CHAPTER II

REVIEW OF LITERATURES

1. General knowledge

Flavonoids, the plant phenolics, are derivatives of the pentose phosphate, shikimate and phenylpropanoid pathways (21-22, 29) and widely distributed in different kinds of plant materials such as leaves, seeds, bark, flowers, fruits and rhizomes (21, 23-27). These compounds play an important role in growth and reproduction (21, 28-30) providing protection against ultraviolet radiation (8, 11), pathogens and herbivores (21, 28, 32-33), besides contributing towards the color and sensory characteristics of fruits and vegetables (24, 31, 34-35). Besides that, it has been proved that they display a wide range of pharmacological and biochemical actions. Most of the beneficial health effects of flavonoids are attributed to their antioxidant activity including scavenging free radicals (21, 26, 37, 39, 41-42), singlet oxygen quenching (25-26, 38, 42), chelate redox-active metals (23, 25, 37, 39, 41-42), modulation of gene expression and interaction with the cell signaling pathways (39) as well as attenuate other processes involving reactive oxygen species (23, 25, 45-47).

Previous studies revealed that the antioxidant of flavonoid compounds depend on structure-activity relationships (SAR), in particular the number and positions of the hydroxyl groups and the nature of substitutions on the aromatic rings which involving its relative orientation of various moieties on the molecule and the stability in different system (21, 23, 46-47). However, these reports concern antioxidant activity of flavonoid in in vitro models. Most of the beneficial health effects of flavonoid antioxidants depend on their bioavailability involving mechanism of absorption and biotransformation which in turn are determined by their structure including their conjugation with other phenolics, degree of glycosylation/acylation, molecular size and solubility through metabolic processes (21, 23, 32, 39, 44, 48-49). Adverse effects due to excessive consumption of phenolic compounds have been limited information

(28, 37, 42, 45, 47, 50). Some of the reported effects of several flavonoids when ingested at high concentrations may be exhibit possible roles in carcinogenicity, genotoxicity, thyroid toxicity (21, 23, 78, 85-87). Thus a biological assay will be needed to determine/confirm the in vitro and in vivo antioxidant activity of flavonoids.

2. Flavonoids Biosynthesis

Flavonoids constitute the largest group of plant phenolics which consisting of phenolic and pyran rings (Fig 2.1). Variations in substitutions patterns result in the major flavonoids classes, i.e., flavonols, flavones, flavanones, flavanols, isoflavones, flavanonols and anthocyanidins. These substitutions may include oxygenation, alkylation, glycosylation/acylation and sulfation. Flavonoids differ in the arrangement of hydroxyl, methoxy and glycosidic side groups and in the conjugation between the A- and B- rings posess different kind of pharmacological and biochemical effects (23, 51-52).

The basic structure of flavonoids consist of two hydroxylated aromatic rings, A and B, joined by a three carbon fragment. Therefore, they contain fifteen carbon atoms in their basic structures and arranged in a C6-C3-C6 configuration. The aromatic ring A is derived from acetate/malonate pathway, while ring B is derived from phenylalanine through the shikimate pathway. The individual carbon atoms are referred to by a numbering system which utilizes ordinary numerals for the A- and C-rings and "prime" numerals for the B-ring (see the numbering systems used for flavonoids, Fig 2.1).

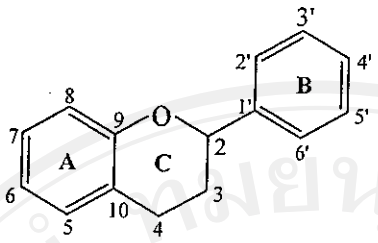


Figure 2.1 The flavonoid skeleton and its numbering system

All flavonoid variants are originated from flavonoid precursors 4-coumaroyl-CoA and malonyl-CoA. Both flavonoid precursors are derived from carbohydrates. Malonyl-CoA is synthesized from the glycolysis intermediate acetyl-CoA and carbon dioxide, the reaction being catalysed by acetyl-CoA carboxylase. The supply of 4-coumaroyl-CoA is more complex. Its involve the "shikimate/arogenate pathway", the main route to the aromatic amino acids phenylalanine and tyrosine in higher plants. This pathway starts from the aromatic amino acid phenylalanine and the key reaction is the deamination of phenylalanine catalysed by phenylalanine ammonialyase (PAL). This enzyme links the primary metabolism with the phenylpropanoid pathway. The product of the reaction, *trans*-cinnamate, is hydroxylated to 4-coumarate by cinnamate 4-hydroxylase, a cytochrome P450 mixed-function monooxygenase. Activation of 4-coumarate by formation of the CoA ester is catalysed by 4-coumarate: CoA ligase.

