

CHAPTER I

GENERAL BACKGROUND

1. Introduction

Excessive free radical production have known to be the cause of various diseases, such as atherosclerosis (Schwenke, 1998), cardiovascular disease (Shao *et al.*, 1999), ischemia reperfusion injury (Ceconi *et al.*, 2003), cataracts (Babizhayev, 1996; Tarwadi and Agte, 2004), brain dysfunction (Parihar and Hemnani, 2004), immune-system decline (Babior, 2000), rheumatoid arthritis (Rall *et al.*, 2000; Jaswal *et al.*, 2003), inflammation (pleiner *et al.*, 2003), carcinogenesis (Borek, 1997), and aging (Ames *et al.*, 1993).

Free radical atom or molecule contains one or more unpaired electrons such as superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($\cdot OH$), nitric oxide ($\cdot NO$), nitrogen dioxide (NO_2^{\cdot}), hydrogen peroxide (H_2O_2), singlet oxygen (1O_2), etc. These free radicals attack biomolecules such as lipid, protein, carbohydrate, and deoxyribonucleic acid (DNA), leading to cell and tissue injury associated with degenerative diseases mentioned above. Reactive oxygen species are produced by sunlight, ultraviolet, ionizing radiation, chemical reactions, and normal metabolism (Yen and Chem, 1995; Pietta *et al.*, 1998; Jung *et al.*, 1999).

In the body, antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase, catalase and antioxidant compounds such as α -tocopherol, ascorbic acid, β -carotene, and polyphenolic compounds reduce or arrest the harmful action of reactive oxygen species (ROS) (Halliwell, 1994; Pietta, 2000).

Fruits and vegetables are good sources of vitamins, minerals, and phytonutrients which may be effective in the prevention of free radical associated diseases (Halliwell, 2000).

The aim of this study was to screen and determine a large number of Thai fruits for their natural antioxidant activity.

2. Literature Review

2.1 Free radicals and active oxygen species

Free radicals are chemical species, which have unpaired electrons. Electrons are present generally in pairs. However, under certain conditions, molecules have unpaired electrons and become free radicals. Thus, free radicals are in general reactive and attack other molecules, although some radicals are not reactive but stable enough to have long life. Some of them have unpaired electrons and are free radicals, but others are not (Halliwell, 1994; Papas, 1999). Table 1 summarizes the active oxygen species, which are relevant to lipid peroxidation and oxidative stress *in vivo*. Nitric oxide and thiyl radical (RS^\cdot), which do not bear unpaired electrons on oxygen, are also included.

Table 1. Active oxygen and related species

Radicals		Non-radicals	
$O_2^{\cdot-}$	superoxide	H_2O_2	hydrogenperoxide
HO^\cdot	hydroxyl radical	1O_2	singlet oxygen
HO_2^\cdot	hydroperoxyl radical	LOOH	lipid hydroperoxide
L^\cdot	lipid radical	Fe=O	iron-oxygen complexes
LO^\cdot	lipid alkoxy radical	HOCl	hypochlorite
LO_2^\cdot	lipid peroxy radical	O_3	ozone
NO	nitric oxide	N_2O_3	dinitrogen trioxide
NO_2^\cdot	nitrogen dioxide	N_2O_4	dinitrogen tetroxide
RS^\cdot	thiyl radical	N_2O_5	dinitrogen pentoxide
P^\cdot	protein radical	ONO_2^-	peroxynitrite
H^\cdot	hydrogen atom	LO_2NO^-	alkyl peroxynitrite
		$O_2NOCO_2^-$	nitrocarbonate
		$ONO_2CO_2^-$	nitrosoperoxy carbonate
		RSH	thiol

2.1.1 Formation of free radicals and active oxygen species

2.1.1.1 Endogenous sources

Free radicals and active oxygen species in the body are by-products of the normal metabolism of mitochondria in aerobic organisms (Fig. 1), phagocytic cells during infection and inflammation, and peroxisomes that are organelles responsible for degrading fatty acids and other molecules (Ames *et al.*, 1993; Borek, 1997).

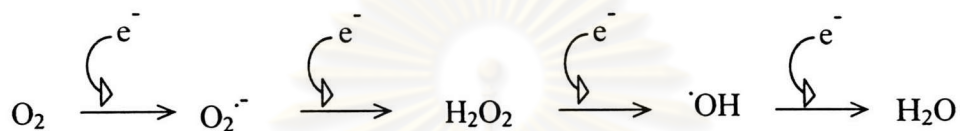


Fig. 1 Oxidants from normal metabolism. The formation of $\text{O}_2^{\cdot-}$, H_2O_2 and $\cdot\text{OH}$ occur by successive additions of electrons to O_2 . Cytochrome oxidase adds four electrons fairly efficiently during energy generation in mitochondria, but some of these toxic intermediates are inevitable by-products.

2.1.1.2 Exogenous sources

Free radicals and active oxygen species are formed by various extrinsic sources such as automobile exhaust, cigarette smoke, radiation, alcohol, herbicides, insecticides, dust, mold, excessive sunlight, ozone, ultraviolet light, noise, air/water pollution, stress, microorganism, etc. (Papas, 1999; Silalahi, 2001).

Free radical and active oxygen species are formed by various ways as summarized in table 2.

