if A• is any antioxidant radical:

$$\begin{aligned} &RO_2^{\bullet} + A^{\bullet} \\ &RO^{\bullet} + A^{\bullet} \longrightarrow \text{non-radical products.} \\ &A^{\bullet} + A^{\bullet} \end{aligned}$$

Many antioxidant compounds shown in Table 1 have properties other than a chain-breaking action. For example, most phenolic antioxidants have metal ion-complexing ability, especially those antioxidant with adjacent —OH groups. However, the chain-breaking action is predominant in peroxidizing lipid systems, causing phenolic antioxidants to be powerful inhibitors of peroxidation process.

Several products of plant origin (some flavonoid, polyphenols) have chain-breaking antioxidant activity; Table 2 shows some examples. Several of these compounds, such as quercetin and catechin, also have metal-binding capacity.

1.2.7 Flavonoid

Flavonoids (or bioflavonoids) are a group of over 4,000 naturally occurring phenolic compounds (polyphenols) sharing a similar chemical structure. They are found in a wide variety of plants, including most common fruits and vegetables. Flavonoids appear to be the active constituents in numerous medicinal plants, and plants that contain flavonoids are widely used in herbal medicine traditions around the world (40).

Flavonoids can be divided into five categories (41).

1. Anthocyanidins, anthochlors and aurones. Anthocynidins are red-blue pigments in plants (such as found in red to blue of different parts in plants). Anthochlors and aurones are yellow pigments found in flowers.

2. Minor flavonoids. Minor flavonoids include flavanones, flavan-3-ols, dihydroflavones, and dihydrochalcones. These are categorized as minor flavonoids due

to their limited natural distribution. Two flavan-3-ols (flavanol) that will be discussed below include (+)-catechin and epigallocatechin 3-gallate (EGCG). Certain flavanols, or flavan-3-ols, are sometimes referred to by trademark term pycnogenols, coined by the French researcher Professor Jack Masquelier. The term pycnogenol means "that which creates condensation," and refers to the tendency of flavanols to create dimers (two identical compounds joined together), oligomers (a few joined together), and polymers (many joined together). One group of flavanol dimer and oligomers are termed proanthocyanidins, and are discussed in item 5.

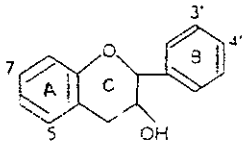
3. Flavones and flavonols. Flavones and flavonols are the most widely occurring flavonoids. Although several hundred flavonol aglycones are known, only quercetin, kaempferol, and myricetin are widely distributed. More than 135 different glycosides of quercetin have been isolated, the most common of which is rutin, a flavonol that has been used to treat capillary fragility. Flavones also occur as glycosides, but in a more limited fashion than flavonols. Baicalin, such as found in *Scutellaria baicalensis* (huang gin), is a common flavone *O*-glycoside used in Chinese medicine.

4. Isoflavonoids. Isoflavonoids are found mostly in the Leguminosae family (legumes), and can be divided into isoflavones, isoflavonones, pterocarpans, isoflavans, and rotenoids. Common isoflavonoids include genistein, daidzein, and biochanin A.

5. Tannins. Tannins include proanthocyanidins gallic acid phenolics (the gallo- and ellagi-tannins). They are characterized by their ability to bind with proteins. Proanthocyanidins are dimers of flavanols. In France, where much of the basic research on flavanols has taken place, they are termed "procyanidols" or in French "oligomeres proanthocyanidoliques" (oligomeric proanthocyanidins in English), or OPC for short. The abbreviation OPC is now used in many countries to refer to proanthocyanidins.

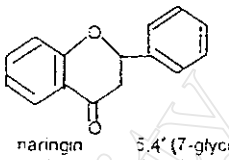
The distribution of these compounds vary between plant species. For example, citrus fruits contain high levels of flavones and flavanones; green tea contains high levels of catechins (17 to 30% of dry weight) and gallic acid phenolics; red, blue, and purple fruits such as berries, grapes contain high levels of anthocyanidins; and pine bark and

Flavan-3-ol



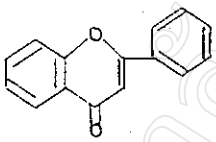
(+)-catechin 3,5,7,3',4'
(-)-epicatechin 3,5,7,3',4'