The key enzyme for the formation of the flavonoid skeleton is chalcone synthase (CHS), which catalyses the stepwise condensation of the three acetate units from malonyl-CoA with 4-coumaroyl-CoA to the C-15 intermediate 4,2',4',6'-tetrahydroxychalcone, (see Fig. 4). The respective 6'-deoxychalcone, isoliquiritigenin, is likewise synthesized from malonyl-CoA and 4-coumaroyl-CoA by chalcone synthase but in coaction with a (reduced nicotinamide adenine dinucleotide phosphate) (NADPH)-dependent reductase. Both chalcone types are the direct

precursors for aurones and other diphenylpropanoids. The 6'-hydroxy- and 6'-deoxychalcone are the intermediate precursors for all flavonoid compounds. The stereospecific cyclization of the chalcone, catalysed by chalcone isomerase, provides a *2S*-flavanone (e.g. naringenin, liquiritigenin) with the typical flavonoid skeleton. Flavanones are the direct precursors for the large class of flavones. Apigenin, for example, is synthesized from flavanones by introduction of a double bond between C-2 and C-3. Two types of enzymes, flavone synthase I, a 2-oxoglutarate-dependent dioxygenase, and flavone synthase II, a cytochrome P450 mixed-function monooxygenase, were found to catalyse this reaction. Besides that, flavanones are also the direct precursors for isoflavones and for the formation of two flavonoid intermediates, the flavan-4-ols and the dihydroflavonoids which are biosynthetic intermediates in the formation of flavonols, catechins, proanthocyanidins and anthocyanidins respectively.

As mentioned earlier, the simple flavonoids, intermediates and end-products, are derived from C15 chalcone intermediate by variety of routes. Modification by hydroxylation of the A-ring and, in particular, the B-ring, methylation of hydroxyl groups, glycosylation, acylation as well as a number of other reactions results in immense diversity of flavonoids found in nature, thus vary in their pharmacological and biological properties (21, 23, 24, 28, 51-52).

Table 2.1 List of enzymes leading to various flavonoid classes

Enzymes	*Acronym*
NON-FLAVONOID PRECUSORS	
I Acetyl-CoA carboxylase	ACC
II Phenylalanine ammonia-lyase	PAL
III Cinnamate 4-hydroxylase	C4H
IV 4-Coumarate:CoA ligase	4CL
V 4-Coumaroyl-CoA -3-hydroxylase	CC3H
FLAVONOID CLASSES	
1 Chalcone synthase	CHS
2 Polyketide reductase	PKR
3 Chalcone isomerase	CHI
4 2-Hydroxyisoflavanone synthase	IFS
5 2-Hydroxyisoflavanone dehydratase	IFD
6 Flavone synthase I	FNS I
Flavone synthase II	FNS II
7 Flavanone 4-reductase	FNR
8 Flavanone 3-hydroxylase	FHT
9 Flavonol synthase	FLS
10 Dihydroflavonol 4-reductase	DFR
11 Leucoanthocyanidin 4-reductase (flavan-3,4-*cis*-diol 4 –reductase)	LAR
12 Anthocyanidin synthase	ANS
13 Flavonoid (anthocyanidin/flavonol) 3-*O* -glucosyl-transferase	FGT