Table 2. Formation of active oxygen species

Active oxygen species	Formation
Superoxide (Hydroperoxyl radical) ($O_2^{\cdot -}$ (HO_2^{\cdot}))	Enzymatic and non-enzymatic one electron reduction of oxygen $O_2 + e \rightleftharpoons HO_2^{\cdot}$ (pK=4.8)
Hydroxyl radical (HO^{\cdot})	Radiolysis of water, metal-catalyzed decomposition of hydrogen peroxide, interaction of NO and superoxide $NO + O_2^{\cdot -} \xrightarrow{H^+} ONOO^- \rightarrow HO^{\cdot} + NO_3$
Alkoxyl and peroxy radicals (LO^{\cdot} , LO_2^{\cdot})	Metal-catalyzed decomposition of hydroperoxides
Hydrogen peroxide (H_2O_2)	Dismutation of superoxide, oxidation of sugars
Iron-oxygen complex (Fe=O, etc.)	Hemoglobin, myoglobin, etc.
Singlet oxygen (1O_2)	Photosensitized oxidation, bimolecular interactions between peroxy radicals reaction of hypochlorite and hydrogen peroxide
Lipid and protein hydroperoxides ($LOOH$, $POOH$)	Oxidation of lipids and proteins
Nitrogen dioxide (NO_2)	Reaction of peroxy radical and NO, polluted air and smoking
Nitric oxide (NO)	Nitric oxide synthase, nitroso thiol, and polluted air
Thiyl radical (RS^{\cdot})	Hydrogen atom transfer from thiols
Protein radical (P^{\cdot})	Hydrogen atom transfer from protein

2.1.2 Physiological function of free radicals

Active oxygen and related species play an important physiological role in the process of energy production, synthesis of biologically essential compounds, and phagocytosis; a critical process of the immune system. They may exert toxic effects as well. They also play a vital role in signal transduction, which is important for cell communication and function (Papas, 1999). On the other hand, increasing evidence have shown that active oxygen species may play a causative role in a variety of diseases including heart disease (Shao *et al.*, 1999), cancer (Borek, 1997), and aging (Ames *et al.*, 1993). The role of antioxidants, which suppress oxidative damage, has received increased attention. It is important to elucidate the mechanisms and dynamics of the oxidative damage in order to understand its biological significance and develop strategies to prevent it. Both active oxygen species and antioxidants are double-edged swords and the balance of their beneficial and toxic effects is determined by the relative importance of many competing biological reactions.

2.1.3 Reaction of free radical and active oxygen species and their reactivity

Free radicals and active oxygen species attack lipids, sugars, proteins, and DNA and induce their oxidation, which may result in oxidative damage such as deterioration of food, membrane dysfunction, protein modification, enzyme inactivation, and DNA strand breaks and modification of its bases. Radical reactions are generally chain reaction as illustrated in Fig. 2. The radicals are generated in a first step called “initiation”; they participate in a sequence of “propagation” reactions; finally they are destroyed in a “termination” process or processes. Although for some molecules extreme conditions are required to form radicals, many others can be transformed into radicals under relatively mild conditions, including those that are encountered in living organisms (Roberforid and Calderon, 1995; Papas, 1999).

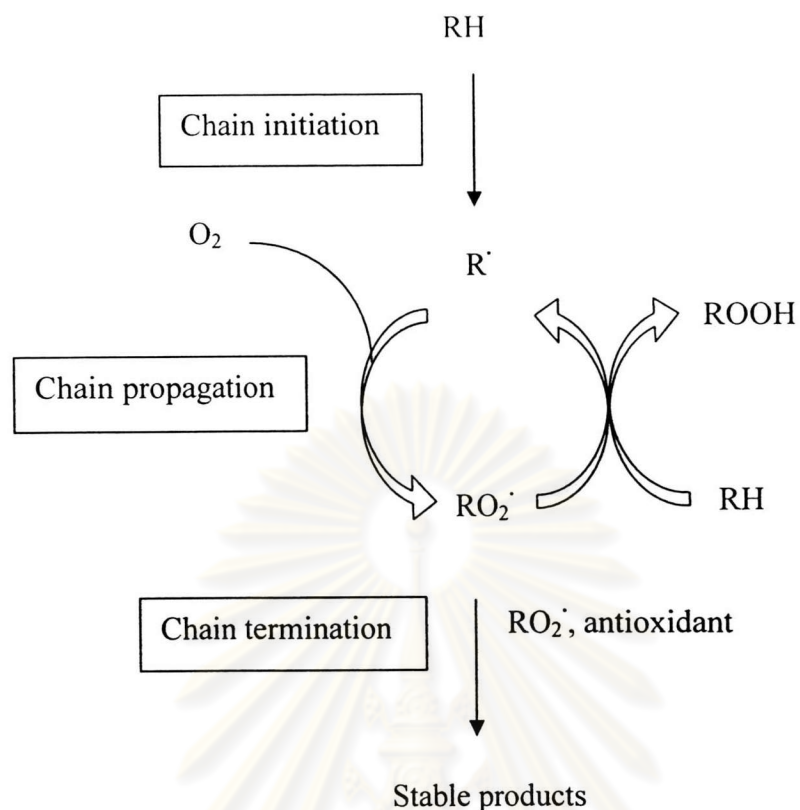


Fig. 2 Chain reaction of free radical (Papas, 1999)

2.1.3.1 Initiation of radical chain reactions

There are three general processes for supplying the energy necessary to generate radicals from initiators: thermal processes, microwave or ultraviolet (UV) radiation processes, and electron transfer (redox) processes. Radicals can also be produced in high energy radiation processes. Moreover, exposure to highly reactive molecular species such as nitric oxide (NO) or singlet oxygen can initiate radical reactions.