Flavanone
(dihydroflavone)



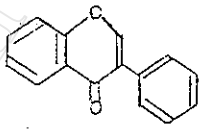
naringin 5,4' (7-glycoside)
naringenin 5,7,4'
taxifolin 3,5,7,3',4'

Flavone



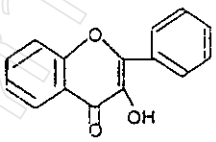
chrysin 5,7
epigenin 5,7,4
rutin 5,7,3',4' (3-rutinoside)

Isoflavone



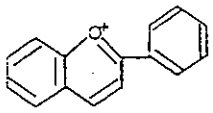
genistin 5,4'
genistein 5,7,4'

Flavon-3-ol



kaempferol 3,5,7,4'
quercetin 3,5,7,3',4'
myricetin 3,5,7,3',4',5'

Anthocyanidin



apigenidin 5,7,4'
perlargonidin 3,5,7,4'
cyanidin 3,5,7,3',4'
peonidin 3,5,7,4' (3'-OMe)
malvidin 3,5,7,4' (3',5'-OMe)

Figure 4: Structures of some flavonoids: flavanols, flavanones, flavones and anthocyanidins.

(From reference 51)

grape seeds contain high levels of OPC. Small molecular weight flavonoids are responsible for the tartness and bitterness of many fruits, whereas large molecular weight flavonoids (tannins), are responsible for their astringency. Some structures of flavonoid are shown in the Figure 4 and 5.

Some researchers question the bioavailability of flavonoids. Approximately half of the ingested flavonoids are absorbed into the bloodstream through the gastrointestinal tract lining, and half are metabolized to other compounds by gastrointestinal microflora (42). However, this varies, depending on the flavonoid.

Research on flavonoids began in 1936 when Szent-Gyorgi and colleagues discovered that crude extracts of vitamin C from lemon juice were more effective than pure vitamin C in treating guinea pigs with experimentally-induced scurvy. Since that time, researcher have identified a number of biochemical actions of flavonoids and polyphenols, including the following (40):

- Anti-allergy actions
- Immunomodulating actions
- Inhibition of platelet aggregation
- Anti-tumor actions

1.2.8 Flavonoid and antioxidant activity

There are some indications that not only endogenous antioxidants but also exogenous antioxidants may offer effective protection from oxidative damages in living systems, in some case, to extend life span in animals. Many dietary antioxidants other than α-tocopherol or vitamin C have been evaluated. Recently, the role of flavonoids acting as antioxidant has been investigated. There are a variety of researches reported the important properties of flavonoid as antioxidant and the various flavonoids found in different kinds of plants. For instance:

- The high antioxidant capacity of flavonoid in red wine after ingestion to increase the antioxidant capacity of serum in vivo (10).

●Inhibitory effects of epicatechin isomers on free radical-induced lysis of RBCs by preventing loss of fatty acids on cell membrane from oxidation (12).

●Flavonoids Inhibit of lipid peroxidation in certain systems (43-46).

●Inhibition of mammalian 5-lipoxygenase and cyclooxygenase by flavonoids (46)

● Flavonoids inhibit the oxidative modification of LDL by macrophages (47).

●Anti-mutagenic action of (+)-catechin against the plant-activated aromatic amine 4-nitro-o-phenylenediamine (48).

●Quercetin and myricetin protect against hydrogen peroxide-induced DNA damage in human lymphocytes (49).

●Antioxidant capability of flavonoids is due to the concomitant activities of scavenging free radicals and chelating iron (50,51).

1.2.9 Properties of some antioxidants derived from plants.

1.2.9.1 Curcumin

Curcumin (diferuloyl methane) from *curcuma longa* has many interesting pharmacological effects including anti-inflammatory and anti-cancer activities (52-54). Those studies have shown that curcumin is a good antioxidant and a potent inhibitor of lipid peroxidation catalysed by iron by which it is its chelator. The study suggested that the phenolic group or the methoxy group on the benzene ring is not important for the inhibition of iron-catalysed lipid peroxidation. The 1,3-diketone system is a potent ligand for metals such as iron. From the spectral studies, it is clear that the curcumin are capable of interaction with Fe^{2+}, and similar results were obtained with Fe^{3+}. The structure of curcumin is shown in Table 2.