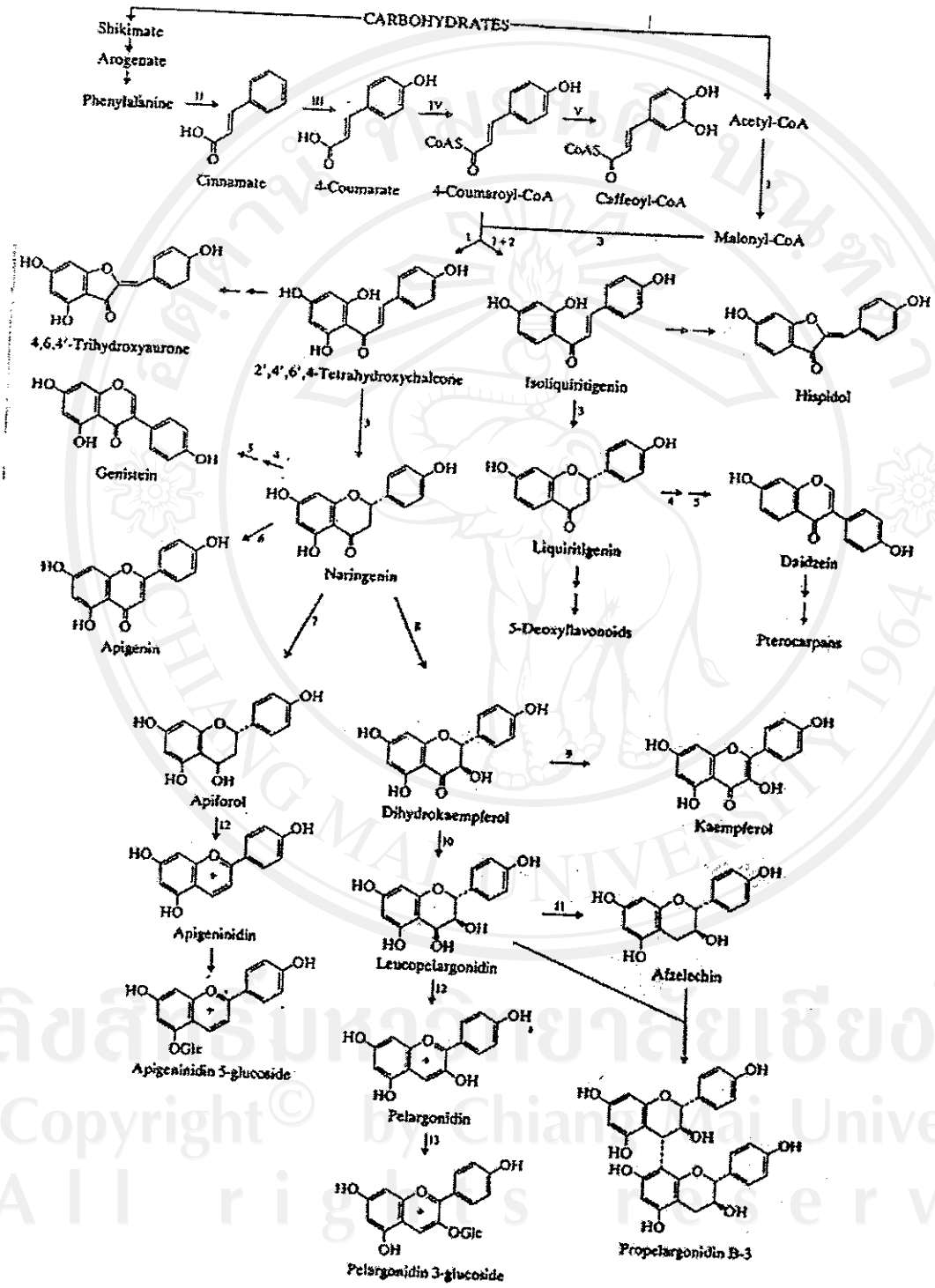


Figure 2.2 Flavonoid biosynthesis pathway

3. Flavonoid antioxidants: Chemistry, Structure-Activity Relationships (SAR) and Mechanism

3.1 Chemistry and Structure-Activity Relationships (SAR)

Flavonoids are secondary metabolites, a large group of phenolic compounds found in plants that are synthesized from both the shikimate and acetate-malonate pathways involving numerous enzymatic steps. These compounds, one of the most widely occurring groups of phytochemicals, are of considerable physiological and morphological importance in plant and also exhibit a wide range of biological effects such as reduction of the risk of cancer (46, 55-59), antiallergenic (21, 26, 41, 60), antiartherogenic (24, 26, 58, 61), anti-inflammatory (21, 26, 28, 40-41), antiviral (45, 59-60, 62), antimicrobial (21, 26, 28), antithrombic (21, 26, 60), anticataracts (63), antidiabetes (39, 41-42, 63), antiarthritis (62-63), antiaging (26, 56, 62, 64) cardioprotective (24, 32, 48-49, 55, 57, 65) and vasodilatory actions (21, 60); many of these biological functions have been attributed to their free radical scavenging and antioxidant activity. The propensity of a flavonoid to inhibit free-radical scavenging and antioxidant activity is governed by its chemical structure, both the number and configuration of H-donating groups are the main structural features influencing the antioxidant capacity of flavonoids and this is referred to structure-activity relationships (SAR).The structure features and mature of substitutions on rings A, B and C which determine the antioxidant activity of them include the following:

3.1.1 Degree of hydroxylation

The configuration and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity by donate hydrogen and electron to free radicals, stabilizing them and giving rise to a relatively stable flavonoid radical. For example, an *ortho*-dihydroxyl (3'-, 4'-OH) structure of ring B of flavonoids such as luteolin, quercetin and catechins results in higher activity as it confers higher stability to the aroxyl radical by electron delocalization, or act as the preferred binding site for

trace metals (21, 23, 41, 44, 56, 65). The presence of hydroxyl groups at the 3'-, 4'-, and 5'- positions or ring B (a pyrogallol group) and/or the presence of 3-OH and 3'-, 4'-OH, e.g. myricetin, epigallocatecin and cyanidin, have been reported to enhance the antioxidant activity of flavonoids for radical scavenging and reducing Fe-binding, compared to those that have a single hydroxyl group However, under some conditions, such compounds may act as pro-oxidants, thus counteracting the antioxidant effect (21, 37, 65).