2.1.3.2 Propagation in radical chain reactions

In a propagation reaction, a radical reacts, firstly, to form a covalent bond, and secondly, to generate a new radical. The three most common propagation reactions are atom abstraction (Fig. 3), β -scission (Fig. 4), and addition to carbon-carbon double bond (Fig. 5) or aromatic rings.

2.1.3.3 Termination of radical chain reactions

In a termination reaction, two radicals interact in a mutually destructive reaction in which both radicals form covalent bonds and the reaction ceases. The two most common termination reactions are coupling and disproportionation.

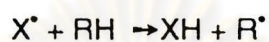


Fig. 3 Atom (or group) abstraction

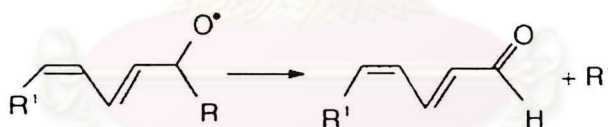
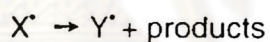


Fig. 4 β -Scission reaction



Fig. 5 Addition reaction

In phagocytosis, activated phagocytes use ROS to kill some strains of invading microorganism. Free radicals and ROS are useful only if they are produced at the right amount, place, and time. Otherwise, they can immediately attack molecules, which are very close to them including lipids, proteins, carbohydrates, and DNA.

Free radical and active oxygen species from various sources can damage biological molecules in membranes and other cellular components by oxidizing lipids, especially polyunsaturated fatty acid, producing a variety of breakdown products, including alcohols, ketones, aldehydes, and ether (Gardner, 1975). In addition, active oxygen species can react with DNA. For example, hydroxyl radical or singlet oxygen can convert a guanine base to 8-hydroxyguanine, which if not excised by DNA repair enzymes results in specific mutations. When the cells divide, the lesion becomes fixated, and mutations and cancer may result (Halliwell, 1994; Borek, 1997) as illustrated in Fig. 6.



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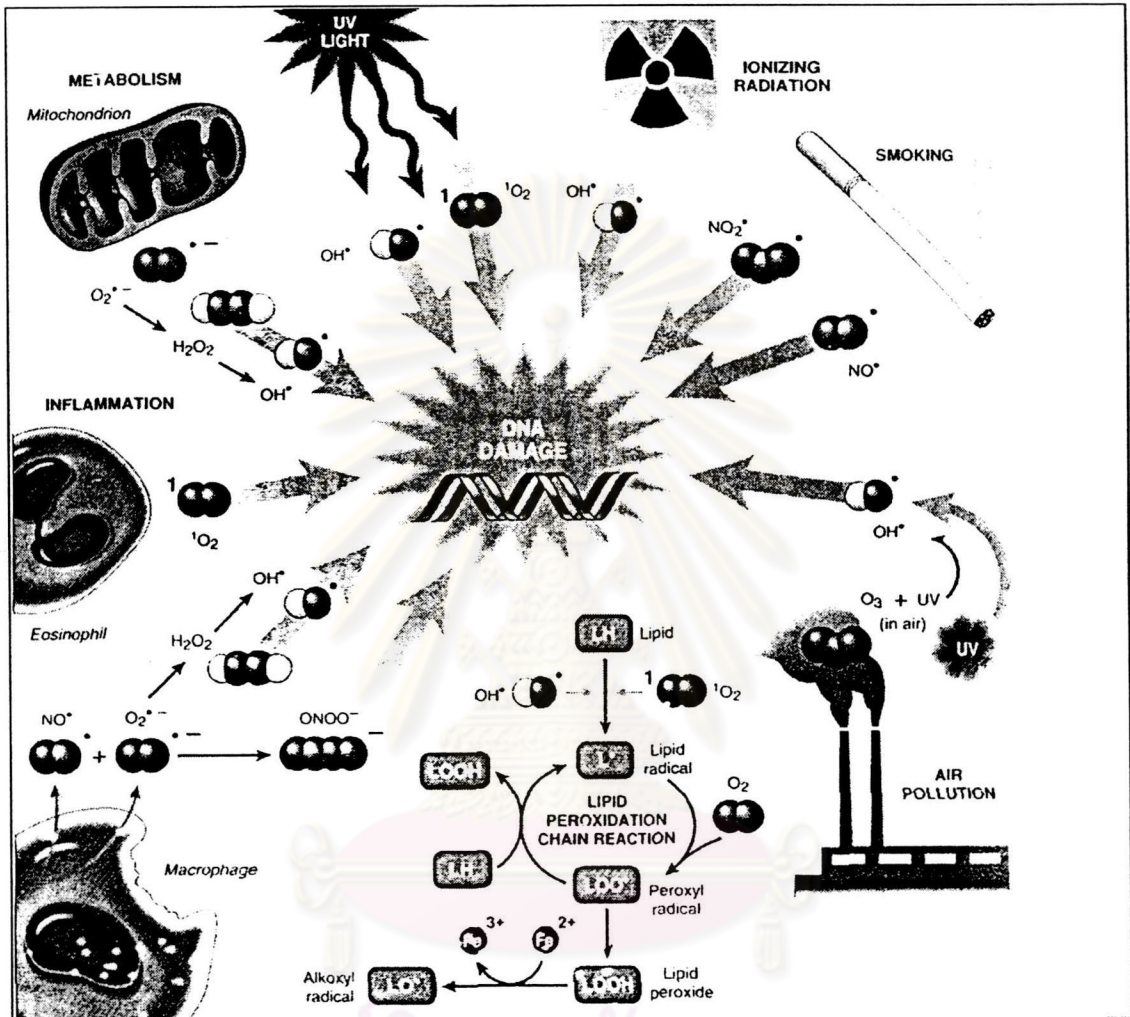