1.2.9.2 Oligomeric proanthocyanidins

Oligomeric proanthocyanidins (OPC) are a complex flavan-3-ol molecular compound, made up of two or three flavan-3-ol molecules bonded to each other (Figure 5). Each single flavan-3-ol molecule is referred to as a "monomer" and is made up of (+)

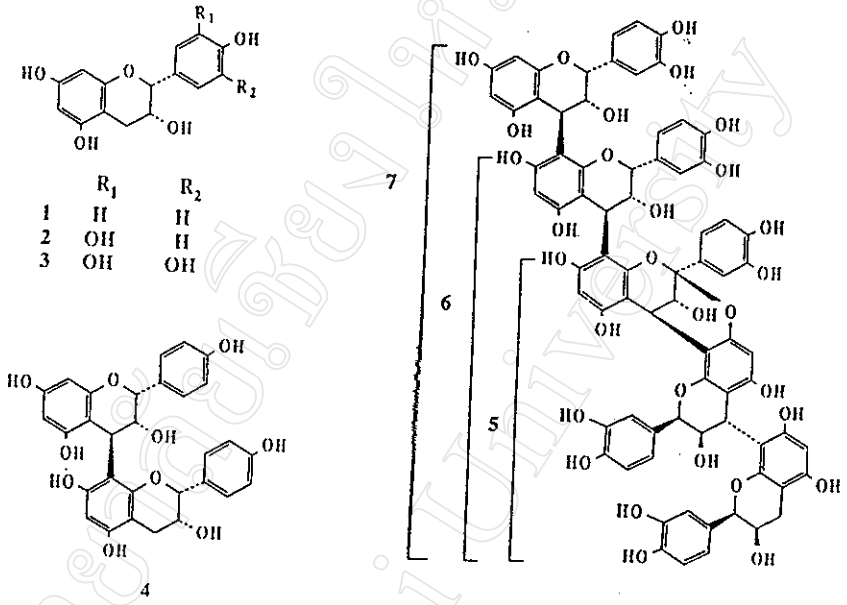


Figure 5: Structures of some known flavan-3-ols and proanthocyanidins. (-)-epiafzelechin (1), (-)-epicatechin (2), (-)-epigallocatechin (3), epiafzelechin-(4β→8)-epiafzelechin (4), epicatechin-(4β→8, 2β→*O*→7)-epicatechin-(4α→8)-epicatechin (5), epicatechin-(4β→8)-spicatechin-(4β→8, 2β→*O*→7)-epicatechin-(4α→8)-epicatechin (6) and epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin-(4β→8, 2β→*O*→7)-epicatechin-(4α→8)-catechin (7).

(From reference 81)

catechin or (-)epicatechin. Two of these flavan-3-ol molecules together constitute a "dimer" and three of these molecules together constitute a "trimer". Monomers are precusors to OPC, but are not biologically active by themselves. When bonded together in dimers and trimers, they become known as "oligomers", and are highly biologically active. When bonded together in higher numbers, the flavan-3-ol molecules become tannins, which offer no known health benefits." Proanthocyanidins" refers to the fact that OPC is colorless, and acquires color (blue or red) through an enzymatic process.

OPC is a natural health-supporting compound found in several parts of plants, such as grape seed and pine bark. OPC is classed with the broad group of antioxidant substances in plants known as polyphenols. Some members of this group are biologically active, and many are not.

OPC is 100 percent bioavailable and active, nontoxic, and colorless. Furthermore, OPC has an affinity for protein, especially collagen. This affinity accounts for much of OPC's extraordinary value to health.

In terms of protective value, OPC, is a superior antioxidant (55), very rapidly absorbed, and is quickly distributed throughout the body. As a free radical fighter, OPC comes to the aid of the body more quickly, thereby reducing the potential for free radial damage and the ravages of aging. OPC also possesses more reactive sites for neutralizing free radicals than other known antioxidants. Furthermore, OPC possesses nucleophilic as well as electrophilic reactive centers, permitting reactivity with both positively charged and negatively charged free radical species. What this means is that OPC can "scavenge" or "quench"(neutralize) a broad variety of types of free radicals.