These observations indicate that among the compounds having the same basic structure, the number of OH groups is the determinant factor for the antioxidant activity but when compared compounds belong to different subclasses, other factors like the structure of the C ring should also concerned (48,73).

3.1.2 *O*-Methylation

The differences in antioxidant activity between polyhydroxylated and polymethoxylated flavonoids are most likely due to differences in both hydrophobicity and molecular planarity. Quercetin and kaempferol, substitution 3-OH group decreases the activity against β-carotene oxidation in linoleic acid. In addition, removal 3-OH effect the conformation of the molecule. Flavonols and flavanols with 3-OH are planar, while the flavones and flavanones, lacking this feature, are slightly twisted. Planarity permits conjugation, electron dislocation and corresponding increase in flavonoid phenoxyl radical stability. Therefore, substitution 3-OH by OCH_3 group may reflect steric effects that perturb planarity and alters the redox potential, which affects the radical scavenging activity, resulting in less antioxidant activity (43, 71). For example, substitution of the hydroxyl groups in position 3' or 4' in ring B by OCH_3 or glucose reduces the antioxidant potency of flavonoids (76).

3.1.3 2, 3 double bond and 4-oxo

Comparison between flavonoids suggested that flavonoid fulfilling with 4-oxo and 2, 3 –double bond distinguishes the better antioxidant. For example, 4-oxo and 2, 3 –double bond combined with 3-OH and 3'-, 4'-OH in ring B (catechol group), e.g. quercetin, myricetin, permits a resonance effect of the aromatic nucleus that lends stability to the flavonoid radical and is therefore critical in optimizing the phenoxy radical-stabilizing effect of a 3'-, 4'- catechol. In addition, combination of 2, 3 –double bond, 3'-, 4'- catechol, 4-oxo and 5-OH is strongly inhibit metal-chelating due to chelating complexes with divalent cations may form between the 5-OH and 4-oxo group or between the 3'- and 4'-OH (43, 70-71). Furthermore, an electron donation group at the position 5 and 7 in ring A protect lipid peroxidation due to the weaken O-H bond making it easier transfer to a lipid peroxyl radical, thus breaking the chain process of lipid peroxidation (23, 65, 78).

3.1.4 Carbohydrate moieties

The position and structure of sugars play an important role in flavonoid antioxidants, aglycones are more potent antioxidants than their corresponding glycosides. Similar to *O*-methylation, *O*-glycosylation interferes the coplanarity of the B-ring with the rest of the flavonoid and the ability to delocalize electrons and/or lending hydrophilicity and altering access to lipid peroxyl and alkoxyl radicals during propagation of lipid peroxidation in membrane e.g. substitution of the 3-OH results with sugar-glycoside increase in torsion angle and loss of coplanarity and subsequent reduced antioxidant activity (21, 23-24, 41, 43, 48).