Fig. 6 Sources and reaction of free radical and active oxygen species (Borek, 1997)

2.2 Antioxidants

Aerobic organisms are protected from oxidative stress by antioxidants. Antioxidants are a diverse group of molecules that protect biological sites from oxidative damage. They usually act by removing or inactivating chemical intermediates that produce free radicals. They are classified on the basis of several criteria such as solubility in lipid or water, chemical and physical characteristics (proteins, enzymatic and non-enzymatic, or small molecules) etc.

It is important to note that individual antioxidants function not in isolation, but as part of systems with significant interdependence and additive or synergistic effects. These systems represent highly specialized strategies developed by various sites in the body for dealing with free radicals and ROS. Humans have evolved with antioxidant systems to protect against free radical and ROS. The system includes some antioxidants produced in the body (endogenous) such as antioxidant enzymes and other obtained from diet (exogenous). In addition, the appropriate antioxidant is generated and transferred to the right site at the right time and at the right concentration when the oxidation stress takes place. This adaptation mechanism is also important in the total defense system (Punchard and Kelly, 1996; Pietta, 2000).

2.2.1 Source of antioxidants

2.2.1.1 Endogenous antioxidants

Endogenous antioxidants are the first defense to suppress free radical and ROS that attacked biomolecules. They are produced by various sites in the body such as superoxide dismutase, catalase, and glutathione peroxides as summarized in Table 3, various kinds of antioxidants with different functions play an important role in these defense systems (Papas, 1999).

Table 3. Defense systems *in vivo* against oxidative damage

1. Preventive antioxidants: suppress the formation of free radicals	
(a) Non-radical decomposition of hydroperoxides and hydrogen peroxide	
Catalase	Decomposition of hydrogen peroxide $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$
Glutathione peroxidase (cellular)	Decomposition of hydrogen peroxide and free fatty acid hydroperoxides $\text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow 2\text{H}_2\text{O} + \text{GSSG}$ $\text{LOOH} + 2\text{GSH} \rightarrow \text{LOH} + \text{H}_2\text{O} + \text{GSSG}$
Glutathione peroxidase (plasma)	Decomposition of hydrogen peroxide and phospholipids hydroperoxides $\text{PLOOH} + 2\text{GSH} \rightarrow \text{PLOH} + \text{H}_2\text{O} + \text{GSSG}$
Phospholipid hydroperoxide, glutathione peroxidase	Decomposition of phospholipids hydroperoxide
Peroxidase	Decomposition of hydrogen peroxide and lipid hydroperoxides $\text{LOOH} + \text{AH}_2 \rightarrow \text{LOH} + \text{H}_2\text{O} + \text{A}$ $\text{H}_2\text{O}_2 + \text{AH}_2 \rightarrow 2\text{H}_2\text{O} + \text{A}$
Glutathione S-transferase	Decomposition of lipid hydroperoxides
(b) Sequestration of metal by chelation	
Transferrin, lactoferrin	Sequestration of iron
Haptoglobin	Sequestration of hemoglobin
Hemopexin	Stabilization of heme
Ceruloplasmin, albumin	Sequestration of copper
(c) Quenching of active oxygen species	
Superoxide dismutase (SOD)	Disproportionation of superoxide $2\text{O}_2^{\cdot -} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$
Carotenoids, vitamin E	Quenching singlet oxygen

Table 3. (cont.) Defense systems *in vivo* against oxidative damage

2. Radical-scavenging antioxidants: scavenge radicals to inhibit chain initiation and break chain propagation

Hydrophilic: Vitamin C, uric acid, bilirubin, albumin

Lipophilic: Vitamin E, ubiquinol, carotenoids

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

Lipase, protease, DNA repair enzymes, transferase

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

2.2.1.2 Exogenous antioxidants

Exogenous antioxidants are derived from diet or nature. Fruits and vegetables contain various antioxidant compounds, such as vitamin C, vitamin E, vitamin A, and carotenoids. Moreover, they contain polyphenolic compounds such as flavonoids, tannins, and lignans (or called secondary metabolite). Phenols occurring in diet and nature are interested in many viewpoints such as antioxidants, astringency, bitterness, browning reactions, color, oxidation substrates, protein constituents, etc. (Papas, 1999; Pietta, 2000). Table 4 summarizes the antioxidant, which found in the dietary.