Highly reactive as an antioxidant in both lipid (fat) and aqueous (water) phases, OPC neutralizes oxygen free radicals and is a valuable protector of healthy cells in a variety of internal conditions. This is an unique property among antioxidants.

OPC provides special protection to connective tissue (to collagen specially) from attack by free radicals, and prevent oxidative damage to cellular membranes. This means that OPC provides protection to vascular membranes, the linings of the stomach

and intestines, sinus and respiratory cavities, and joints and vertebral spaces. As a result OPC helps to prevent or reverse diseases and degeneration in these areas.

1.2.10 The calcium pump of the erythrocyte membrane

1.2.10.1 The role of calcium in biological system

Calcium is one of the most abundant metals in living organism and accounts for 2% of the total body weight in vertebrates. Most of it is mineralized in bones and teeth as hydroxyapatite and only 1% is formed in solution in the extra- and intracellular fluids. The calcium is absorbed in the proximal small intestine and this process is regulated by vitamin D_3 (1,25-dihydroxy cholecalciferol). Parathyroid hormone, calcitonin and vitamin D are involved in the complex control of renal calcium and phosphate excretion, skeletal calcium mobilization and homeostasis of the cellular calcium level. The concentration of Ca^{2+} in the cytoplasm is kept low, at 10^{-7} M to 10^{-8} M, whereas the extracellular calcium concentration is around 10^{-3} M (56).

External stimuli such as hormones, growth factors, light, and electricity, upon activating specific cellular plasma membrane receptors, trigger the generation of the second messengers inositol(1,4,5)-triphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ then opens the specific Ca^{2+} -channel and allow Ca^{2+} to enter the cytoplasm either from outside the cell or internal organelles such as the sarcoplasmic reticulum in muscle or the endoplasmic reticulum in non-muscle cells. An increased cellular calcium level triggers many intracellular processes, including fertilization, cell growth, transformation, secretion, smooth muscle contraction, sensory perception, neuronal signaling, DNA replication, DNA repair, gene transcription and cell volume recovery (57-60).

The calcium signal is transduced into an intracellular response, in part by calcium-binding proteins that are thought to be involved in the regulation of many cellular activities. These proteins may be subdivided into two groups, with distinct structural features: (1) the proteins with EF-hand motif such as calmodulin, troponin-c, parvalbumin and (2) the annexin family (61). These calcium-activated proteins may influence cellular activities by affecting phosphorylation of the key enzymes in the metabolic pathways (60).

In the erythrocytes, calcium exerts significant effects such as regulation of cell shape and plasticity, control of potassium transport via the Gardos-effect and the Na^+/K^+-pump (62). *In vitro* studies in Ca^{2+} -enriched human erythrocytes showed deleterious effects including degradation of cytoskeletal proteins with changes in cell morphology, perturbation of phospholipids asymmetry of the membrane bilayer, loss of deformability, and membrane vesiculations (63,64).

1.2.10.2 The mechanism of calcium transport across erythrocyte membrane

The physiological calcium concentration in human erythrocytes is between 10 and 20 mol/l and most of calcium is attached to the cell membrane. Cytoplasmic calcium concentration is in the range of 10^{-6} to 10^{-7} M, about three order of magnitude smaller than that in the plasma. Any intracellular buffering of calcium makes this large, inwardly directed gradient even steeper and maintaining this calcium distribution requires powerful defense mechanisms. These are the low passive permeability of the red cell membrane for calcium and the ATP-dependent, active extrusion from the cell interior (62).

Generally, eukaryotic cells possess several calcium transporting system (Figure 6). Only three systems, viz, Ca^{2+} channels, Ca^{2+}-ATPase, and Na^+/Ca^{2+} exchanger, are found in the plasma membranes. The Ca^{2+} channels are responsible for a controlled influx of calcium ions from extracellular space. They are the voltage sensitive channels. The Na^+/Ca^{2+} exchanger is active in excitable tissues such as heart, brain and kidney. It is a large capacity but low affinity calcium exporting system of the plasma membrane. The Ca^{2+}-ATPase has a high affinity for calcium (Kd <0.5 μM) and is believed to play the most important role in the maintenance of the 10^4 fold gradient of Ca^{2+} between cells and medium (65).