3.1.5 Degree of polymerization

The high antioxidant activity of the procyanidin dimmers is attributed to their hydroxyl functions that are potent hydrogen donators. Procyanidins, increasing degree of polymerization enhances the effectiveness to against variety of free radicals. Furthermore, the extensive conjugation between 3-OH and 3'-, 4'- catechol in ring B together with abundant $\beta_{4\rightarrow8}$ linkages endow a polymer with significant radical scavenging properties by increasing the stability of its radicals (23, 44).

From several reports of flavonoid antioxidants, in particularly SAR, most researchers agree that the presence of hydroxyl groups and especially catechol moiety, 3-OH and 2,3-double bond showed to be the most important factors in determining high antioxidant activity. However, the possibility of hydrogen bond interactions between hydroxyl groups as donators and 4-oxo moiety as acceptor or kinetic factors which may influence the antioxidant activity should also considered (23, 43, 70-71, 75).

Apart from SAR, antioxidant activity of flavonoids also depend on their antioxidant functions, lipophilicity, amphiphilic characteristic and partial affinity for intracellular membrane systems (70, 73) e.g. lipid peroxidation, the reaction takes place in membranes and the scavenging of lipid peroxyl radicals can only be accomplished by the fraction of antioxidants that is present in the membrane, this means that not only lipophilicity but also amphiphilic character of compounds seem to play a role in this mechanism (43, 65, 72). Several in vivo studies indicated that flavonoids are modified on absorption through metabolic processes in small intestine and in liver or can be modified and cleavaged by the enzymes of the gut micro flora in colon resulting in significant alteration in their redox potential (43, 79, 82). Most ingested flavonoids are extensively degraded to various phenolic acids, some of which still possess a radical-scavenging activity, which is evidenced experimentally by the increase of the plasma antioxidant status (43, 79, 82). However, the antioxidant efficacy in vivo of flavonoids is still limited and somewhat controversial. Data on

biological markers such as blood levels of flavonoids and their metabolites are not widely available, thus making it difficult to determine role of the flavonoid antioxidants. It is, therefore, essential to know the nature of the main flavonoids ingested, their dietary origin, the amounts consumed in different diets, their bioavailability and the factors controlled their bioavailability (79-83, 88).

From the accumulative evidences, the structural heterogeneity of flavonoids, their multiple mechanism of action and the diverse experiment methods use to evaluate their antioxidant activity pose challenges in assembling a collective hierarchy of SAR. Appropriate application of SAR to human nutrition requires systematic comparisons of homologs that differ in a single structural attribute. Although SAR of flavonoid metabolites warrants further research, modifications that ensue during metabolism are known to include hydroxylation, *O*-methylation, cleavage of heterocycle, deglycosylation and scission of polymeric species into monomeric units. Structure-acitivity relationships among naturally occurring flavonoids thereby offer preliminary insight into the impact of these metabolic alterlations on various mechanisms of antioxidant activity (75, 78-81, 88).

3.2 Antioxidants and its mechanism

Antioxidants

Antioxidant is defined as any substance that when present at low concentration compared to those of an oxidizable substrate (e.g. lipid, proteins and DNA) significantly delays or presents oxidation of that substrate (25, 47, 59, 66). These definitions therefore includes not only chain-breaking antioxidant e.g. ascorbic acid, tocophero, uric acid, glutathione (66-67, 89-91) but also enzymatic systems e.g. superoxide dismutase, catalase, glutathione peroxidase (66-67, 78-79) and proteins used to sequester metals capable to HO° production e.g. transferring, ferritin, ceruloplasmin, nemopexin, haptoglobin and albumin (79,84).