Table 4. Antioxidants in the dietary

Dietary and exogenous antioxidants include;

- tocopherol and tocotrienols (vitamin E)
- ascorbate (vitamin C)
- vitamin A and carotenoids (β -carotene, lycopene, lutein, etc.)
- Se (and other metals essential for the function of antioxidant enzymes)
- phytochemicals (polyphenolic compound, flavonoids, tannins, etc.)
- dietary and other supplements (Coenzyme Q₁₀, glutathione, lipoic acid, etc.)
- food antioxidants (Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT), propylgallate, Tertiary butyl hydroquinone (TBHQ), rosemary extract, etc.)

2.2.2 Dietary antioxidants

Dietary antioxidant that derived from fruits, vegetables, nuts, and grains can decrease the risk of several diseases. For example, flavonoids can reduce free radical and ROS that cause of coronary heart disease (Hertog *et al.*, 1993), cardiovascular disease (Blache *et al.*, 2002), rheumatoid arthritis (Ostrakhovitch and Afanas'ev, 2001; Jaswal, 2003), gastric lesions (Casa *et al.*, 2000), etc.

Flavonoids are a group of polyphenolic compounds diverse in chemical structure and characteristics. They found ubiquitously in plants such as in fruits, vegetables, nuts, seeds, flowers, and bark. They are a class of plant secondary metabolites derived from the condensation of a cinnamic acid with three malonyl-CoA groups. All flavonoids arise from this initial reaction, which is catalyzed by the chalcone synthase enzyme. The chalcone is usually converted rapidly into a phenylbenzopyran, and further modification leads to the flavones, isoflavanes, flavonols or anthocyanins. Additional structural elaboration, mainly through glycosylation but also via acylation or alkylation, gives us the huge variety of flavonoid structures seen throughout the plant kingdom (Bloor, 2001). Flavonoids are categorized into 8 subgroups (Cook and Samman, 1996; Hollman, 2001).

2.2.2.1 Chalcones (Fig. 7)

2.2.2.2 Flavones (Fig. 8): Lutrolin, Apigenin

2.2.2.3 Flavonols (Fig. 9): Quercetin, Kampferol, Myricetin, Isorhamnetin, Morin, Rutin

2.2.2.4 Flavanones (Fig. 10) : Hesperetin, Naringenin, Eriodictyol

2.2.2.5 Flavan-3-ols (Fig. 11): (+)-Catechin, (+)-Gallocatechin, (-)-Epicatechin, (-)-Epigallocatechin, (-)-Epicatechin 3-gallate, (-)-Epigallocatechin 3-gallate, Theaflavin, Theaflavin 3-gallate, Theaflavin 3'-gallate, Theaflavin 3,3' digallate, Thearubigins

2.2.2.6 Anthocyanidins (Fig. 12): Cyanidin, Delphinidin, Malvidin, Pelargonidin, Peonidin, Petunidin

2.2.2.7 Isoflavones (Fig. 13): Genistein, Daidzein

2.2.2.8 Neoflavones (Fig. 14)

The biosynthesis pathway of flavonoids are illustrated in Fig. 15.