The mechanism of calcium uptake in the erythrocyte is poorly known. The process is suspected to be mediated by a carrier because the influx curve is a saturable type. It can be inhibited by 8 mM Sr^{2+} or 8 mM Ba^{2+} or 150 μM Co^{2+}. The Ca^{2+} influx is

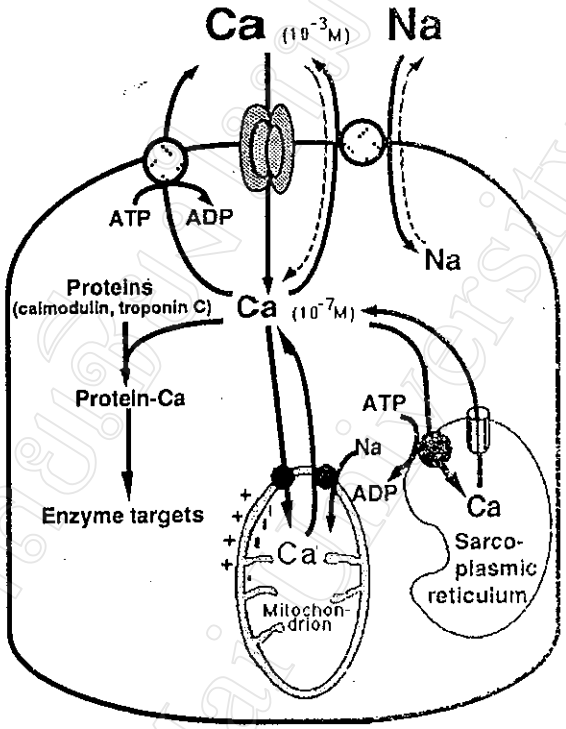


Figure 6: Calcium transporting system in eukaryotic cells. The figure shows the seven transporting systems; three in the plasma membrane (Ca^{2+}-ATPase, Na^{+} /Ca^{2+}-exchangers which normally importes Ca but sometimes also exports it, and Ca^{2+}-channels); two in the endo- (sarco-) plasmic reticulum (the Ca^{2+}ATPase and the still undefined release channel); two in the inner membrane of mitochondria (the electrophoretic uptake uniporter and the Ca-releasing Na^{+}/Ca^{2+}-exchanger). The figure also shows the soluble calcium binding proteins. (From reference 70)

sensitive to Verapamil suggesting that the transport system in erythrocytes is similar to identical to the slow Ca^{2+} channel of excitable tissues. Moreover, the inward transport of Ca^{2+} can be inhibited by quinidine, suggesting that the gradient of K^+ ions may play a role in the system (66).

1.2.10.3 The plasma membrane Ca^{2+}-ATPase

The membrane Ca^{2+}-ATPase, or Ca^{2+}/Mg^{2+}-ATPase (ATP phosphohydrolase, EC 3.6.1.3) is the only active Ca^{2+}-extrusion system in the erythrocytes (62,67). The enzyme belongs to the P-class of the ion-motive ATPase, i.g. it forms an aspartyl phosphate intermediate during the reaction cycle and is inhibited by vanadate. The pump is also inhibited by La^{2+} ($Ki \approx 1$ μM). In the erythrocyte, it represents about 0.01-0.1 percent of the total membrane proteins. The enzyme is a single polypeptide with a molecular mass about 138,000 dalton and does not contain carbohydrate. It has high affinity for Ca^{2+} (Km <0.5) and ATP (Km 1-2.5 μM) and is stimulated by Na^+ or K^+ (66).

The plasma membrane Ca^{2+}-ATPase is stimulated by direct interaction with calmodulin (CaM) (Kd about 1 nM). Other activating factors have been suggested, e.g. polyunsaturated fatty acid, acidic phospholipids, kinase-mediated phosphorylation (PKA, PKC), limited proteolysis and oligomerization.