Free radicals and active oxygen species

Free radicals are chemical species, which have unpaired electrons (67-69). Free radicals, generally, always occur as intermediates in metabolic and physiological processes and also are as old entities as life itself (77, 89-91). Active oxygen and related species are essential for production of energy, phagocytosis, regulation of cell growth and intercellular signaling and synthesis of biologically essential compounds and phagocytosis, a critical process of immune system (79, 89-91). However, under certain conditions such as temperature, light, air, diet, alcohol, aging etc. (47, 69) the efficiency of such protecting system decrease, resulting in disturbances of the redox equilibrium established under health conditions. Over-production of free radicals initiates an uncontrolled chain reaction that lead to oxidation stress, damaging in cell membranes, proteins in tissue or enzymes carbohydrates and DNA resulting in aging and several degenerative diseases (74, 79, 89-91).

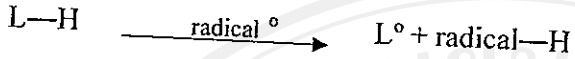
Mechanism of Antioxidants

As mentioned earlier, free radicals-generated reactions take place in mitochrondria, which is considered to be the energy storehouse of living cell. The oxygen-mediated radicals can be countered by the body's natural defense using various types of radical scavengers, here call endogenous antioxidants such as glutathione peroxidase, superoxide dismutase (SOD), catalase and melatonin. However, different external factors as mentioned in previous paragraph initiate imbalances of these systems, thus lead to oxidative stress, resulting in various diseases. Therefore, dietary antioxidants such as ascorbic acid, carotenoids and flavonoids are need for diminishing the cumulative effects of oxidative damage over the life spans. These external antioxidants can protect oxidative stress induced by various active oxygen species by abstract the lone electron from free radical molecules and help humans to keep control on these injurious species. The preventive mechanisms of antioxidant action consist of three steps. The first line defense is suppressing the formation of free radicals and active oxygen species. The radicals

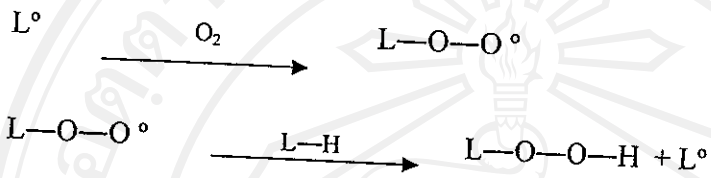
scavenging antioxidants are responsible in the second defense line and inhibit chain intiations and or/break the chain propagation. Then, the antioxidant enzymes such as phospholipase, proteases, DNA repair enzymes and transferases act as the third line defense. In addition, the appropriate antioxidant is generated and transfer to right site at the right time with the right concentration when oxidative stress occur. This adaptation mechanism is also important in the total defense system (79, 89).

As we know, oxidation is essential to many living organisms for production of energy to fuel biological processes. However, an imbalance caused by excess oxidants, particularly Reactive Oxygen Species (ROS) e.g. hydroxyl radicals (HO•), superoxide ($O_2^{\circ-}$), peroxyl radicals (ROO•). These toxic substances are capable oxidizing protein cells, nucleic acid and lipids, thus lead to pathophysiological conditions and/or diseases. Among the free-radical reactions, lipid peroxidation is important, for example, food deterioration and oxidative modification of low density lipoprotein (LDL) which is accepted as a key initial event in the progression of atherosclerosis (41, 89). The chain reaction can be summarized as shown in Fig 2.3. It consists of three steps, namely, chain initiation, chain propagation and chain termination. In chain initiation step, the free radical is formed and then attack the lipid, thus generate lipid radicals. The lipid radical L° reacts with oxygen molecule quite rapidly to give lipid peroxyl radicals. Then the lipid peroxyl radicals attack another lipid molecule and abstracts hydrogen atom to yield lipid hydroperoxide and at the same time another lipid radical which react with oxygen and continues the second oxidation sequence. Thus, the chain is propagated and one molecule of chain-initiating radical may cause the oxidation of many molecules. The chain oxidation is terminated when lipid radical or lipid peroxyl radical is scavenged by and antioxidants such as vitamin E and C or when two lipid peroxyl radicals react to give non-radical products such as alcohols and ketones. The characteristic of oxidation that proceeded by chain reaction illustrated in Fig. 2.3.