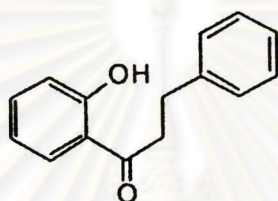


Fig. 7 Structure of chalcone

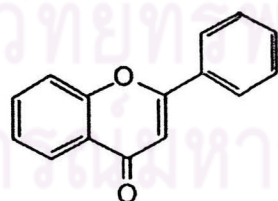


Fig. 8 Structure of flavones

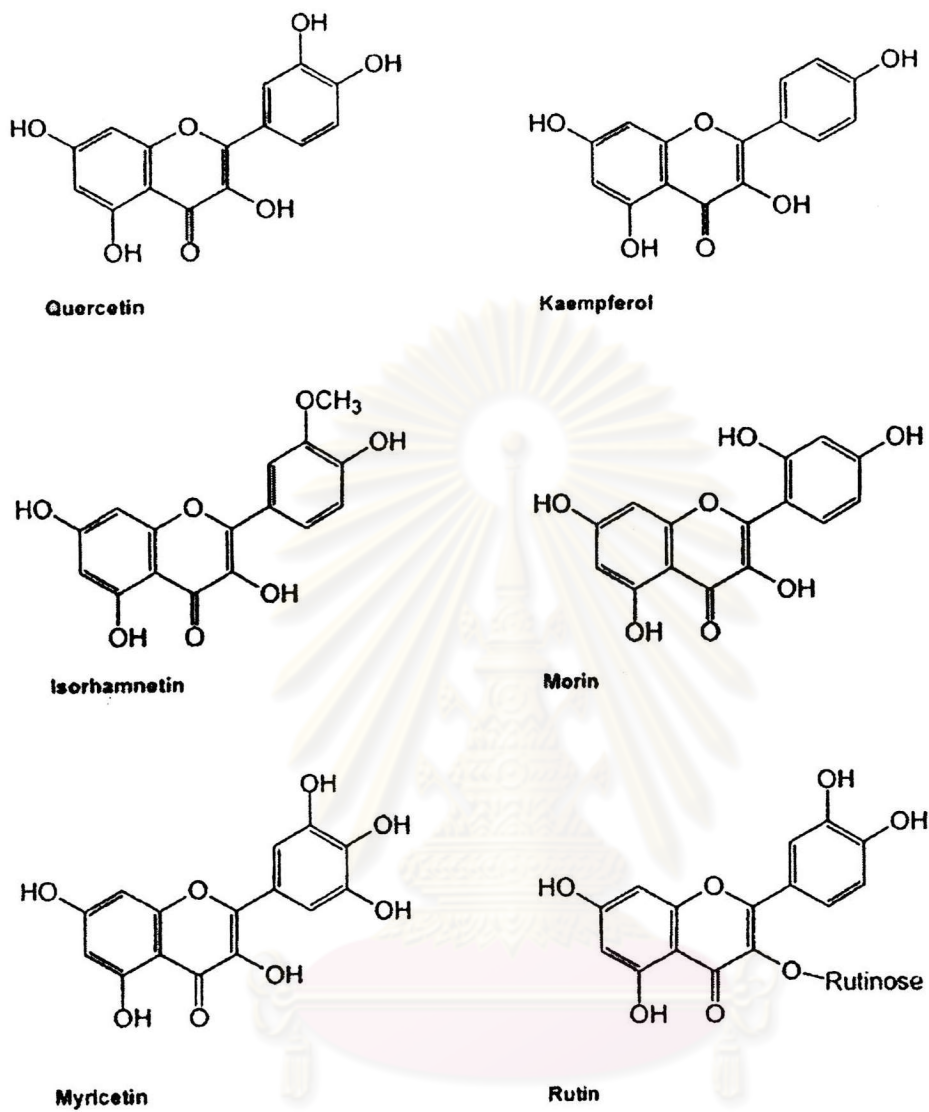


Fig. 9 Structures of flavonols

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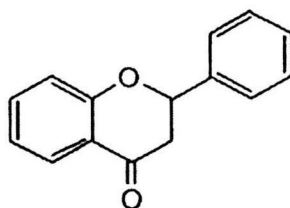