The stoichiometry between transported Ca^{2+} and hydrolyzed ATP approaches 1.0 in plasma membrane Ca^{2+}-ATPase in contrast to 2.0 in the Ca^{2+} pump of sarcoplasmic reticulum (68). The proposed mechanism for the reaction cycle is shown in Figure 7. The enzyme in the Ca^{2+}-high affinity conformer (10 μM free Ca^{2+} = E1 state) after binding to Mg/ATP and Ca^{2+}, is phosphorylated at a specific aspartyl residue (residue number 475). Then, the phosphoenzyme intermediate of E1 transforms to the Ca^{2+}-low affinity conformer (E2-state) from which the bound Ca^{2+} ion is released. The phosphoenzyme intermediate of E2 conformer is then dephosphorylated; the mechanism may be associated with the incoming ATP molecule which also accelerates E2 to E1 transition. It has been proposed that vanadate (VO_4^{3-}) inhibition involves the step of E2 to E1

transition by interacting with the E2 conformer and thus blocks the last step of the reaction cycle. Lantanum ion (La^{3+}) inhibits the Ca^{2+} pump of the red cell by arresting the proteins in a phosphorylated form, that is, La^{3+} blocks the transition between E1-P and E2-P (69).

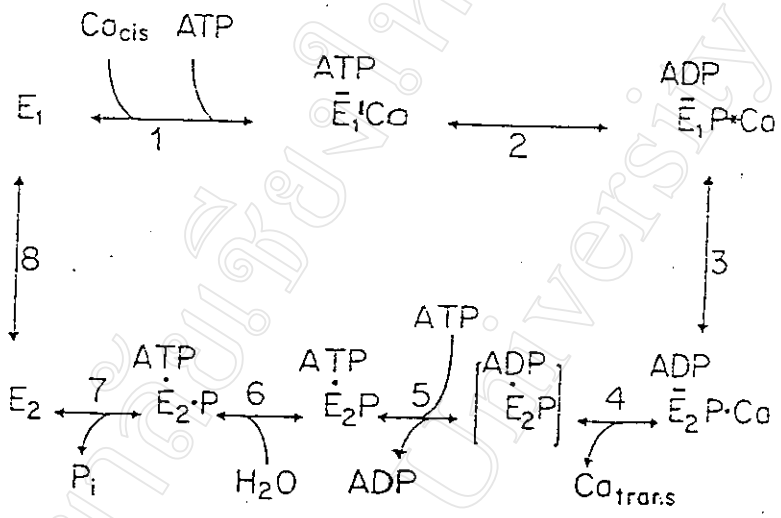


Figure 7: Proposed reaction cycle of the Ca^{2+} pump in plasma membrane and sarcoplasmic reticulum. E1, E2: conformations of pump protein; translocation occurrence step 3; EP, covalent bond: (.) low and (-) high affinity non-covalent ATP, ADP of Ca^{2+} bond; (*) "occluded" Ca^{2+}; [ ], "ADP-insensitive" E. In forward mode (clockwise): invading arrows, preceding E has increased affinity; emerging arrows, preceding E has decreased affinity. Mg^{2+} is not shown: it probably enters at step 1 and is liberated at step 7; step 3 is decisive for acceleration by Mg^{2+}. It is unclear what happens between ATP•E2 and ATP/E1 (step 8). Step 6 (7,8?) is decisive for acceleration by ATP at low affinity site. (From reference 68)

1.3 Experimental designs

The present study is to investigate the in vitro antioxidant properties of the polyphenol mostly contained in aqueous extract obtained from seed coat of tamarind, *Tamarindus indica* Linn. The antioxidant activity of the extract compounds have been semipurified and compared to standard and known antioxidants: vitamin E, vitamin C, Trolox and curcumin. Furthermore, the investigation has also determined the specificity of inhibitory effect of the extract to different kinds of reactive oxygen species. Chemically, the extract was analyzed and classified according to its heat stability and reactive properties.

Since Ca^{2+}-ATPases are responsible for the regulation of calcium homeostasis in eukaryotic cells. One report has indicated that erythrocyte membranes exposed to oxidative stress by either thalassemic condition of patients or the given generated condition in vitro; the activity of the damaged Ca^{2+}-ATPase would not respond to calmodulin stimulation after oxidative damage (70). Therefore, the study has been also designed to assay the attributes of the extract as an antioxidant practical to prevent lipid peroxidation and Ca^{2+}-ATPase on erythrocyte membrane from oxidative damage under free radical-generating system, as compared to vitamin E and curcumin. Lipid peroxidation was assayed by Thiobarbituric Acid Reactive Substances (TBARS) reaction determining malondialdehyde (MDA), a consequence of lipid peroxidation. The enzyme, Ca^{2+}-ATPase, was determined calmodulin response by the coupled enzyme method.