Chain initiation:



Chain propagation:



Chain termination:

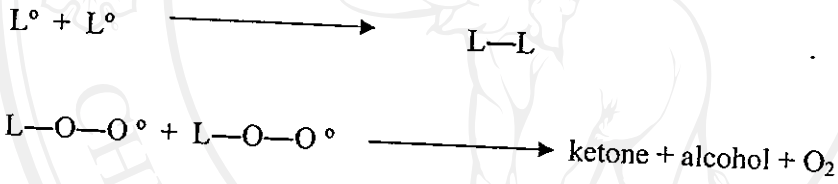
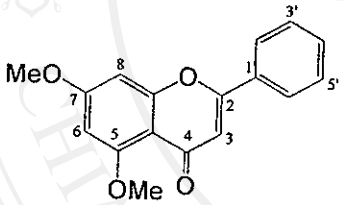
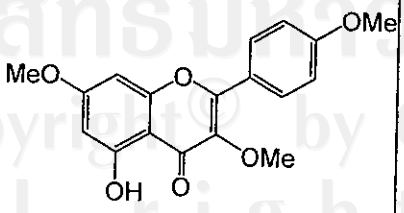


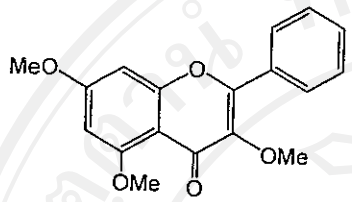
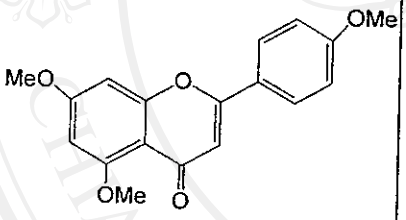
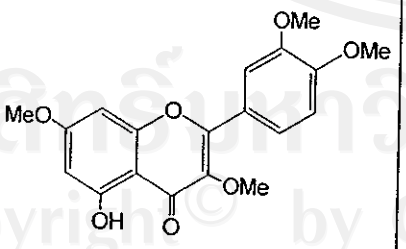
Fig. 2.3 Chain reaction of Lipidperoxidation

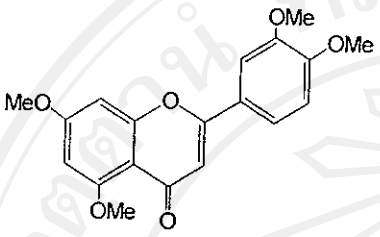
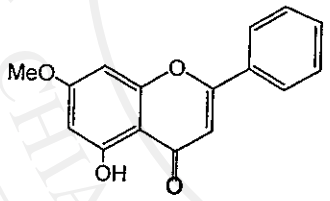
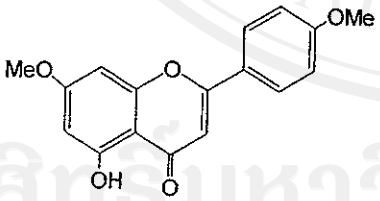
4. The chemistry and biological activities of *K. parviflora* Wall. ex Bak.

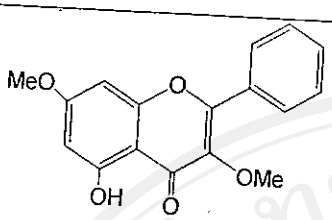
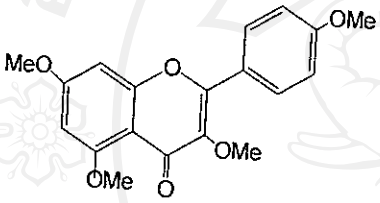
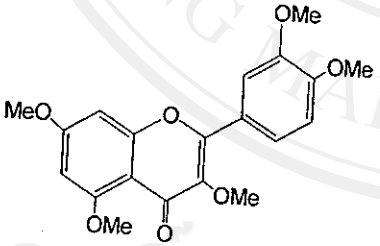
Chemical constituents isolated from *K. parviflora* were reported as flavones, flavanones and chalcones. List of the compounds and their biological activities found in rhizome of *K. parviflora* is shown in Table 2.2.