Fig. 10 Structure of flavanones

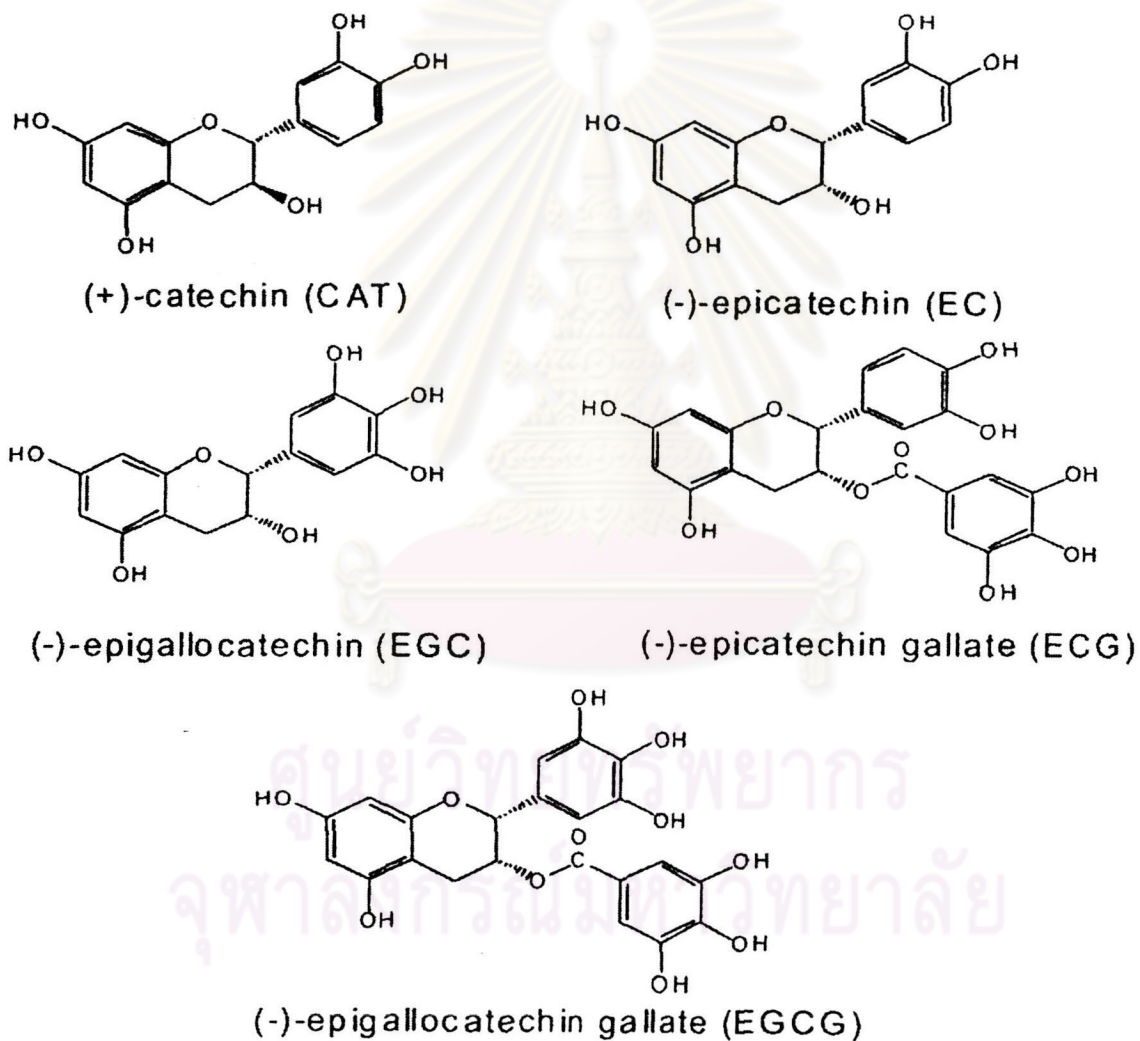


Fig. 11 Structures of flavan-3-ols

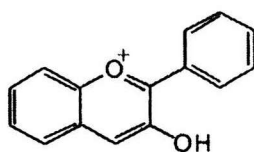


Fig. 12 Structure of anthocyanidins

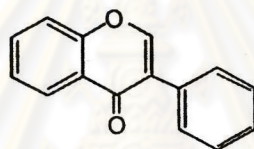


Fig. 13 Structure of isoflavones

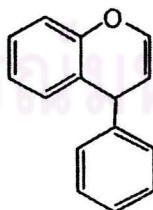


Fig. 14 Structure of neoflavone

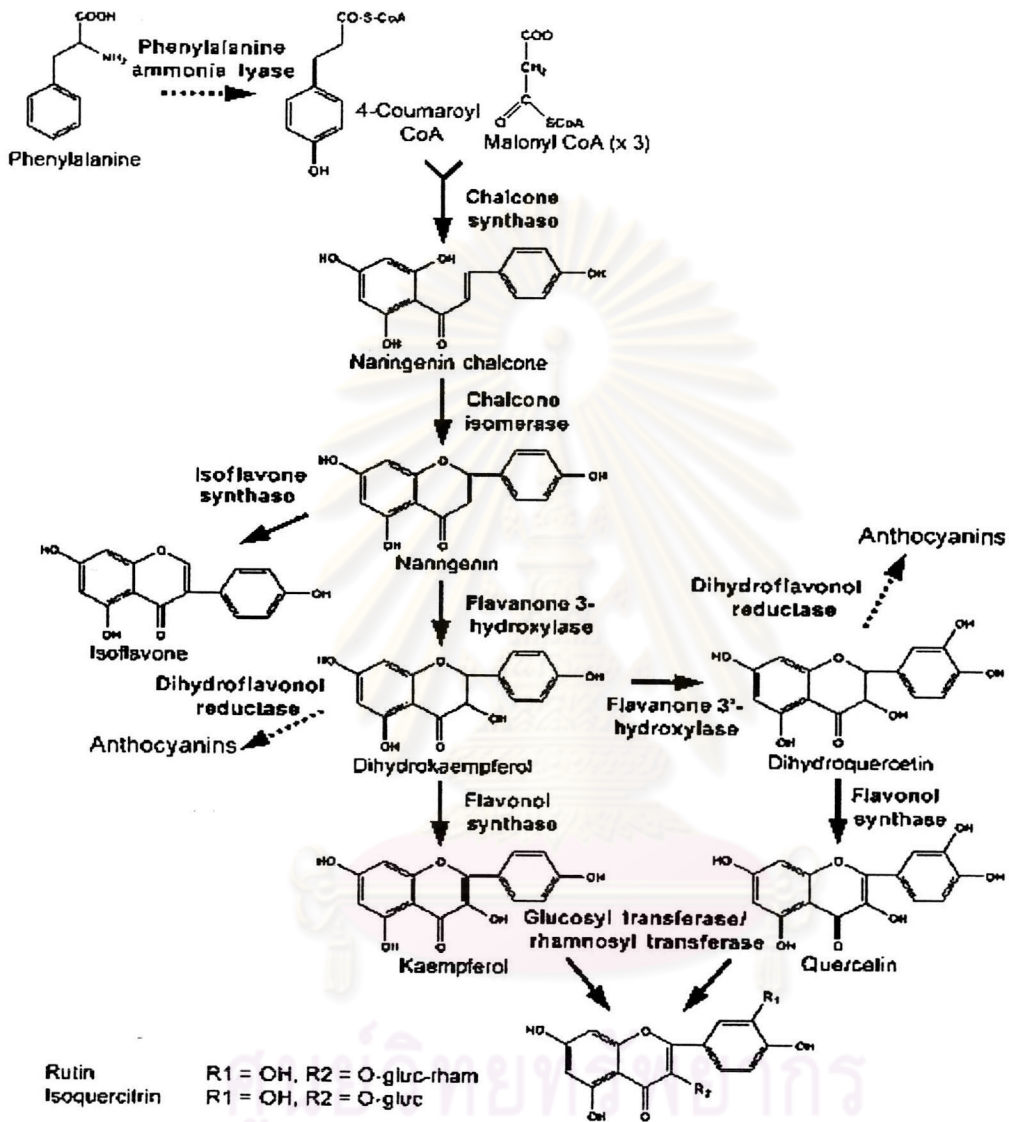


Fig. 15 Flavonoids biosynthetic pathway.