The whole experimental designs and procedures are described in details in Chapter 2, and the protocol is shown in the Figure 8.

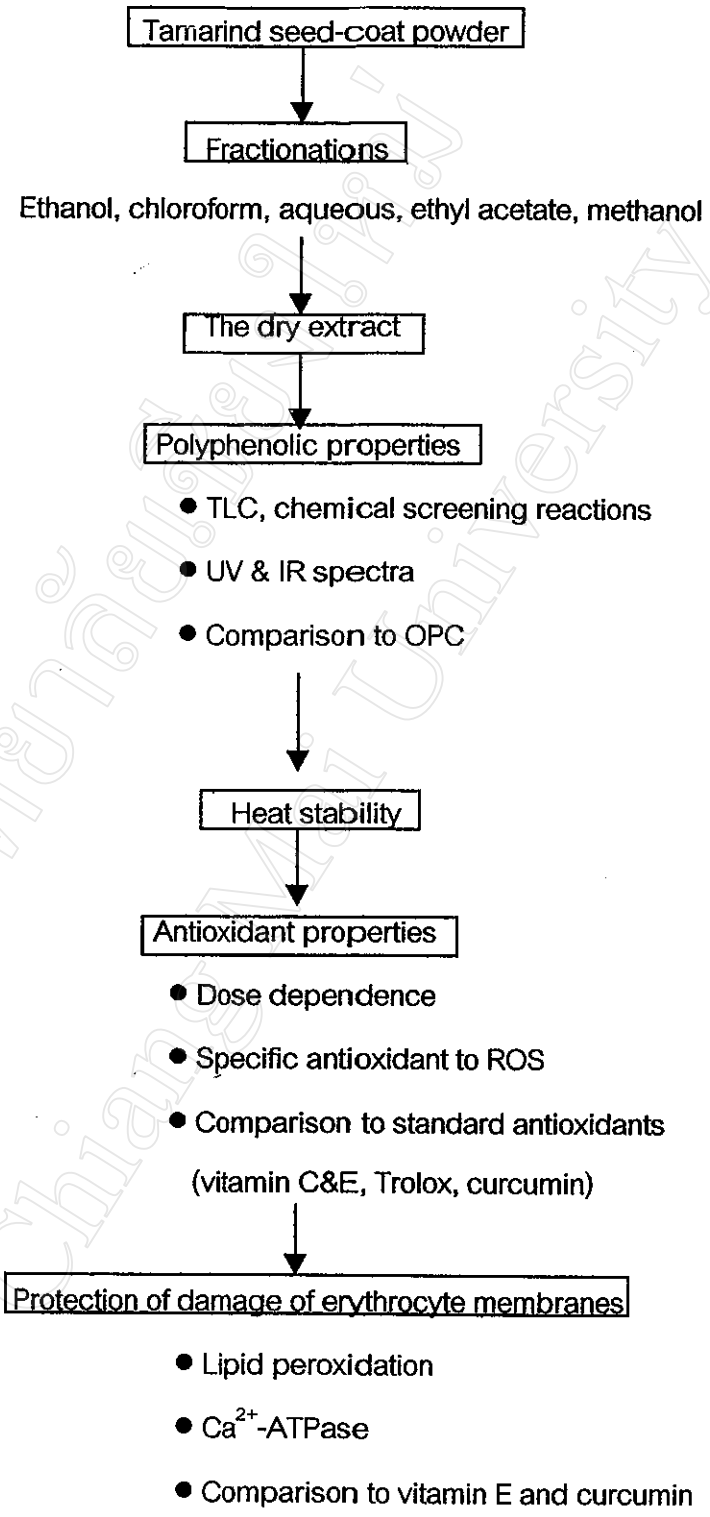


Figure 8: Experimental designs for this study

1.4 Objectives

To study the chemistry and antioxidative effects of the polyphenolic compound extracted from seed coat of *Tamarindus indica* Linn., by measuring the in vitro antioxidant capacity in the system of ABTS/H_2O_2/White-radish peroxidase, ABTS/H_2O_2/Metmyoglobin, Fenton reaction and Neo-tetrazolium method and its inhibitory effects to prevent lipid peroxidation and the damaged Ca^{2+}-ATPase on erythrocyte membranes under oxidative-generating condition.