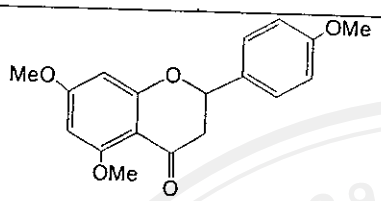
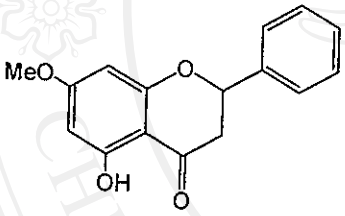
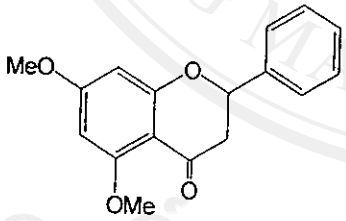
Table 2.2 Chemical constituents and biological activities of *K. parviflora*:

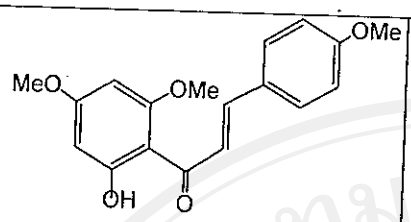
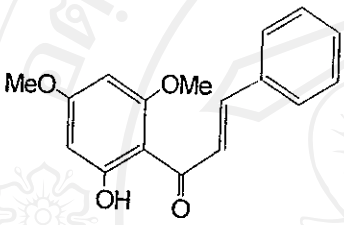
Category	Chemical compounds	Biological activity	References
1. Flavones	1. 5,7-dimethoxyflavone 	- antiinflammatory, antipyretic and analgesic effect - low toxicity in animal	4,8
	2. 5-hydroxy-3,7,4'-trimethoxyflavone 	-	4

Category	Chemical compounds	Biological activity	References
	3. 3,5,7-trimethoxyflavone 	-	4
	4. 5,7,4'-trimethoxyflavone 	- antiplasmodial - antifungal - antimycobacterial	4, 11
	5. 5-hydroxy-3,7,3',4'-tetramethoxyflavone 	-	4

Category	Chemical compounds	Biological activity	References
	6. 5,7,3',4'-tetramethoxyflavone 	- mild antifungal, - antimycobacterial	4, 11
	7. 5-hydroxy-7-methoxyflavone 	-	5
	8. 5-hydroxy-7,4'-dimethoxyflavone 	-	4
	9. 5-hydroxy-3,7-dimethoxyflavone	-	4

			
Category	**Chemical compounds**	**Biological activity**	**References**
	10. 3,5,7,4'-tetramethoxyflavone 	-	5
	11. 3,5,7,3',4'-pentamethoxyflavone 	- increase the accumulation of rhodamine 123 and daunorubicin in LLC-GA5 COL 150 cells	5
2. Flavanone	1. 5-hydroxy-7,4'-dimethoxyflavanone	-	4

			
Category	**Chemical compounds**	**Biological activity**	**References**
	2. 5-hydroxy-7-methoxyflavanone 	-	4
	3. 5,7-dimethoxyflavanone 	-	4
3. Chalcone	1. 2'-hydroxy-4,4',6'-trimethoxychalcone	-	5

			
	2. 2'-hydroxy-4',6'-dimethoxychalcone 	-	5

Apart from the investigation in chemical constituents as mentioned above, biological activity of flavonoid compounds obtained from Zingiberaceous plants revealed that anti-inflammatory activity of flavonoids depended upon the present of methoxy groups at C5 and C7 at ring-A and the pyrano at ring-C of flavones and flvanones molecule. Flavanones, absent of 2,3-double bond in ring-C, possessed a comparable bronchodilator effect as aminophylline on animal model. Whereas chalcone, the minor component, ring-B opened, not showed inflammatory effect (9). In addition, methoxyflavone compounds promoted nitric oxide production in human umbilical vein endothelial cells (13), increased the accumulation of rhodamine 123 and daunorubicin in LLC-GA5-COL150 cells (14) and inhibited HIV-1 protease, HCV and HCVM protease (15). On the other hand, the ethanolic extract of *K. parviflora* possessed gastroprotective effect (16), potented anti-allergic effect (17) as well as increase weights of seminal vesicle and stimulating spermatogenesis in male rats (18).

Beside that, Rhizomes powder displayed low acute toxicity on rat model (10). Biologically fermented juice, tonic drink, possessed antibacterial against *Lactobacillus spp.* (11).