Flavonoids and other plant phenolics are important in the plant for normal growth development and defense against infection and injury (Harborne, 1975). They are commonly found in both edible and nonedible plant. They have been reported to exhibit a wide range of biological effects, including antioxidant, anti-inflammatory (Carlo *et al.*, 1999; Wang *et al.*, 1999), antimicrobial activity (Palma and Taylor, 1999; Yildirim *et al.*, 2001; Vardar-Unlu *et al.*, 2003), vasodilatory actions (Duarte *et al.*, 1993), antiallergic (Carlo *et al.*, 1999; Mastuda *et al.*, 2002), and antiviral (Raza *et al.*, 1996; Du *et al.*, 2003).

Polyphenols from the seeds of *Vitis vinifera* L. cv. Shiraz berries consisted of flavan-3-ol monomers ((+)-catechin and (-)-epicatechin-3-O-gallate and procyanidins (Kennedy *et al.*, 2000).

Grape marc (*Vitis vinifera*) contained mainly quercetin, catechin, epicatechin, kampherol, and procyanidin (Bonilla *et al.*, 1999). Methanol extract from grape pomace showed highest antioxidant activity, using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) model system. This methanol extract showed 71.7, 73.6, and 91.2 % inhibition using the thiobarbituric acid method, hydroxyl radical scavenging activity, and LDL oxidation, respectively, at 200 ppm (Murthy *et al.*, 2002).

Red wine has several flavonoids such as catechin, gallic acid, epicatechin, myricetin, quercetin, and resveratrol. Moreover, red wine can reduce the risk of cardiac disease and protect red blood cells from oxidation (Frankel *et al.*, 1995). White wine has less flavonoid than red wine (Tedesco *et al.*, 2000).

Antioxidant activity of orange juices (*Citrus sinensis* L.) were determined by bleaching of the stable DPPH radical and peroxidation, induced by 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH), on mixed dipalmitoylphosphatidyl choline/linoleic acid unilamellar vesicles (Rapisarda *et al.*, 1999).

The ethyl acetate soluble fraction from star apple (*Chrysophyllum cainito*) showed high antioxidant activity in the DPPH assay ($IC_{50} = 22 \mu\text{g/ml}$). The polyphenolic antioxidant constituents in star apple are (-) -epicatechin, (+) -catechin, (+) -gallocatechin, (-)-epigallocatechin, quercetin, quercitrin, isoquercitrin, myricitrin, and gallic acid (Luo *et al.*, 2002).

Quercetin O-glycoside, kaempferol O-glycosides, and xanthone C-glycosides were found in mango (*Mangifera indica* L. cv. "Tommy Atkins") peels (Schieber *et al.*, 2003).

Apple (*Malus pumila*) has several flavonoids such as flavanols, quercetin, rutin, (-)-epicatechin, and (+)-catechin (Lotito *et al.*, 2004).

Guyot *et al.* (1998) reported that procyanidins were the predominant phenolic constituents in cider apple fruits (*Malus domestica* var. kermerrien).

Edible plant materials, especially aronia and crowberry contain high antioxidant activity and high total phenolic content (GAE > 20 mg/g) (Kahkonen *et al.*, 1999).

The antioxidant properties, and DPPH radical scavenging activities of broccoli (*Brassica oleracea* L. var. *italica* Plenca) cultivated in Taiwan were tested by Guo and colleagues. The methanol extract of broccoli showed the highest DPPH radical scavenging activity (> 90%) followed by the water extract (43%) and acetone extract, respectively (Guo *et al.*, 2001).

The amount of total phenolic compounds, the DPPH scavenging activities and inhibitory effect on erythrocytes hemolysis of an oolong tea water extract (OTE) were evaluated. The OTE was separated into different fractions with higher amounts of phenolics compounds (lower molecular weight) have stronger antioxidative activities (Zhu *et al.*, 2002).

Resveratrol (3,5,4'-trans-trihydroxystilbene), a natural phytoalexin present in grapes and red wine, and its analogues, 3,4-dihydroxy-trans-stilbene (3,4-DHS), 4,4'-dihydroxy-trans-stilbene (4,4'-DHS), 4-hydroxy-trans-stilbene (4-HS), and 3,4-dihydroxy-trans-stilbene (3,4-DHS), were studied for their inhibitory effect of AAPH or Fe²⁺/ascorbate induced peroxidation of rat liver microsomes (Cai *et al.*, 2003).

The antioxidant activity of methanolic extract of mulberry leaves (*Morus alba* L.) was studied by Yen *et al.* It exhibited 78.2% inhibition on peroxidation of linoleic acid, greater than that of α -tocopherol (72.1%) but equal to that of butylated hydroxyanisole (Yen *et al.*, 1996).

The antioxidant activity, using DPPH model system and inhibitory effect of rat liver homogenates on lipid peroxidation of Pomegranate (*Punica granatum*) peel and seed extracts were determined. The methanol extract of peels and seeds showed 81% (at 50 ppm) and 23.2% (at 100 ppm) DPPH radical scavenging activity, respectively. The methanol extract showed 56% inhibition of lipid peroxidation at 100 ppm (Singh *et al.*, 2002).